

Studies on the genetic and environmental basis of endometriosis

**Ruth M. Hadfield
St. Hilda's College
D.Phil. Thesis**



Table of Contents

ABBREVIATIONS.....	vi
ACKNOWLEDGEMENTS.....	vii
PUBLICATIONS.....	viii
ABSTRACT.....	ix
 1. General Introduction	
1.1 INTRODUCTION	1
1.2 PATHOGENESIS OF ENDOMETRIOSIS	2
<i>Introduction</i>	2
<i>Endometriosis and Müllerian anomalies</i>	3
<i>The role of the immune system in the pathogenesis of endometriosis</i>	5
1.3 CLINICAL CHARACTERISTICS OF ENDOMETRIOSIS	6
<i>Diagnosis of endometriosis and the classification of disease severity</i>	6
<i>Treatment of endometriosis</i>	7
<i>Prevalence</i>	8
<i>Risk factors for endometriosis</i>	11
<i>Endometriosis and ovarian cancer</i>	14
<i>CA125</i>	15
1.4 EVIDENCE FOR AN ENVIRONMENTAL ROLE IN DISEASE DEVELOPMENT	16
1.5 EVIDENCE THAT ENDOMETRIOSIS IS A GENETIC DISEASE	17
1.6 AIMS OF THIS WORK.....	22
1.7 CHAPTER SUMMARY	22
 2. Risk Factors for Endometriosis in the Rhesus Macaque	
2.1 INTRODUCTION	24
<i>Non-human primates as a model for the study of human endometriosis</i>	24
<i>The rhesus macaque as a model for the study of human endometriosis</i>	25
<i>Reproductive biology of the rhesus macaque</i>	25
<i>Studies of spontaneous endometriosis in non-human primates</i>	26
<i>Length of time in captivity as a risk factor for endometriosis</i>	27
<i>Hysterotomy as a risk factor for endometriosis</i>	28
<i>Oestradiol as a risk factor for endometriosis</i>	28
<i>Laparoscopy as a risk factor for endometriosis</i>	29
<i>Environmental risk factors</i>	29
Dioxin.....	29
Ionizing radiation	31
<i>Aims of this work</i>	32
2.2 METHODS.....	32
<i>Animals</i>	32
<i>Archived colony records</i>	33
<i>Clinical details</i>	35
<i>Definition of cases and controls</i>	35
<i>Details of animals with endometriosis</i>	36
<i>Age of cases and controls</i>	37
<i>Matched control group</i>	37
<i>Statistical methods</i>	39
<i>Conditional logistic regression</i>	40
2.3 RESULTS	40

Prevalence of endometriosis in the colony.....40

Analysis of all cases and controls 41

Analysis of matched case and control groups 43

Risk factors for endometriosis: univariate analysis 43

Risk factors for endometriosis: logistic regression analysis 46

Familial relationships between animals.....46

2.4 DISCUSSION 48

Prevalence of endometriosis in the colony.....48

Risk factors for endometriosis 50

The effect of pregnancy on endometriosis 52

Conclusion 53

Summary.....54

3. Does Endometriosis have a Familial Component in the Rhesus Macaque?

3.1 INTRODUCTION 55

3.2 MRI IN A RHESUS MACAQUE PEDIGREE TO DIAGNOSE ENDOMETRIOSIS 56

Introduction.....56

 Basic principles of MRI 56

 Image interpretation 58

 Advantages and disadvantages of MRI 59

 Accuracy of MRI for endometriosis diagnosis.....59

 MRI diagnosis of endometriosis in non-human primates.....60

Methods 60

 Identification of families for analysis.....60

 Identification of animals for MRI.....61

 MR imaging 62

Results 62

 Cohort of macaques MRI scanned 62

 Magnetic resonance images.....63

 Results of MR imaging 63

 Family groups identified 64

Discussion 64

3.3 SEGREGATION ANALYSIS 66

Introduction.....66

 The direct method.....68

 The proband method 68

 The general proband method and the singles method 68

Methods 70

 Creation of M-link format file.....70

Results 72

Discussion 75

3.4 STUDIES ON THE FEASIBILITY OF LINKAGE ANALYSIS 76

Introduction.....76

Methods.....77

Results 78

 Genotyping.....78

Discussion 80

3.5 CONCLUSION & SUMMARY 81

4. Familial Endometriosis

4.1 INTRODUCTION 82

4.2 COLLECTION OF AFFECTED FAMILIES 83

Introduction.....83

Methods 83

Results 84

 Participants in the OXEGENE study.....84

 Characteristics of stage A versus stage B endometriosis.....85

 Familial endometriosis 88

Discussion 90

4.3 MRI STUDIES 92

 Introduction..... 92

 Methods 92

 Results 93

 Discussion 95

4.4 DELAY IN THE DIAGNOSIS OF ENDOMETRIOSIS 97

 Introduction..... 97

 Methods 98

 Results 98

 Comparison of UK and USA cohorts..... 98

 Discussion 99

4.5 ENDOMETRIOSIS IN TWIN SISTERS 102

 Introduction..... 102

 Methods 102

 Zygoty determination 102

 Results 104

 Discussion 105

4.6 CONCLUSION..... 107

5. Candidate Gene Association Studies

5.1 INTRODUCTION 108

5.2 SUBJECTS STUDIED..... 110

 Population Controls..... 110

 Normal Controls..... 111

 Sporadic Cases 111

 Familial Cases 111

 Mothers of affected women..... 112

 DNA extraction 112

5.3 GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE 113

 Introduction..... 113

 Methods 115

 Results 116

 Discussion 117

5.4 CYTOCHROME P450 1A1 (CYP1A1)..... 118

 Introduction..... 118

 Methods 121

 Results 122

 Discussion 123

5.5 GLUTATHIONE-S-TRANSFERASE *M1* AND *T1* NULL MUTATIONS 124

 Introduction..... 124

 Methods 126

 Multiplex PCR for genotyping of GSTM1 and GSTT1 126

 Linkage analysis..... 129

 Familial DNA samples for linkage analysis 130

 Results 130

 Association studies..... 130

 CYP1A1 MspI polymorphism/GSTM1 null genotype combination..... 133

 Linkage studies..... 133

 Discussion 135

5.6 CONCLUSION..... 137

6. Genome-Wide Search for Endometriosis Susceptibility Loci: Chromosome One

6.1 INTRODUCTION 138

 Genetic analysis of complex traits..... 138

 Linkage analysis..... 139

Power calculations.....141

Markers for linkage analysis.....142

6.2 METHODS.....144

Subjects 144

Laboratory methods 144

Genotyping.....145

Data analysis.....147

 Single point analyses.....147

 Multipoint analysis.....148

 Comparison of linkage methods.....149

6.3 RESULTS 149

6.4 DISCUSSION 153

7. Summary

7. SUMMARY.....154

8. References

8. REFERENCES.....158

Abbreviations

bp	base pair
CI	confidence interval
cm	centimetres
cM	centimorgan
COREC	Central Oxford Research Ethics Committee
EDTA	ethylenediaminetetraacetic acid
ER	oestrogen receptor
HLA	human leukocyte antigen complex
kb	kilobase
kg	kilograms
ml	millilitres
MRI	magnetic resonance imaging
μl	microlitres
PCR	polymerase chain reaction
PIC	polymorphism information content
ppt	parts per trillion
rAFS	revised American Fertility Society Classification system
RFLP	restriction fragment length polymorphism
SD	standard deviation
vs.	versus
WWW	World Wide Web

Acknowledgements

I am completely indebted to my supervisor, Stephen Kennedy, for his excellent guidance, constant enthusiasm, ideas and encouragement. His efforts in proof-reading this thesis were also much appreciated. I thank Professor David Barlow and Helen Mardon for their advice and interest in this work.

I am very grateful to Dan Weeks, Department of Human Genetics, University of Pittsburgh, for his help and advice on statistical genetics, particularly in the segregation analysis presented in Chapter Three.

I thank the staff of the Wisconsin Regional Primate Research Center for their help and assistance over the years and for making my visit there especially enjoyable. In particular, I am indebted to Joe Kemnitz, Joan Scheffler, Chris Coe (Harlow Primate Laboratory), Hideo Uno and Rikki Colman. I am also grateful to Pat Yudkin for her advice and guidance on the statistical analysis of data presented in Chapter Two. I thank Stephen Golding, Cathy Westbrook, Tal Jacobson and Euan Laird for their part in the MRI studies. I am grateful to Sanjiv Manek for providing tissue for the association studies presented in Chapter Five, and also to Soma Mukherjee and Satoshi Nakago for their help and Lynda Tyfield for providing controls. Thanks also to Sarah Wolfensohn, Department of Physiology, for providing samples for the rhesus microsatellite marker study.

The work presented in Chapter Six would not have been possible without the assistance of Oxagen Ltd. I thank all staff for their helpfulness during the four months I spent there learning the techniques of genetic analysis, in particular Trevor Nicholls, Mark Edwards, Simon Bennett, Gina Kearsey, Alison Walsall, Kay Davies, Jan Pullen, Linda Collins, Bhal Sidhu and Jemma Masson. I also thank Oxagen for allowing me to analyse and include the preliminary chromosome one data in this thesis. The contribution of international collaborators who collected affected families and blood samples is also gratefully acknowledged.

I thank the laboratory staff of the Wellcome Trust Centre for Human Genetics for being very helpful and allowing me to use their facilities for the linkage studies presented in Chapter Five.

Financial support for parts of this work was provided by Oxagen Ltd., an MRC-ROPA grant and an Oxford and Anglia Regional Health Authority grant.

Much of this work would not have been possible without the help of the Endometriosis Associations of many countries. I thank all the gynaecologists who have referred patients and provided copies of surgical records and, most importantly, patients and their families for participating.

The advice, support and friendship of NDOG staff, past and present, has been much appreciated, and I would especially like to acknowledge Karen, Zsuzsa, Shanthi, Sailesh, Isabella and Esther.

Lastly, my thanks go to Graeme for his unfailing optimism and encouragement and my family for their support, confidence in me and for enduring my absence from New Zealand for even longer than any of us ever anticipated.

Publications

The work presented in this thesis has been published previously, as follows:

Absence of a relationship between endometriosis and the N314D polymorphism of galactose-1-phosphate uridyl transferase (GALT) in a UK population (1999) Hadfield, R., Manek, S., Nakago, S., Mukherjee, S., Mardon, H., Barlow, D. and Kennedy, S. *Molecular Human Reproduction*, in press.

Magnetic resonance imaging to assess familial risk in relatives of women with endometriosis (1998) Kennedy, S., Hadfield, R., Westbrook, C., Weeks, D.E., Barlow, D. and Golding, S. *The Lancet*, 352, 9138, 1440-1441.

Beyond providing information: the Internet as a research tool in reproductive medicine. (1998) Suchard, M., Hadfield, R., Elliott, T. and Kennedy, S. *Human Reproduction*, 13, 1, 6-7.

Endometriosis in monozygotic twins (1997) Hadfield, R., Mardon, H., Barlow, D. and Kennedy, S. *Fertility & Sterility*, 68, 5, 941-942.

The use of MRI in genetic studies of endometriosis. (1997) Kennedy, S.H., Hadfield, R., Barlow, D.H., Weeks, D.E., Laird, E. and Golding, S. *J.Am.Med.Genet.*, 71, 371-372.

Risk factors for endometriosis in the rhesus monkey (*Macaca mulatta*): a case-control study (1997) Hadfield, R., Yudkin, P., Coe, C., Scheffler, J., Uno, H., Barlow, D., Kemnitz, J. and Kennedy, S. *Human Reproduction Update*, 3, 2, 109-115.

Delay in the diagnosis of endometriosis - a survey of women from the United States of America and the United Kingdom (1996) Hadfield, R., Mardon, H., Barlow, D. and Kennedy, S. *Human Reproduction*, 11, 4, 878-880.

Age of onset of pain symptoms in non-twin sisters concordant for endometriosis (1996) Kennedy, S., Hadfield, R., Mardon, H. and Barlow, D. *Human Reproduction*, 11, 2, 403-405.

Studies on the Genetic and Environmental Basis of Endometriosis

Ruth M. Hadfield
St. Hilda's College
D.Phil. Trinity 1999

There is now considerable evidence that endometriosis is likely to be a complex multifactorial trait, such as diabetes or asthma, in which a number of susceptibility loci interact with each other, and the environment, to produce the disease phenotype. This thesis presents studies on the genetic and environmental basis of endometriosis in both a non-human primate model and in women.

The study of the autopsy records of 399 female rhesus monkeys identified 81 (20%) with spontaneous endometriosis. Age, exposure to ≥ 3 oestradiol implants (relative risk 9.7, $P < 0.001$) or ≥ 1 hysterotomy (relative risk 5.8, $P = 0.006$) were significant risk factors as determined by conditional logistic regression. Living descendants of the affected animals had MRI scans which suggested that 8/113 (7%) had at least one endometriotic lesion >1 cm in diameter. Segregation analysis was conducted on the resulting 12 pedigrees, which contained 64 half sib-pairs, 2 full sib-pairs and 11 mother-daughter pairs.

Human, affected sib-pairs and families were recruited for the OXEGENE study to conduct sib-pair analysis using microsatellite markers at 10cM resolution across the entire genome. MRI studies of the first-degree relatives of women with rAFS stage III-IV disease estimated that the relative risk (λ_R) may be as high as 14 (95% CI 4.8 - 30.3). Candidate gene studies, comparing the frequency of the GALT N314D polymorphism, the CYP1A1 MspI polymorphism and the GSTM1 and T1 null mutations in two case groups, with either sporadic disease or a family history of endometriosis, and two control groups, did not show evidence of association. Linkage analysis using three microsatellite markers and 50 affected sib-pairs in the region to which GSTM1 maps (1p13) did not show evidence of linkage to this region. However, there was an apparent relationship between the presence of both the GSTM1 null mutation and the CYP1A1 MspI polymorphism and an increased risk of endometriosis. The initial findings of a sib-pair analysis, using 29 microsatellite markers across chromosome one in 128 affected sib-pairs, did not reveal evidence of linkage.

These findings provide some insight into the aetiology of endometriosis in women.

Chapter One

General Introduction

1.1 INTRODUCTION

Endometriosis is defined histologically as the presence of endometrial glands and stroma in sites outside the uterus. Endometriosis may present as dysmenorrhoea, dyspareunia, a pelvic mass, infertility or pelvic pain, or it may be an incidental finding at surgery in an asymptomatic woman. Although not life-threatening, endometriosis represents an important public health problem through lost working hours, hospitalisation and as one of the leading reasons for hysterectomy. This disease often results in considerable pain and distress to sufferers, although few data on the true impact to these women's lives are available. In a recent survey of 4,000 American Endometriosis Association members, whilst probably representing a biased population, 79% reported that the disease meant that they could not always carry out normal work activities and 25% were incapacitated for between two to six days. Adding to the burden of this illness, 47% of members had to see a doctor five or more times before receiving a diagnosis or referral.

Despite the fact that endometriosis affects millions of women worldwide, very little is known about its aetiology, pathogenesis or exact prevalence. There is, however, increasing evidence for a genetic basis to endometriosis. It is likely that endometriosis is a complex, multifactorial trait, such as asthma and diabetes, in which multiple gene loci interact with each other and the environment to produce the disease phenotype. As a result of recent advances in high throughput genetics technology it is now possible to screen the entire genome for susceptibility loci for a disease, even when there are no postulated candidates. For a disease such as endometriosis, where the basic mechanisms of disease development remain to be elucidated, this approach is a particularly attractive option. For such a genome-wide screen to be conducted a large number of affected pedigrees must be identified and blood samples collected for DNA

isolation. Studies of candidate gene loci and the parallel investigation of an animal model are commonly adopted to complement this strategy.

The purpose of this chapter is to introduce briefly the disease endometriosis and summarise current understanding of its aetiology. Evidence for the role of genetic susceptibility loci and the environment in disease development will also be presented here. Lastly, an overview of the work presented in this thesis will be given.

1.2 PATHOGENESIS OF ENDOMETRIOSIS

Introduction

It is not known what mechanisms interact to cause endometriosis. Probably the most generally accepted theory is that viable endometrial cells enter the peritoneal cavity through the fallopian tubes because of retrograde menstruation (Sampson, 1927). There is considerable evidence for this theory. Firstly, it has been shown that there are viable endometrial cells present in the tissue lost during menstruation (Te Linde & Scott, 1950). Studies have also observed retrograde menstruation in up to 76% of women and 62% of female baboons (D'Hooghe *et al.*, 1996, Liu & Hitchcock, 1986). The fact that endometriotic lesions are more frequently located close to the fallopian tubes in comparison to more distal sites is compatible with this theory. There is also evidence that endometriosis and eutopic endometrium are very similar in the expression of proteins, including growth factors and their receptors, steroid receptors and integrins (Thomas, 1995) suggesting the endometriotic tissue originates from the endometrium. Nevertheless, the observation that retrograde menstruation occurs in most women, whether they have endometriosis or not, suggests that other mechanisms must also be involved in disease development.

Other explanations for the transport of viable endometrial tissue into the peritoneum include dissemination through the lymphatic system or at the time of surgery. There are case reports of endometriosis in caesarean section scars (Kale *et al.*, 1971, Kennedy *et al.*, 1988), an increased risk of endometriosis has been observed in rhesus monkeys exposed to hysterotomy (Hadfield *et al.*, 1997b) and there is evidence that if care is

taken to avoid spillage of endometrial cells into the abdominal cavity, the risk of scar endometriosis is diminished (Martin & Higginbottom, 1973).

The other principal theory is differentiation of the mesothelial cells lining the peritoneum into endometrial glands and stroma (Novak, 1926). This, the metaplastic theory, may explain the rare occurrence of endometriosis in men treated with oestrogen for prostate cancer (Schrodt *et al.*, 1980), and in women with Müllerian agenesis arising in the absence of functioning endometrium (Ugur *et al.*, 1995). Metaplasia must occur in conjunction with hormonal effects to account for the lack of endometriosis in males and the preponderance of endometriotic lesions close to the ovary. Finally, it is possible that these mechanisms may act simultaneously and it is almost certain that other factors are involved.

Adenomyosis is defined as the presence of endometrial glands and stroma within the myometrium and may cause abnormal bleeding and dysmenorrhoea. There may or may not be a relationship between endometriosis and adenomyosis and as for endometriosis, the factors which cause adenomyosis remain elusive. It is possible that some of the underlying mechanisms in disease development are common for both conditions.

Endometriosis and Müllerian anomalies

In the past, women with Müllerian anomalies have been studied to determine the prevalence of endometriosis and to gain evidence for or against the theories of retrograde menstruation and coelomic metaplasia. Müllerian anomalies are abnormalities in the upper vagina, cervix, fallopian tubes and/or uterus which result due to abnormalities in their formation from the Müllerian ducts during foetal development. The different classes of Müllerian anomaly are illustrated in Figure 1.1. Hypothetical experiments using a mathematical model to calculate flow rates have shown that over 50% of menstrual flow becomes retrograde when the cervical os diameter is < 0.5 mm (Barbieri *et al.*, 1992) indicating that this may be an important factor in the development of endometriosis. Hence, an increase in the frequency of endometriosis in women with obstructive Müllerian anomalies, *i.e.* those which prevent menstrual outflow and therefore may increase retrograde menstruation, would provide evidence for the Sampson theory.

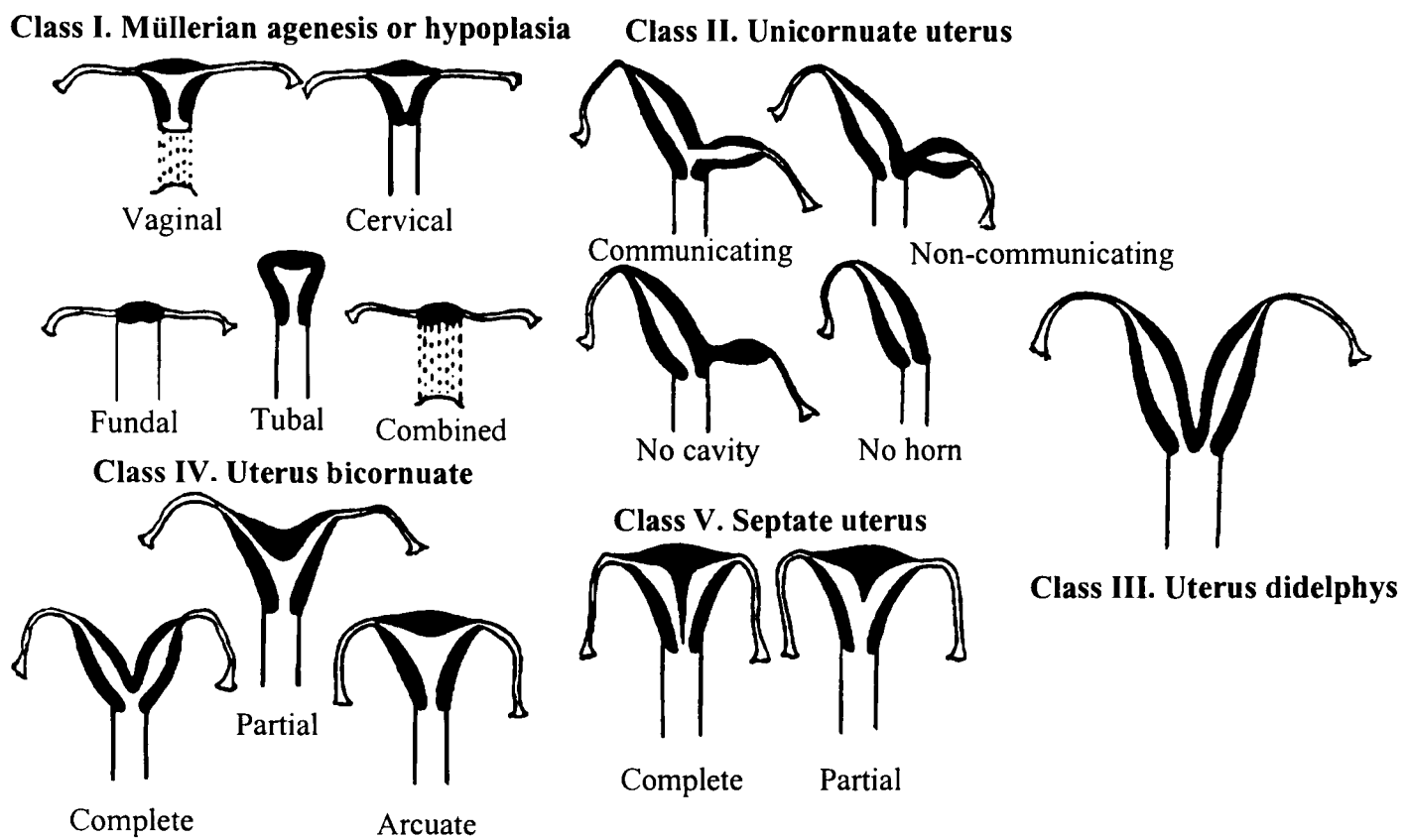


Figure 1.1. Classification of Müllerian anomalies [adapted from Fedele *et al.*, 1992].

In a study of 186 women with Müllerian anomalies, 37 (20%) had endometriosis (Ugur *et al.*, 1995). The cases of endometriosis had Müllerian agenesis (38%), unicornuate uterus (8%), didelphys uterus (5%), bicornuate uterus (22%) and septate uterus (27%). No significant difference was found between the class of Müllerian anomaly and the prevalence of endometriosis. Of the women studied, 41/186 (22%) lacked functioning endometrium. When these patients were excluded from the analysis it was found that endometriosis occurred in 58% of patients with obstructive anomalies and 18% with non-obstructive anomalies ($P < 0.001$). Similarly, Olive & Henderson (1987) found that 10/13 (77%) of women with outflow obstruction had endometriosis compared to 16/43 with no obstruction. In another study of 198 women with non-obstructive anomalies, the risk of endometriosis was in fact significantly diminished in comparison to the control group (Fedele *et al.*, 1992). These data, which demonstrate a positive relationship between obstructive anomalies and endometriosis, provide evidence for the theory of retrograde menstruation.

The epidemiological relationship between non-obstructive anomalies and endometriosis may not be solely due to increased exposure to retrograde menstruation; for example, genetic factors may play a role. There is evidence for familial clustering of Müllerian anomalies (Ergun *et al.*, 1997, Verp *et al.*, 1983) and identification of chromosomal

regions to focus on, in the search for endometriosis genes, may be useful. One case study has reported Müllerian dysgenesis in a patient with mental retardation and a karyotype showing extra chromosomal material on the short arm of chromosome 13 (Maier & Koch, 1990). An association between the N314D polymorphism of galactose-1-phosphate uridyl transferase (GALT), which results in decreased enzyme activity in the breakdown of galactose, and vaginal agenesis has been observed (Cramer *et al.*, 1996a) in a small sample size. The gene for GALT is located on chromosome 9p21 (Sparkes *et al.*, 1980).

The role of the immune system in the pathogenesis of endometriosis

Retrograde menstruation appears to be a common phenomenon, yet not all women develop endometriosis. One explanation for this could be an inadequate or abnormal immunological response, in some women, to the presence of endometrial cells in the peritoneum.

There is clear evidence that the immune system is in some way involved in the development of endometriosis, and there is a large body of literature devoted to the study of this aspect of the disease (reviewed by Hill, 1997). For example, when an immunosuppressive state was induced in baboons by treatment with steroids and azathioprine, the progression of endometriosis in animals with spontaneous disease was increased. Functional changes in natural killer (NK) cells, cytotoxic T-lymphocytes, B cells, peripheral blood monocytes (PBMs) and peritoneal macrophages (PMs) have been connected with endometriosis. *In vitro*, these cells display a decreased cytotoxicity to autologous endometrial cells. PBMs suppress the growth of endometrial cells from eutopic endometrium in culture, but promote the growth of endometrial cells from endometriosis. PBMs and PMs are activated and secrete higher levels of cytokines such as TNF- α and prostaglandins in endometriosis. There is increased expression of markers associated with activation of PMs, such as HLA DR and CD14 and increased production of pro-inflammatory molecules such as IL-1, IL-6, IL-8 and TNF- α . Activated PMs have also been shown to release molecules which appear to enhance the adhesion of endometrial cells, increase vascularisation to endometrial implants and promote proliferation of endometriotic implants. It has been demonstrated that NK cell-mediated cytotoxicity is impaired in the peritoneal fluid of women with endometriosis. Endometriosis is also more resistant to PBM and PM mediated

endometrial cell lysis and apoptosis when compared to eutopic endometrium. These changes in immunity suggest that endometrial cells, within menstrual debris entering the peritoneum, are not recognised and cleared in the same way in women with endometriosis compared to women without the disease (Dmowski *et al.*, 1994, Grosskinsky & Halme, 1993).

An auto-immune mechanism has also been suggested and anti-endometrial auto-antibodies have been identified in the peritoneal fluid and the serum of women with endometriosis, but also in women with a normal pelvis. Numerous studies into the auto-immune nature of endometriosis have failed consistently to identify significantly raised levels in women with the disease (reviewed by D'Hooghe & Hill, 1995).

There is substantial evidence that the immune system plays some role in the development of endometriosis, but it is uncertain whether they represent a primary role of the immune system in disease aetiology or whether the presence of endometriosis itself initiates these phenomena.

1.3 CLINICAL CHARACTERISTICS OF ENDOMETRIOSIS

Diagnosis of endometriosis and the classification of disease severity

Endometriosis can be accurately diagnosed by laparoscopy, laparotomy or at hysterectomy. More recently, the less invasive tool of magnetic resonance imaging (MRI) has been successfully used to diagnose of endometriotic cysts and implants over one centimetre in diameter. This technique is discussed in greater detail in Chapter Three.

A classification system, revised from a previous version, has been devised by the American Fertility Society (The American Fertility Society, 1985) to enable the severity of disease to be determined at the time of diagnosis (Table 1.2). The classification system was designed for use with infertility patients to determine their chances of achieving a pregnancy. Therefore, the stage of disease determined using this system does not necessarily correlate with symptoms such as dysmenorrhoea or dyspareunia. Throughout this thesis, minimal and mild stages of disease (stage I - II) according to the revised American Fertility Society classification system (rAFS) have

Table 1.2. The revised American Fertility Society Classification of Endometriosis: 1985 (The American Fertility Society, 1995). Stage I (minimal) disease scores 1-5, stage II (mild) disease scores 6-15, stage III (moderate) disease scores 16-40 and stage IV (severe) disease scores >40.

PERITONEUM	ENDOMETRIOSIS		< 1cm	1 - 3cm	> 3cm
	Superficial		1	2	4
	Deep		2	4	6
OVARY	R	Superficial	1	2	4
		Deep	4	16	20
	L	Superficial	1	2	4
		Deep	4	16	20
	POSTERIOR CULDESAC OBLITERATION		Partial		Complete
			4		40
OVARY	ADHESIONS		<1/3 Enclosure	1/3- 2/3 Enclosure	> 2/3 Enclosure
	R	Filmy	1	2	4
		Dense	4	8	16
	L	Filmy	1	2	4
		Dense	4	8	16
TUBE	R	Filmy	1	2	4
		Dense	4*	8*	16
	L	Filmy	1	2	4
		Dense	4*	8*	16

* If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16.

been grouped together and are often referred to as 'stage A' disease. Similarly, moderate and severe stages of disease (stage III - IV) have been grouped together and referred to as 'stage B' disease.

Treatment of endometriosis

The primary treatment for endometriosis is surgical excision. Most drug treatments rely on the fact that endometriotic tissue becomes atrophic in response to low circulating oestrogen or high androgen concentrations. The treatments can be divided into four main groups: Danazol, gonadotropin-releasing hormone (GnRH) agonists, progestagen only regimes and combined oestrogen-progestagen regimes. Danazol is a testosterone derivative (Figure 1.3) and exerts its action in multiple ways. It binds to androgen receptors and acts as an androgen agonist (*i.e.* stimulates receptor system pathways) and it also binds to progesterone receptors and acts as both a progesterone agonist and antagonist. Danazol binds to sex hormone binding globulin (SHBG) with a higher affinity than testosterone resulting in higher concentrations of free testosterone, which is the reason for the androgenic side effects of this drug. Danazol also acts by inhibiting enzymes involved in steroidogenesis, by suppressing oestrogen production in the ovaries. Thus, the net effect of danazol is to decrease oestrogen levels and increase androgen levels which in turn causes atrophy in endometriotic implants.

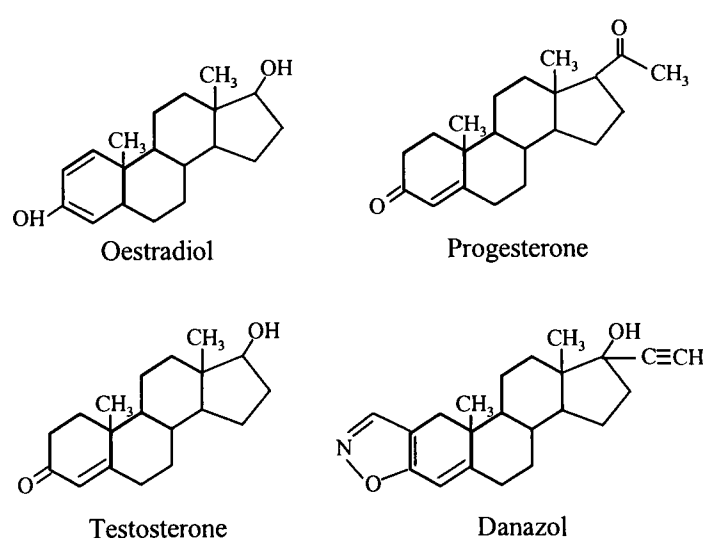


Figure 1.3. Structures of oestradiol, progesterone, testosterone and danazol.

GnRH is a 10 residue peptide (Figure 1.4) released by the hypothalamus which controls the secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) by the pituitary. GnRH has a short half-life because of its susceptibility to degradation by endopeptidases. However, the half-life of synthetic forms of the peptide, e.g. nafarelin

and buserelin, is considerably longer due to their resistance to these enzymes. Nafarelin and buserelin differ from GnRH by only one amino acid (Figure 1.4) and they bind with high affinity to GnRH pituitary receptors, thereby acting as GnRH agonists. Initial treatment results in an upregulation of LH and FSH production, followed by desensitisation causing a decrease in LH, FSH and oestradiol concentrations.

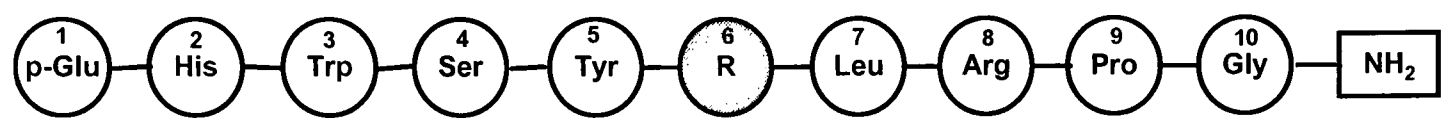


Figure 1.4. Amino acid sequence of gonadotropin releasing hormone (GnRH) (R=glycine). In synthetic agonists such as nafarelin (R=D-naphthylalanine) and buserelin (R=CH₂OC(CH₃)₃) the glycine at position 6 is replaced by a different group.

Finally, progestagens either combined with oestrogen or used alone, may be used to inhibit LH and FSH production, thus decreasing ovarian oestrogen production and causing decidualisation of the endometrium. The method of action of agents used to treat endometriosis illustrates the important role of oestrogen in maintaining this disease.

Prevalence

There are inevitable difficulties in assessing the prevalence of endometriosis. Firstly, the disease can only reliably be diagnosed at open or laparoscopic surgery. This makes it impossible to calculate the prevalence in a sample chosen randomly from the general population because it would not be ethical to subject such women to an unnecessary surgical procedure. More recently, MRI has been successfully used to diagnose endometriosis, but the limits of this technique may preclude the diagnosis of small (<1cm) endometriotic implants. The availability of MRI has opened up the possibility of scanning a group of representative women, although to date such a study has not been conducted.

Estimating the prevalence based upon the number of women surgically diagnosed has also been problematic mainly because not all women with endometriosis will have had a surgical diagnosis. Some gynaecologists will decide to treat the disease empirically without a surgical diagnosis and other women with endometriosis will simply not have received a diagnosis, either because they have not reported their symptoms to a doctor,

or because of the doctor’s failure to diagnose it. The accuracy of any surgical diagnosis may vary from practitioner to practitioner. More thorough examinations of the peritoneal cavity to find small endometriotic lesions may be conducted in symptomatic women compared to controls (*i.e.* women undergoing sterilisation), biasing prevalence estimates. Another consideration is that the prevalence of endometriosis may be related to age hence the importance of knowing the mean age of women in studies. The chance of being accurately diagnosed is almost certainly higher now than it was 10 or 20 years ago, which gives the impression that the incidence is rising. Finally, it is likely that all women have a degree of endometriosis at some time in their reproductive lives and it is therefore important to take into account the severity of disease in prevalence studies.

There have been a number of studies attempting to determine the prevalence of endometriosis, but their accuracy is questionable because of the factors listed above. Many of these studies report the prevalence of disease in women undergoing laparoscopy for tubal sterilisation. These studies are therefore based upon a biased sample of mostly fertile women; a major flaw in a disease known to be associated with infertility. However, these data may provide some idea of the frequency in the normal population. The prevalence observed in these studies has varied between 2% and 43%; these data are summarised in Table 1.5. Only studies containing more than 50 subjects were included in this analysis.

Table 1.5. The prevalence of endometriosis in women undergoing tubal sterilisation.

Reference	Number studied	Number affected	%
Dodge <i>et al.</i> , 1986	76	14	18.4
Kirshon & Poindexter, 1988	566	42	7.4
Kresch <i>et al.</i> , 1984	50	7	14.0
Liu & Hitchcock, 1986	75	32	42.7
Mahmood & Templeton, 1991	598	37	6.2
Moen, 1987	108	19	17.6
Moen, 1991	206	42	20.4
Moen & Muus, 1991	107	24	22.4
Sangi-Haghpeykar & Poindexter, 1995	3384	126	3.7
Strathy <i>et al.</i> , 1982	200	4	2.0
Trimbos <i>et al.</i> , 1990	200	5	2.5

The prevalence of moderate to severe stage endometriosis (stage B) in the seven studies presented in Table 1.5 was much lower at only 0.5% when combined (range 0% - 2.9%). A consistent prevalence for stage B disease across all of these studies is in contrast to the prevalence when all stages of disease are included. This highlights the fact that many women have a small amount of endometriosis detectable by skilled surgeons. Whilst it is likely that these prevalence figures represent a slight underestimate of the true frequency, due to the bias towards women with proven fertility, one of the authors (Moen, 1991) was able to demonstrate that the group of women studied closely resembled a normal population. The conclusion was based on the fact that, in Norway, laparoscopic sterilisation is universally available to women over the age of 25 years free of charge. In addition, previous studies had shown no significant difference between social characteristics of the women studied and the female population in general, and no significant difference was observed between the number of births in the women studied compared to the general population.

The prevalence of endometriosis has also been assessed in groups of women reporting infertility. In a study of 100 infertile patients 21% had endometriosis, and 10% had moderate to severe disease (Strathy *et al.*, 1982). Another study found a similar prevalence in 654 infertility patients with 133 (20%) cases of endometriosis of whom 34 (5%) had stage B endometriosis (Mahmood & Templeton, 1991). In a group of 156 women with pelvic pain, a prevalence of 15% was observed, with 2% having stage B endometriosis (Mahmood & Templeton, 1991).

Population studies of the number of women with surgically diagnosed endometriosis, have also been conducted. Moen & Schei, 1997 found that in Norwegian women aged 40-42 the self-reported prevalence of diagnosed endometriosis was 2% (79/4034) and the surgically diagnosed prevalence was 1.1% (43/4034). There have also been several estimates of the incidence of endometriosis from hospital-based studies ranging from 1.8% - 10.3% (reviewed by Eskenazi, 1997).

It can be concluded that the population prevalence of endometriosis remains unknown, and that the frequency in women of reproductive age varies enormously from study to study when all stages of endometriosis are included. However, the prevalence of stage

B disease, which varies less between studies, can be estimated at 0.5% of women undergoing tubal sterilisation.

Risk factors for endometriosis

There have been a large number of studies investigating possible risk factors for endometriosis, but their findings have often been contradictory. This is, in part, due to bias in the choice of cases and controls investigated. Nevertheless, there have been several consistent risk factors identified in these studies, including age and exposure to menstruation.

Age is an element which has reproducibly been shown to play an important role in endometriosis and is probably the most significant factor identified to date. The risk of endometriosis in women attending a family planning clinic aged between 40-44 years was 6 times higher than for women in the 25-29 age group (Vessey *et al.*, 1993). Another study found that women aged 36 years or over were twice as likely to be diagnosed with endometriosis at the time of tubal sterilisation compared to women under 25 years (Sangi-Haghpeykar & Poindexter, 1995). It can be concluded from these and other studies that the risk of endometriosis increases with age, and does not begin to decline until after the age of 44 (Houston *et al.*, 1988). However, it is interesting to note that no correlation between age and severity of disease has been observed.

Apart from age, no other sociodemographic characteristics have been reproducibly related to endometriosis. Most studies investigating the prevalence of endometriosis have focused on Caucasian women and there is a lack of data on the frequency of the disease in other races. Several studies have demonstrated no difference in the frequency of endometriosis in black compared to white women, and two studies have suggested higher prevalences in Asian and Japanese women (Eskenazi & Warner, 1997, Houston *et al.*, 1988). Another study demonstrated that the prevalence of endometriosis was 51% (104/202) in Asian infertility patients from Kuala Lumpur compared to 22% (101/464) in Caucasian infertility patients from Aberdeen (Arumugam & Templeton, 1992). Lastly, the prevalence of disease has been shown to be very low (1.1%) in an ultra-orthodox Jewish population (Bocker *et al.*, 1994).

One of the most striking observations in epidemiological studies of endometriosis is the positive relationship with exposure to menstruation and therefore menstrual debris. The hypothesis put forward to explain this relationship is that the total exposure of the peritoneum to menstrual debris through retrograde menstruation is increased if menstruation is more frequent, longer in duration or heavier. Increased exposure may also occur because a woman has not been pregnant, if she does not breast feed or if there is a long delay between pregnancies or before the first pregnancy. A study of 75 women undergoing laparoscopic tubal sterilisation at the time of menstruation found a relationship between endometriosis and retrograde menstruation. Thirty-two (43%) women were found to have endometriosis and retrograde spillage was observed in 31/32 (97%) compared to only 26/43 (60%) women without endometriosis (Liu & Hitchcock, 1986). Other studies have shown that women with endometriosis are more likely to have a menstrual flow lasting ≥ 6 days per month and a heavy menstrual flow (Eskenazi & Warner, 1997, Sangi-Haghpeykar & Poindexter, 1995, Vercellini *et al.*, 1997).

Fewer pregnancies and length of time since last pregnancy, both factors which increase exposure to menstruation, have also been associated with endometriosis. Women with fewer than two live births had an increased risk of endometriosis when compared to controls ($P = 0.001$). Women in whom ≥ 6 years had elapsed since their last pregnancy were at increased risk ($P = 0.001$), and a significant trend was observed between increased time since last childbirth and risk of endometriosis ($P = 0.016$). Lastly, in three studies the age at first childbirth for cases was not significantly different from controls (Moen, 1987, Moen, 1991, Sangi-Haghpeykar & Poindexter, 1995). This finding may in part be due to the fact that patients were undergoing tubal sterilisation and formed a biased sample of the population of women with endometriosis.

It could be expected that exposure to oral contraceptives could either increase the risk of endometriosis, due to increased hormone concentrations, or decrease the risk due to reduced exposure to menstruation. Interestingly, three separate studies have failed to find a significant difference in oral contraceptive use amongst women with endometriosis compared to controls (Kirshon & Poindexter, 1988, Moen, 1987, Vessey *et al.*, 1993). However, Vessey *et al.* observed that the relative risk of endometriosis was low in women who were currently taking oral contraceptives (relative risk 0.4)

compared to women who had taken oral contraceptives more than 4 years previously (relative risk 1.8). These risk values were calculated relative to those women with endometriosis who had never taken oral contraceptives. These data may represent a trend towards past oral contraceptive use as a risk factor for endometriosis, and current use as a protective factor; however, the difference was not significant and sample sizes were small. In another study, multivariate analysis confirmed a significant protective effect of current oral contraceptive use ($P = 0.03$) (Sangi-Haghpeykar & Poindexter, 1995). In this analysis, oral contraceptive users were compared to women using no method of contraception, which introduces the possibility of biasing the data towards infertile women. If this comparison had been made with women using another form of contraception the difference might have been less significant. The odds ratios for endometriosis reported in this study were 0.4 for the oral contraceptive, 0.6 for other methods of contraception and 1.0 for no contraception. Another study demonstrated a significant elevation in the mean length of time oral contraceptives were taken by women with endometriosis compared to controls (Mahmood & Templeton, 1991). The study was based on large sample sizes (227 cases and 1315 controls); however, cases and controls were not matched which may have introduced bias. It is difficult to study this factor in relation to endometriosis as some women would have been prescribed the oral contraceptive for non-contraceptive purposes, such as to treat menorrhagia or amenorrhoea. Intra-uterine contraceptive device (IUCD) use has also been analysed in several studies (Kirshon & Poindexter, 1988, Mahmood & Templeton, 1991, Matorras *et al.*, 1995, Moen & Muus, 1991, Sangi-Haghpeykar & Poindexter, 1995, Vessey *et al.*, 1993) and no significant effect observed in all but one (Sangi-Haghpeykar & Poindexter, 1995).

A large number of other variables have been investigated as possible risk factors for endometriosis although results tend to be conflicting. A protective effect of smoking one or more packs per day before the age of 17 was shown in one study (Cramer *et al.*, 1986), and three subsequent studies failed to reproduce any association with smoking (Matorras *et al.*, 1995, Moen & Schei, 1997, Sangi-Haghpeykar & Poindexter, 1995). Cramer *et al.* (1986) also observed that over three hours exercise per week started before the age of 26 had a protective effect. Both smoking and exercise may lower oestradiol concentrations (Baker *et al.*, 1981, Bonen *et al.*, 1981), and it has been postulated that this may be the mechanism behind their protective effect against

endometriosis. An association between caffeine consumption and the presence of endometriosis has been observed and in young women and it has been shown that endometriosis is inversely related to waist-to-hip ratio (Eskenazi & Warner, 1997).

While many studies investigating risk factors in endometriosis have been conducted, only a few have been carried out in an unbiased way with adequate controls. The only clear risk factors for endometriosis in women which have emerged to date are age and increased exposure to menstruation.

Endometriosis and ovarian cancer

There have been a number of observations which provide evidence that a relationship between ovarian cancer and endometriosis exists. For example, it has been reported that ovarian endometriosis occurs in epithelial ovarian cancer in 15% of Japanese women (Jimbo *et al.*, 1997b). The authors observed that the frequency of endometriosis in ovarian cancer was 41% for clear cell and 23% for endometrioid cell types. Another USA based study reported that 28/91 (31%) of patients with endometrioid adenocarcinoma of the ovary had endometriosis. There have been a number of other studies reporting a similar relationship (for example Erzen & Kovacic, 1998, Heaps *et al.*, 1990, Kline *et al.*, 1990, McMeekin *et al.*, 1995, Sainz de la Cuesta *et al.*, 1996, Toki *et al.*, 1996, Vercellini *et al.*, 1993). The co-existence of endometriosis and ovarian epithelial cancer may be due to the common risk factors the diseases share such as infertility, age and hormonal effects. However, it has also been proposed that malignant transformation of endometriosis into ovarian cancer may occur and allelotyping of adjacent endometriosis and ovarian cancer has revealed evidence of a common lineage (Jiang *et al.*, 1998).

Other types of cancer have also been associated with endometriosis. It has been shown that the risk of ovarian cancer, breast cancer and non-Hodgkin's lymphoma was significantly elevated following discharge from hospital with a diagnosis of endometriosis (Brinton *et al.*, 1997). This apparent relationship between endometriosis and increased cancer risk again may result from common hormonal, immune or genetic risk factors acting singly or in combination.

Although endometriotic tissue is regarded as a benign tumor, it has a number of features in common with malignant tumours: endometriosis is oestrogen dependent, it tends to recur and it exhibits progressive, invasive growth. It has also been shown that endometriotic cysts are monoclonal in origin, and may arise from a single progenitor endometrial cell (Jimbo *et al.*, 1997a). The majority of normal endometriotic glands, however, have also been shown to be monoclonal (Jiang *et al.*, 1996).

Loss of heterozygosity in endometriosis at candidate ovarian tumour suppressor gene loci has also been described. Loss of heterozygosity was detected on chromosomes 9p (18%), 11q (18%) and 22q (15%) (Jiang *et al.*, 1996). These are all sites at which loss of heterozygosity in endometrioid ovarian cancer has also been reported. The authors highlighted the need for further studies to investigate whether the affected regions include genes which may be involved in the initiation and development of endometriosis.

Lastly, Shin *et al.* (1997) reported chromosomal aberrations in a small number of endometriotic samples, with significant increases in the number of cells with monosomy for chromosome 16 observed in two out of four patients. Increased monosomy for chromosome 17 and trisomy for chromosome 11 was also observed in isolated cases. Interestingly, candidate tumour suppressor genes have been mapped to all three of these chromosomes. More extensive studies are required to investigate whether aneuploidy is present in endometriosis more often for certain chromosomes.

CA125

A relationship between CA125 and endometriosis has also been observed. CA125 is an antigen recognised by a monoclonal antibody raised using an ovarian cancer cell line originating from a serous cystadenoma. This antibody recognises an epitope on a glycoprotein closely related to the Lewis blood group antigens. CA125 is expressed by a variety of tissues, including normal endometrium, and is also present in the serum of women without endometriosis. It has been shown that the endometrium is the main source of circulating CA125 and levels are correlated to the growth activity of this tissue.

Particularly high serum concentrations of CA125 (over 35 IU/ml) have been detected in the majority of women with ovarian cancer. The levels of CA125 are significantly raised in women with stage B endometriosis and this finding has been reproduced by a number of investigators. The performance of CA125 measurement in the detection of endometriosis has been assessed in a meta-analysis of 23 published studies (Mol *et al.*, 1998). It has a limited role in diagnosing all disease stages but for an 89% specificity the test has 47% sensitivity to detect stage B disease.

In the foetus, the CA125 antigen is present in coelomic epithelium and in the Müllerian ducts, which are eventually derived from this tissue. The Müllerian ducts give rise to the fallopian tubes, the uterus, the cervix and the upper vagina later in gestation. Other tissues which are derived from the coelomic epithelium, and therefore also express CA125, include the pleura, pericardium, peritoneum and ovarian surface epithelium. It has been proposed that tissues derived from the coelomic epithelium have the potential to differentiate into Müllerian-directed epithelium and stroma (Fujii, 1991). The presence of CA125 on these tissues, in the foetus and the adult, provides evidence for the metaplastic theory of endometriosis.

1.4 EVIDENCE FOR AN ENVIRONMENTAL ROLE IN DISEASE DEVELOPMENT

Several environmental agents have been implicated in the development of endometriosis. Most of these studies have been conducted in non-human primates, including the baboon (*Papio* species) and the rhesus macaque (*Macaca mulatta*). These species provide a good model for human endometriosis because they develop the disease spontaneously; the disease is morphologically identical to the human disease and there is a high degree of homology between non-human primates and humans at the genetic level.

An increase in the prevalence of endometriosis amongst macaques fed a diet containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) compared to those fed a normal diet has been reported (Rier *et al.*, 1993). The exact mechanism of dioxin action remains to be elucidated, however it is known to be a carcinogen and has an anti-oestrogenic effect. In support of these findings in non-human primates, there is a high occurrence of endometriosis in Belgium, where it has been noted that there are also

high levels of dioxin pollution (Koninckx *et al.*, 1994). Another study is currently underway to investigate whether there is a higher than expected prevalence of endometriosis in Seveso, Italy following high level dioxin exposure due to an accident in 1976 (Bois & Eskenazi, 1994). The relationship between endometriosis and dioxin is presented in greater detail in Chapter Two.

It has also been reported that exposure to ionizing radiation increased the prevalence of endometriosis in the rhesus macaque in a dose-dependent manner (Fanton & Golden, 1991, Wood, 1991, Wood *et al.*, 1983). Other environmental factors for which a relationship with endometriosis has been discovered in a non-human primate model include diethylstilbestrol (DES) exposure *in utero*, exposure to hysterotomy and exposure to oestradiol implants (Hadfield *et al.*, 1997b, Hendrickx *et al.*, 1988).

The mechanisms behind the involvement of environmental factors in the development of endometriosis is uncertain. These factors may play an indirect role in disease development by, for example, altering the immune response to ectopic endometrial cells. Alternatively, a more direct mechanism may be involved, for instance the environmental agent may act directly to cause somatic genetic damage.

1.5 EVIDENCE THAT ENDOMETRIOSIS IS A GENETIC DISEASE

There are numerous case studies in the literature which report familial aggregation of endometriosis. The largest of these presented a collection of 100 families with two or more affected members (Kennedy *et al.*, 1995). There are also case reports of endometriosis in monozygotic twins. Moen reported that amongst 515 patients with endometriosis, 8 reported having a monozygotic (MZ) twin sister and two a dizygotic (DZ) twin sister. Of the MZ pairs, 6/8 (75%) were also affected by endometriosis but neither of the DZ pairs were concordant (Moen, 1994). A collection of 16 MZ twin sisters, of whom 88% were concordant for endometriosis and 56% were concordant for stage B disease, has also been published (Hadfield *et al.*, 1997a).

Recently, a more comprehensive study of twin-pairs in Australia has been carried out (Treloar *et al.*, 1999). The authors surveyed over 3,000 twin sisters and found that the prevalence of concordance for surgically confirmed endometriosis amongst MZ twins

was 2.1% (18/841) and 1.6% (3/485) for DZ twins. In comparison, the frequency of discordance (*i.e.* one twin affected and the other not) was 10.3% (87/841) for MZ and 9.9% (49/485) for DZ twins. The tetrachoric twin pair correlations for surgically confirmed endometriosis were 0.52 ± 0.08 for MZ and 0.19 ± 0.16 for DZ twins. These results provide substantial evidence for the involvement of a genetic component in the development of endometriosis and Treloar *et al.* suggest that there is evidence for at least 51% of the variance in latent liability to endometriosis being of genetic origin.

An increased prevalence of disease has been noted in the first-degree relatives (*i.e.* mothers, sisters and daughters) of affected women in a number of studies. One problem with these studies has been defining a suitable control population and, although several different strategies have been adopted, all have shortcomings.

One study used a control group made up of the relatives of patients' best friends, while another used the relatives of the patients' husbands. Two studies have used a control group made up of the relatives of women with a normal pelvis. Ideally, the prevalence in first-degree relatives would be compared to an established population prevalence, however, as discussed earlier in this chapter, these data are unavailable for endometriosis.

In a study of 43 American women with endometriosis, Lamb *et al.* found that 34.9% of mothers and 21.2% of sisters of affected women had the disease. In comparison, for the control group of patients' best friends, 4.6% of mothers and 1.8% of sisters were affected. The relative risk, calculated in comparison to the controls, was 11.6 for a sister of an affected woman, 7.5 for the mother of an affected woman and 8.9 for mothers and sisters combined. Although some women had daughters, the majority were under 12 years old and, therefore, these data were excluded from the analysis. For second-degree relatives (*i.e.* grandmothers, aunts, nieces and granddaughters) the risk was 5.1, approximately one half of the risk of 8.9 for a first-degree relative (Lamb *et al.*, 1986). This halving of risk is what one would expect for a genetic disease, as second-degree relatives share an expected 25% of genes compared to first-degree relatives who share 50%. The severity of disease in these patients was not presented.

Coxhead and Thomas (1993) studied 64 first-degree relatives of women with endometriosis in a British population and compared the prevalence of the disease to a control group consisting of the 128 first-degree relatives of women with a normal pelvis. This group found that the relative risk for mothers and sisters of women with endometriosis was 6.0 (Coxhead & Thomas, 1993).

Another study found a similar increased risk for relatives of 515 affected women in a Norwegian population (Moen & Magnus, 1993). In this study, the control group consisted of 149 women with a normal pelvis, determined at laparoscopy. The authors found that the relative risk of endometriosis and/or adenomyosis was 8.4, 6.0 and 7.2 respectively for sisters, mothers, and mothers and sisters combined. It was also noted that women with a positive family history of endometriosis/adenomyosis were significantly more likely to have severe endometriosis compared to those with a negative family history ($P < 0.01$). The risk of endometriosis in first-degree relatives of the 203 women with stage B disease only was 8.6 (Moen, personal communication).

In an earlier USA based study, using the relatives of affected women's husbands as controls, the same trend was observed. Of 123 patients, 9/153 (5.8%) sisters and 10/123 (8.1%) mothers had endometriosis. In the control group, only 1/104 (1.0%) sisters and 1/107 (0.9%) mothers were affected. Thus, the relative risk of endometriosis was 6.1 in sisters, was 8.7 in mothers and 7.3 in all first-degree relatives (Simpson *et al.*, 1980). The authors also observed that 62% of women with an affected relative had severe endometriosis compared to only 23% of women without an affected relative.

A more accurate assessment of the prevalence of endometriosis amongst the first degree relatives of women with the disease could be obtained using MRI. Such a study has been conducted, and 56 relatives of 29 women with stage B endometriosis were MRI scanned. The prevalence of endometriosis was 5/35 (15%) in first-degree relatives and 1/12 (8%) in more distant relatives (Kennedy *et al.*, 1998). No control group of normal women who have undergone pelvic MRI scans was available for comparison. This study is presented in detail in Chapter Four.

These data are summarised in Table 1.6. The relative risk values in a sister, compared to the general population (λ_s) and the risk in a first-degree relative (λ_R) are tabulated.

As the population frequency of endometriosis is unknown, the values from the respective controls were used to calculate the risk.

λ_S	λ_R	Reference
6.1 (0.8 - 49.0)	7.3 (1.7 - 31.5)	(Simpson <i>et al.</i> , 1980)
-	6.0 (1.2 - 30.6)	(Coxhead & Thomas, 1993)
8.4 (1.6 - 45.1) [§]	7.2 (2.1 - 24.3) [§]	(Moen & Magnus, 1993)
11.6 (1.5 - 93.4)	8.9 (2.6 - 30.5)	(Lamb <i>et al.</i> , 1986)
15.0 [†] (5.1 - 31.9)	14.0 [†] (4.8 - 30.3)	(Kennedy <i>et al.</i> , 1998)

Table 1.6. The risk of endometriosis in the sisters (λ_S), and first-degree relatives of affected women (λ_R) compared to controls. The 95% confidence intervals are given in parentheses. [§]Disease defined as endometriosis and/or adenomyosis. [†]Included probands with stage B disease only and compared the frequency in relatives to an estimated population disease frequency of 1%.

The true λ_S and λ_R for endometriosis are not known, and there are inherent difficulties in calculating these values. For example, the age at which relatives should be included in these figures is debatable as a young woman found to have a normal pelvis at laparoscopy may later go on to develop the disease. Another consideration is that women who have a relative with disease may be more likely to get a diagnosis than those without an affected relative. Finally, many of the factors which make the prevalence of endometriosis difficult to determine also apply here, such as the fact that the diagnosis must be made surgically and that minimal endometriosis may not represent a disease state.

Nevertheless, collectively, these reports provide strong evidence that endometriosis has a genetic component and give an indication of the order of magnitude of λ_S and λ_R . As shown in Table 1.6, it is likely that first-degree relatives of a woman with endometriosis are at a 6-9 times increased risk of developing the disease themselves.

The increased risk in first-degree relatives of endometriosis patients is comparable to the increased risk seen in the first-degree relatives of patients with other complex traits such as rheumatoid arthritis and insulin dependent diabetes mellitus (IDDM). For comparison, λ_R values for other diseases are presented in Table 1.7.

Disease	λ_R	Reference
Breast cancer	2.0	(Unic <i>et al.</i> , 1997)
Prostate cancer	2.4	(Spitz <i>et al.</i> , 1991)
Rheumatoid arthritis	4.4	(Koumantaki <i>et al.</i> , 1997)
Insulin dependent diabetes mellitus	9.6 [§]	(Lorenzen <i>et al.</i> , 1994)
Inflammatory bowel disease	10.0 [†]	(Orholm <i>et al.</i> , 1991)

Table 1.7. The relative risk (λ_R) of disease in a first-degree relative of a patient diagnosed with the disease. For comparison, independent studies have suggested that λ_R for endometriosis is between 6-9. [§]Life-time risk up to age 60. [†]Relative risk in siblings only (λ_S).

Other evidence that endometriosis is likely to have a genetic component includes the fact that adenomyosis also appears to have a familial tendency (Arnold *et al.*, 1994, Arnold *et al.*, 1995). Adenomyosis is defined as the presence of endometrial glands and stroma within the myometrium. It may represent another manifestation of endometriosis or it may be a separate disease.

There have also been reports of a preponderance of women with red hair diagnosed with endometriosis (Woodworth *et al.*, 1995), and scoliosis appears to be common among patients (Cramer *et al.*, 1996b) compared to controls. Endometriosis has been associated with dysplastic naevi and melanoma (Hornstein *et al.*, 1997). Several investigations have been conducted into whether there is a relationship between HLA type and endometriosis and there was no significant difference compared to population frequencies (Maxwell *et al.*, 1989, Simpson *et al.*, 1984). More recently, association between endometriosis and polymorphisms and mutations in a number of genes has been described (Baranov *et al.*, 1996, Baranova *et al.*, 1997, Baranova *et al.*, 1999, Cramer *et al.*, 1996b). These association studies are introduced and discussed in detail in Chapter Six.

In conclusion, it seems likely that endometriosis is a complex, multifactorial trait with several different genes interacting either with each other and/or the environment to produce the disease phenotype.

1.6 AIMS OF THIS WORK

The work presented in this thesis has involved studies into the genetic and environmental basis of endometriosis with the principal aim of identifying susceptibility genes for the disease. The records from a large colony of rhesus macaques with a naturally high prevalence of endometriosis were examined to discover whether environmental and/or familial factors play a role in disease development. In women, families were identified for the Oxford Endometriosis Gene (OXEGENE) study, blood samples collected and DNA isolated. The goal of this study was to genotype all individuals at multiple microsatellite loci across the entire genome, conduct sib-pair analysis and identify regions where there is statistical evidence for nearby existence of predisposing loci. To complement this approach a number of candidate gene loci were also studied in these families and in another independent set of cases and controls.

1.7 CHAPTER SUMMARY

The work presented in this thesis includes studies on the nature of endometriosis in both humans and the rhesus macaque. Studies in the animal model are included in Chapters Two and Three, and human studies in Chapters Four to Six. Studies on the environmental basis of endometriosis in the rhesus macaque are presented in Chapter Two. The suitability of this non-human primate species as an animal model, and existing evidence for an environmental role in disease development, are discussed in detail. By systematic audit of the necropsy reports of female animals from a large colony of captive macaques, potential risk factors for endometriosis such as age, hysterotomy and oestradiol treatment were investigated. In Chapter Three the familial nature of endometriosis in this species is explored. MRI scans were conducted to identify living animals with disease and segregation analysis performed in the pedigrees identified. Chapter Four includes studies on the familial nature of endometriosis in women and describes the patients and families collected for the OXEGENE study. In Chapter Five the results of association studies in groups of affected individuals and

groups of controls are presented. Four different candidates are investigated: the galactose-1-phosphate uridyl transferase N314D polymorphism, the glutathione-S-transferase M1 and T1 null mutations and the cytochrome P450 1A1 MspI polymorphism. In Chapter Six the results of sib-pair analysis, using a set of microsatellite markers spanning chromosome one, are presented. Lastly, Chapter Seven contains a general summary of the results presented in this thesis.

Chapter Two

Risk factors for endometriosis in the rhesus macaque (Macaca mulatta)

2.1 INTRODUCTION

There is increasing evidence that endometriosis is likely to be a complex trait, with both genetic and environmental factors contributing to the disease phenotype. Familial clustering of endometriosis has been observed, but to what degree this clustering can be attributed to environmental factors is unknown. As well as sharing many genes, siblings also tend to experience very similar environments by living in the same location, eating foods from the same source etc. Separating the environmental and genetic factors from each other is difficult and would ideally take the form of a large cohort twin study comparing monozygotic and dizygotic twins. A useful alternative is to examine an animal model such as the rhesus macaque (*Macaca mulatta*). Endometriosis occurs spontaneously in this species and animals kept in large colonies, such as at the Wisconsin Regional Primate Research Center (WRPRC), provide an ideal opportunity for such a study as the animals have been exposed to a well-characterised environment. The WRPRC has constantly housed a population of over 800 rhesus macaques since 1972 and comprehensive records of the animals' lives and the findings at necropsy are available.

Non-human primates as a model for the study of human endometriosis

Most non-human primates have regular menstrual cycles, external menstruation and similar gynaecological anatomy to women. Cases of spontaneous endometriosis have been described in many non-human primate species in both captivity and the wild (Table 2.1). The disease is morphologically and histologically identical to the human disease (MacKenzie & Casey, 1975) and hence non-human primates, particularly the rhesus macaque and the baboon (*Papio* species), have been utilised for endometriosis research.

Table 2.1. Species of non-human primates in which spontaneous endometriosis has been reported. The reference list is not exhaustive.

Species	Common Name	Literature Reference
<i>Cerocebus albigena</i>	Grey-cheeked mangaby	(Schmidt & Hartfiel, 1978)
<i>Ceropithecus aethiops</i>	African green	(Cary <i>et al.</i> , 1982)
<i>Ceropithecus neglectus</i>	De Brazza’s monkey	(Binhazim <i>et al.</i> , 1989)
<i>Gorilla gorilla</i>	Gorilla	(Dore & Lagace, 1985)
<i>Macaca cyclopis</i>	Taiwan rhesus	(Chin, 1994)
<i>Macaca fascicularis</i>	Cynomolgus monkey	(Fanton & Hubbard, 1983)
<i>Macaca mulatta</i>	Rhesus monkey	(McCann & Myers, 1970)
<i>Macaca nemestrina</i>	Pigtailed macaque	(Waterton <i>et al.</i> , 1992)
<i>Papio anubis</i>	Olive baboon	(D’Hooghe <i>et al.</i> , 1991)
<i>Papio cynocephalus</i>	Yellow baboon	(Cornillie <i>et al.</i> , 1992)
<i>Papio doguera</i>	Kenya baboon	(Folse & Stout, 1978)
<i>Papio hamadryas</i>	Hamadryas baboon	(Shalev <i>et al.</i> , 1992)

The rhesus macaque as a model for the study of human endometriosis

Spontaneous endometriosis in the rhesus macaque has been observed both in the wild and in captivity. The disease occurs particularly in colonies of captive rhesus (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) (Ami *et al.*, 1993, McCann & Myers, 1970).

Rhesus macaques with endometriosis may show signs of abdominal discomfort, anorexia and constipation at the time of menstruation. The disease is often severe, may involve all the abdominal regions and occasionally results in death due to bowel obstruction (Schaerdel, 1986). Rhesus macaques have also been used as a model for experimentally induced endometriosis, initiated by surgical transplantation of endometrial tissue into the peritoneum, which is usually mild. However, the studies presented in this thesis are only of spontaneously occurring disease.

Reproductive biology of the rhesus macaque

The macaques are an Old World species and members of the family cercopithecidae. This family contains nine genera which can be divided into three groups:

- a) African long-tailed monkeys

b) Mangabeys

c) Macaques, baboons and baboon-like monkeys

In the wild, the rhesus macaque is distributed across central Asia (from Afghanistan to China). The animals live in large, multi-male and female groups which tend to have a majority of females. They move in an integrated group and travel large distances daily.

Menarche occurs in the rhesus macaque at approximately 2-3 years of age. Sexual maturity is reached between 2.6-3.5 years and the first pregnancy is usually between 3-5 years. The menstrual cycle length is 26-30 days with menstruation lasting 4-5 days. In the wild, and when housed in captivity outdoors, the rhesus macaque is a seasonal breeder with births occurring during Spring (March to June). The length of gestation is 165-175 days (*i.e.* 23-25 weeks), normally giving rise to a single offspring, and infants are weaned at 7-14 months. The inter-birth interval for feral animals is usually one year. Rhesus macaques frequently continue to menstruate until death in the wild and, although cyclical irregularity may occur at 25-30 years, few appear to reach a menopause. The average life span is 29 years, with some animals living well into their late 30's (Hendrickx & Dukelow, 1995).

Rhesus macaques have a close phylogenetic relationship with humans which is reflected at the genetic level. Rhesus monkeys have a diploid chromosome number of 42, in comparison to 46 in humans, and a chromosomal banding pattern which corresponds closely. There is 92.7% sequence homology with humans in noncoding DNA sequences.

Studies of spontaneous endometriosis in non-human primates

The prevalence of endometriosis in a large colony of rhesus monkeys has not been reported previously. However, there are reports of the frequency in groups of baboons and cynomolgus monkeys (*Macaca fascicularis*) and in small groups of rhesus macaques.

In a group of 52 captive baboons, 13 (25%) were found to have minimal endometriosis (D'Hooghe *et al.*, 1991) staged using an adapted version of the revised AFS classification system (The American Fertility Society, 1985); histological

confirmation was obtained in 75% of cases. In an investigation of 94 female cynomolgus monkeys, 21 (22%) had endometriosis (excluding cases with adenomyosis alone) (Ami *et al.*, 1993). Affected animals were aged between 11-23 (mean 15.3) years and all had ovarian endometriosis >1cm, often with accompanying adenomyosis.

In another study, gynaecological pathology in 38 captive rhesus macaques (mean age approximately 8.5) was compared with 17 free-ranging animals (mean age approximately 8) (DiGiacomo, 1977). Twelve of the captive animals (32%) had endometriosis compared with two (12%) of the free-ranging animals. It was also observed that the 12 captive animals had more severe disease than the two free-ranging animals which both had minimal - mild disease.

In a further group of 35 female rhesus monkeys, 9 (26%) cases of endometriosis were identified (Bertens *et al.*, 1982). Finally, two other reports have discussed the prevalence of endometriosis in infertile rhesus macaques; 16/95 (17%) infertile animals (only some of which were selected for investigation) had endometriosis in one study (Rippy *et al.*, 1996), and 21/63 (33%) in the other (McCann & Myers, 1970).

Length of time in captivity as a risk factor for endometriosis

It is possible that duration of captivity increases the chance of an animal developing the disease. There are a number of potential explanations for this including increased stress, fewer pregnancies and increased frequency of menstruation (which would provide greater opportunity for retrograde menstruation). In a study of 104 baboons housed in captivity for varying lengths of time it was found that animals in captivity for more than two years were significantly more likely to have endometriosis than those captive for less than one year ($P = 0.03$) (D'Hooghe, 1994, D'Hooghe *et al.*, 1996) although these animals were not matched for age. In rhesus monkeys, those in captivity for between 1 - 10.5 (mean 4.5) years were significantly more likely to have endometriosis than free-ranging animals (DiGiacomo, 1977); the mean ages of the two groups were not significantly different. It is likely, therefore, that captivity increases the chance of developing the disease. However, it is not clear whether the

cause is the environment of captivity or experimental procedures such as hysterotomy, laparoscopy and drugs to which animals might have been exposed.

Hysterotomy as a risk factor for endometriosis

Previously, in three small, rhesus macaque studies, hysterotomy (*i.e.* any incision in the uterus, including caesarean section, often conducted to facilitate experimental procedures e.g. foetal studies) and treatment with ovarian steroids were identified as possible risk factors for endometriosis. In the first study of 21 rhesus monkeys with endometriosis, 91% had undergone one or more hysterotomies (McCann & Myers, 1970). In the second study, 6 out of 9 (67%) rhesus monkeys with endometriosis had had one or more caesarean sections, of these, 5 (56%) had also had treatment with ovarian steroids (Bertens *et al.*, 1982). The third study demonstrated that endometriosis was significantly associated with number of hysterotomies ($P < 0.01$) as was adenomyosis (DiGiacomo, 1977). Similar observations have also been made in other non-human primate species. In the baboon, 9/13 (69%) animals with endometriosis had had one or more hysterotomy, compared to only 5/33 (15%) with a normal pelvis (D'Hooghe *et al.*, 1991). In the cynomolgus monkey, an association was not observed with 5/27 (19%) animals with endometriosis having one or more hysterotomy compared to 11/67 (16%) without endometriosis (Ami *et al.*, 1993).

Hysterotomy is not considered a risk factor for the development of pelvic endometriosis in women, but there is evidence implicating seeding of endometrial cells following hysterotomy as a cause of endometriosis in abdominal scars (Chatterjee, 1980, Kale *et al.*, 1971, Kennedy *et al.*, 1988).

Oestradiol

Treatment with oestradiol has also been implicated as an important factor for both spontaneous, and experimentally induced endometriosis in the rhesus monkey (Dizerega *et al.*, 1980). In the rhesus monkey, endometrium was seeded into the peritoneal cavity of 26 castrated cynomolgus monkeys. The animals were divided into four groups, receiving either no treatment (controls), oestradiol only (*via* silastic capsules), progesterone only, or oestradiol and progesterone simultaneously. At laparotomy 16 weeks later, it was found that only animals with steroid treatment had viable endometriotic implants. This is consistent with reports of radioactive ^3H -

oestradiol accumulation in rhesus endometriotic tissue (Eisenfeld *et al.*, 1971), and disease regression following treatment with GnRH agonists that suppress oestradiol production (Moody *et al.*, 1989).

Laparoscopy

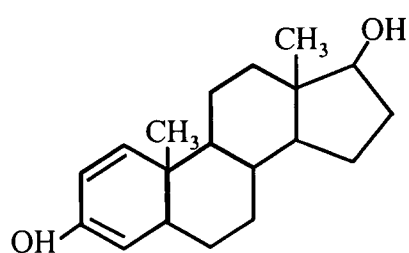
There are no studies of the effect of repeated laparoscopy on the risk of developing endometriosis in the rhesus macaque. However, it has been suggested that repetitive laparoscopies in the baboon may have facilitated the development of the disease by peritoneal trauma and/or irritation (D'Hooghe *et al.*, 1992). Each animal was exposed to three laparoscopies over a one year period in D'Hooghe's study.

Environmental risk factors

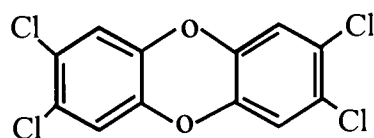
The rhesus macaque has been used in research on the effect of exposure to environmental agents such as dioxin and ionizing radiation. Some of these agents have been shown to increase the chance of developing endometriosis compared to unexposed control animals. The animals in the present study have not been knowingly exposed to such environmental agents. These observations may, however, provide clues to the mechanisms behind the development of endometriosis.

Dioxin

In 1993, the first evidence that exposure to environmental agents, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin, Figure 2.2) caused endometriosis in the rhesus macaque was published (Rier *et al.*, 1993). In this study, captive female rhesus macaques were divided into three groups and given a diet containing either no dioxin, 5 parts per trillion (ppt) dioxin or 25 ppt dioxin for a period of 4 years. Ten years later, following the deaths of three animals and the observation of widespread endometriosis at autopsy, the remaining animals were laparoscoped. Five out of seven (71%) animals exposed to the highest dioxin concentration had endometriosis, compared to 3 of 7 (43%) in the 5 ppt group and 33% in the control group. The severity of disease was also correlated with the dose of dioxin received.



Oestradiol

2,3,7,8-tetrachlorodibenzo-*p*-dioxin**Figure 2.2.** Structures of oestradiol and dioxin

Since these initial findings, it has been shown that dioxin exposure increases the growth of surgically induced endometriotic sites in both rat and mouse models (Cummings *et al.*, 1996). It has also been reported that dioxin concentrations in blood were higher in women with endometriosis than in matched controls (18% vs. 3%), although severity of disease was not correlated to the presence of dioxin (Mayani *et al.*, 1997).

Although it is known that dioxin is a toxin and a carcinogen, the mechanism by which dioxin might cause endometriosis is unknown. Potential mechanisms, which may act singly or in combination, include:

- I. Dioxin binds to and activates the aryl hydrocarbon receptor (AhR) which then forms a complex with the AhR nuclear translocator protein (Arnt) and the complex is translocated to the nucleus. The AhR-Arnt complex activates transcription of target genes by binding to 5' flanking specific gene regulatory sequence elements. Known targets include cytochrome P450, involved in the phase I detoxification system to produce reactive, carcinogenic intermediates, and growth regulatory genes such as plasminogen activator inhibitor-2 (PAI-2).
- II. Dioxin has been shown to reduce oestradiol receptor (ER) levels in the uterus resulting in an anti-oestrogenic effect; this response is mediated by the AhR (DeVito *et al.*, 1992, Kharat & Saatcioglu, 1996, Safe *et al.*, 1991). The precise mechanism is unknown; initially it was thought that the hydroxylation of oestradiol by cytochrome P450 (pathway 5, Figure 2.3) may be responsible. However, more recent findings in animal models have shown that dioxin exposure

does not alter oestradiol levels and reduces ER levels, so other mechanisms must also operate. Potential mechanisms are outlined in Figure 2.3. Mechanisms may include direct action of the AhR-Arnt complex to: a) inhibit transcription of ER mRNA (pathway 1), or b) inhibit transcription of oestradiol regulated genes by direct action (pathway 3) or c) indirect action via activation of transcription of other proteins (pathways 2 and 4).

III. Dioxin acts as an immunosuppressant, is an inhibitor of T-lymphocyte function and alters cytokine production by leukocytes (Neubert *et al.*, 1991).

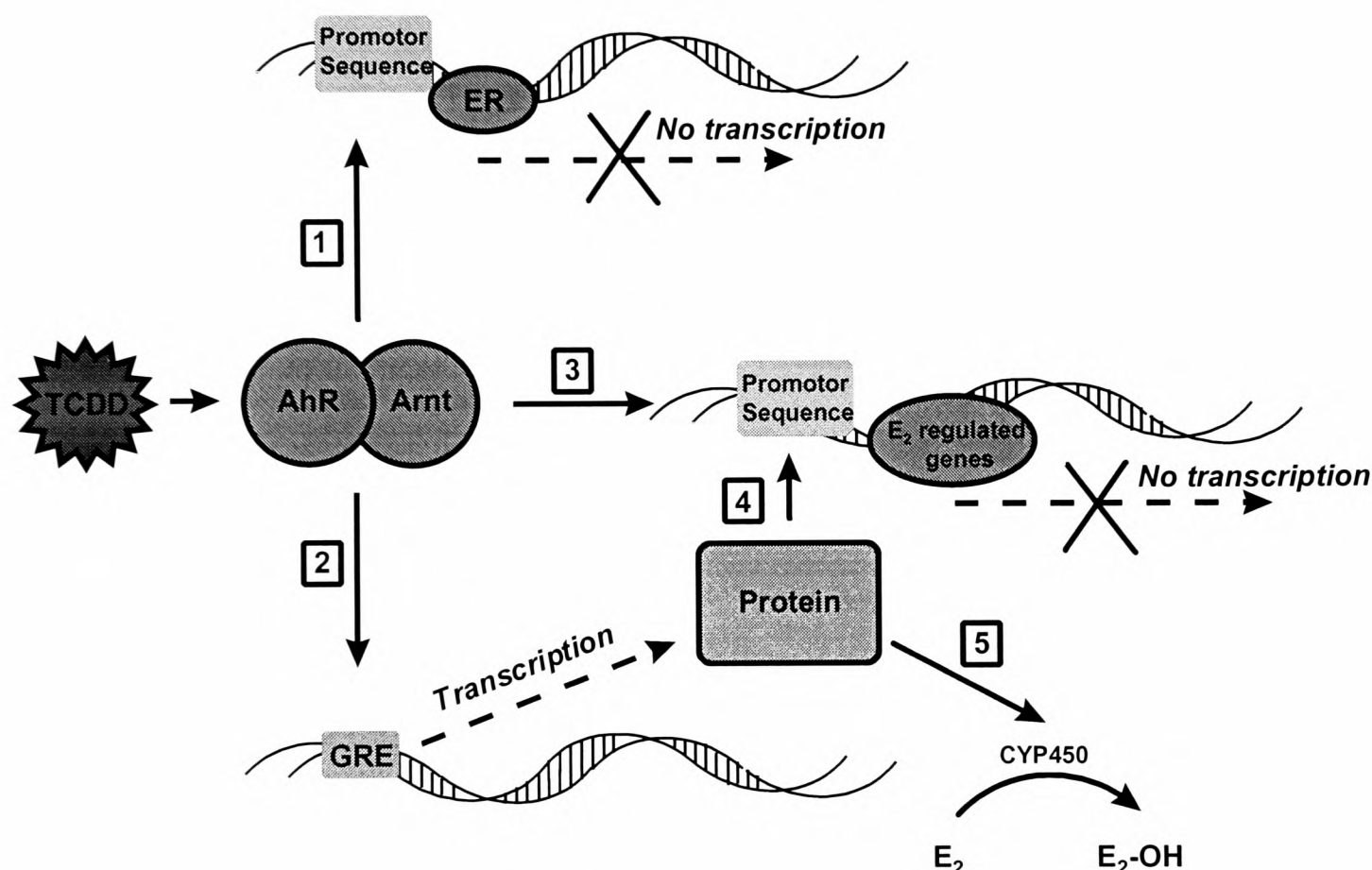


Figure 2.3. Diagram illustrating some of the possible mechanisms of dioxin (TCDD) action as an anti-oestrogen. AhR = aryl hydrocarbon receptor, Arnt = AhR nuclear translocator protein, ER = oestrogen receptor, E₂ = oestradiol and GRE = gene regulatory elements. [Adapted from Safe, 1991].

Ionizing Radiation

It has been shown that ionizing radiation induces endometriosis in the rhesus macaque. Female rhesus macaques (n = 128) were exposed to ionizing radiation in the form of single-energy protons, mixed-energy protons, X-rays or electrons and compared to an unexposed control group (n = 23). Of the exposed animals, 74/128 (58%) developed endometriosis compared to 6/23 (26%) of controls (Fantom & Golden, 1991, Fantom *et al.*, 1986, Wood, 1991). Furthermore, higher whole-body

doses led to a higher proportion of affected animals. The majority of the animals (85%) had moderate to severe disease and the age range of affected animals was 8 - 25 years. The mechanism of action of ionizing radiation in causing a higher frequency of endometriosis is unknown.

Diethylstilboestrol (DES)

Diethylstilboestrol (DES) is a potent synthetic oestrogen which was used up until the early 1970's as a method, now known to be ineffective, of preventing miscarriage in high risk pregnancies. A large number of women were exposed to DES as fetuses *in utero* resulting in a well documented increased risk of developing adenocarcinoma. A study was conducted in the rhesus macaque exposing 19 pregnant females to DES and investigating the 8 female offspring until death (Hendrickx *et al.*, 1988). The investigators observed vaginal adenosis in 5 of 8 (63%), and endometriosis in 4 of 8 (50%) of the exposed animals.

Aims of this work

The first aim was to determine the prevalence and nature of spontaneous endometriosis by auditing the necropsy records of a large colony of rhesus macaques. Secondly, in a case-control study, the aim was to determine which factors affected the risk of developing endometriosis. The animals had, over their lifetimes, been exposed to a variety of experimental procedures including laparoscopy, hysterotomy (including caesarean section) and oestradiol treatments. This is the first time an analysis of risk factors for endometriosis has been conducted using a large group of affected rhesus macaques and matched controls without the disease.

2.2 METHODS

Animals

The majority of monkeys evaluated had lived their entire lives at the Wisconsin Regional Primate Research Center (WRPRC). A colony of rhesus macaques has been maintained at the WRPRC since the early 1970's and the population at any one time has remained fairly constant at around 800 animals (Figure 2.4). Housing conditions ranged from individual caging to social groups of up to 60 monkeys. Most of the monkeys lived in a carefully controlled laboratory environment (12 hour on and 12

hour off lighting regime, 21°C temperature), although a few lived in an indoor/outdoor facility. All animal protocols had received institutional approval. The WRPRC is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

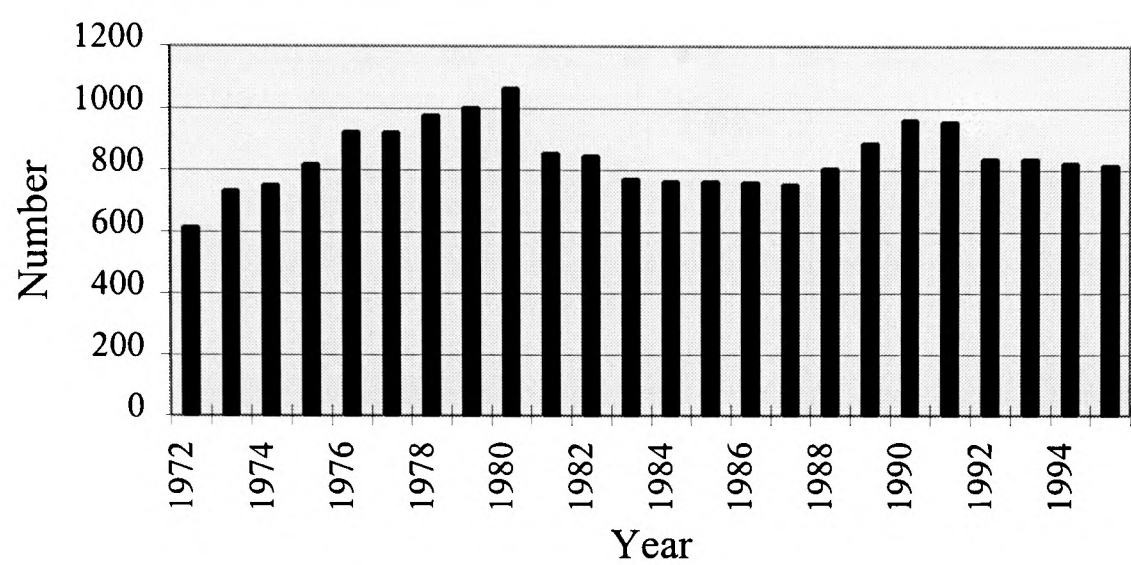


Figure 2.4. Annual population of rhesus macaques at the WRPRC (1972 - 1995)

Archived colony records

It is a requirement of U.S. Federal law that all colony animals are autopsied and that full records are kept. The colony UNIX based database contained details of all animal procedures and treatments. Records of all familial relationships were also kept, and a program ‘seekin’ was available to determine whether two animals were related, and the nature of the relationship. In most cases both parents were known, although occasionally the identity of the father was unknown. Some of the animals studied were feral and therefore neither parent was known.

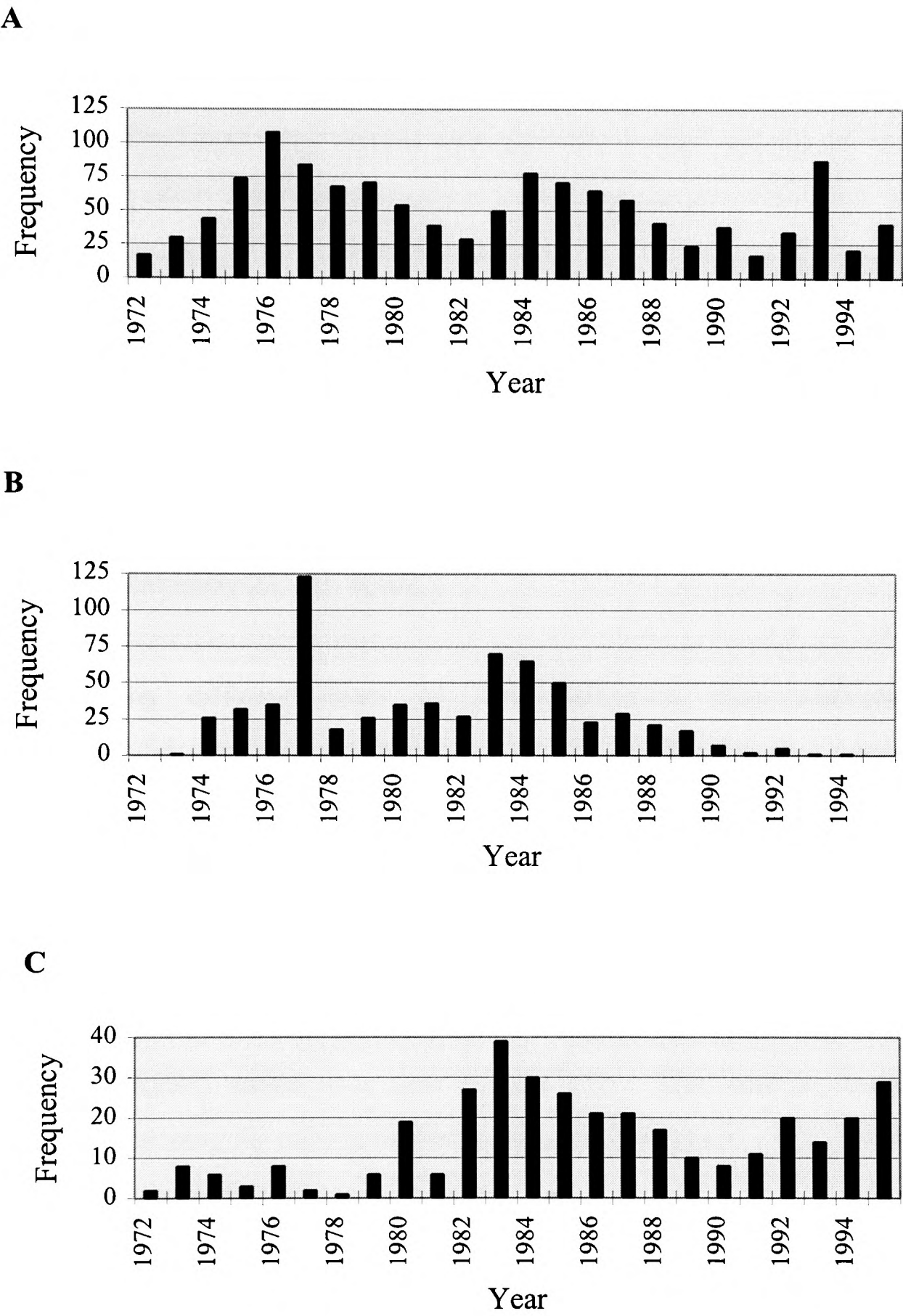


Figure 2.5. Annual figures for three procedures studied.

Graph A: The number of animals that received $\geq$ one laparoscopy.

Graph B: The number of animals that received $\geq$ one oestradiol treatment.

Graph C: The number of animals which had a hysterotomy (no animal had more than one hysterotomy in a given calendar year).

Clinical details

Laparoscopies were performed for experimental purposes (Dierschke & Clark, 1976, Hutz *et al.*, 1985) and not for the diagnosis or treatment of endometriosis or other pelvic pathology. The laparoscopy records were generally limited and did not usually include information about observations made at the time of the procedure such as the presence of endometriosis. The laparoscopy experiments were to investigate the use of the technique in the rhesus monkey and to observe ovarian folliculogenesis.

Hysterotomies were conducted during pregnancy usually to facilitate foetal experiments (Perelman *et al.*, 1986). The mean gestational age at which hysterotomy was performed was 19 weeks (range 4 - 26 weeks); the normal gestation for this species being approximately 23 - 25 weeks.

Implants containing different doses of 17- β oestradiol were administered subcutaneously via Silastic capsules for varying lengths of time during a variety of experiments over 25 years (Dierschke *et al.*, 1985, Hutz *et al.*, 1986). Experiments involving oestradiol were conducted to investigate follicular atresia in the rhesus macaque and to elucidate mechanisms controlling reproductive behaviour.

The procedures above were conducted at different frequencies because the research interests at the WRPRC varied over time (Figure 2.5). This was an important observation in determining the criteria for matching controls to cases, and is described in detail later in this section.

Definition of cases and controls

The autopsy records of the 399 female rhesus macaques that died between 1980 and 1995 were audited. Only the records of animals aged ≥ 4 years were audited for this study, based on the fact that the rhesus macaque does not reach sexual maturity until this age. The autopsies were nearly all performed by the same pathologist who routinely recorded macroscopic evidence of endometriosis including the site, extent of disease and presence of adhesions. Serial sections were cut through the uterus if hemorrhagic or nodular changes in the myometrium were observed. Animals that underwent oophorectomy more than one year before death were excluded from the study (2 cases and 122 controls) based on the observation that ectopic endometrial

tissue is not viable in castrated monkeys (Dizerega *et al.*, 1980). Animals with adenomyosis alone ($n = 2$) were also excluded.

Cases ($n = 81$) were defined as those animals with macroscopic evidence of endometriosis; 52/81 (64%) of the autopsy records also gave histological confirmation of disease. Controls ($n = 318$) were defined as those animals documented as having a normal pelvis at autopsy. The severity of disease was assessed retrospectively as minimal-mild or moderate-severe according to the rAFS classification system (The American Fertility Society, 1995) using the description contained in the autopsy record. This staging system has similarly been used in other endometriosis studies in non-human primates (D'Hooghe *et al.*, 1992, Schenken *et al.*, 1987), and its use presented little practical difficulty because animals tended to have either a few isolated foci or a severely affected pelvis.

Details of animals with endometriosis

Of the 81 animals with endometriosis, 11 (13.6%) were classified as having minimal-mild, and 63 (77.8%) moderate-severe disease. Disease severity could not be determined reliably from the autopsy notes in 7 (8.6%) of the affected animals. There was no significant difference in the mean ages ($\pm$ standard deviation, SD) at death between animals with minimal-mild (23.6 years $\pm$ 6.9) and moderate-severe disease (20.2 years $\pm$ 5.3): difference between the two groups 3.4 years (95% CI -0.2 to 7.0).

The disease sites for the 74 animals in which disease severity could be determined are presented in Table 2.6. No information on ovarian involvement was available in 9 (12%) animals. Particularly severe endometriosis, resulting in a 'frozen' pelvis, was found in 27 (36%) of the cases. Adenomyosis accompanying endometriosis was found in 16 (22%) of the cases.

Thirty-one (38%) of the 81 animals with endometriosis died spontaneously of natural causes and 5 (6%) died as a result of surgical or anaesthetic complications. A total of 42 animals were sacrificed, 7 (9%) for experimental reasons and 35 (43%) for non-experimental/humane reasons. The reason for death was not stated in 3 (4%) cases.

Table 2.6. Sites of endometriosis in the 74 cases in which disease severity could be assessed.

Site of disease	Number of cases (n = 74)
Ovaries	34 (46%)
Colon/Large intestine	26 (35%)
Small intestine	21 (28%)
Pouch of Douglas	17 (23%)
Bladder	9 (12%)
Liver	6 (8%)
Kidney	2 (3%)
Spleen	6 (8%)

In the control group, 69 (22%) died for experimental reasons and 244 (77%) for non-experimental or natural reasons. The reason for death was not stated in five of the autopsy reports.

Age of cases and controls.

No age at death was recorded for 18 controls; however, it was noted in the autopsy records that these animals were ‘adults’. The mean ages at death for the 81 cases and the 300 controls in whom age was recorded were 20.7 ± 5.5 (range 10 - 35) and 13.4 ± 7.7 (range 4 - 37) years respectively, (difference 7.3; 95% CI 5.5 to 9.1). The differences in the age at death distribution between the two groups are illustrated in Figure 2.7.

Matched control group

Because of the age difference between cases and controls, and since age is clearly a factor influencing the probability of developing endometriosis, affected animals were matched to controls by age at death (to within one year). They were also matched for date of death (to within two years) because experimental procedures had been conducted with a different frequency over the study period (Figure 2.5), thereby affecting the probability that an animal would be exposed. Matching was achieved using a computer program which minimised the average difference between age at death and year of death between cases and controls. Animals that had been included

in studies which involved manipulation or removal of the pituitary or central nervous system were excluded as matched controls.

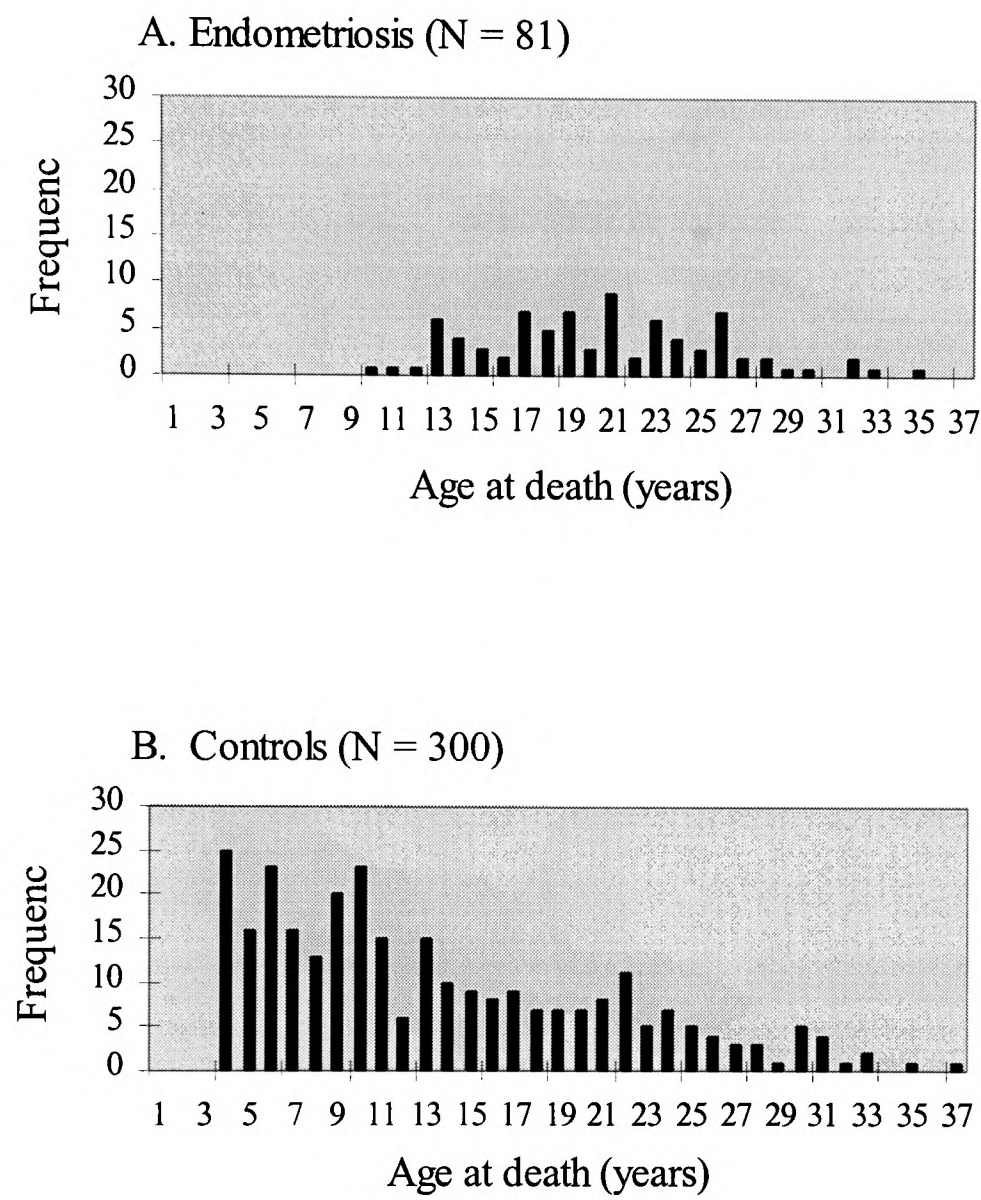


Figure 2.7. Graphs showing the different age at death distributions for animals without endometriosis at autopsy ($n = 300$) and animals with endometriosis at autopsy ($n = 81$). No animals < 10 years old had endometriosis at autopsy.

Table 2.8. The maximum number of legitimate matches possible between one case and one control under various criteria. The strictest criteria (age at death to within one year and year of death to within 2 years) were used in this analysis.

Age at death matched to within:	Year of death matched to within:	Number of matches
± 1 year	± 2 years	62
± 1.5 years	± 2 years	65
± 1 year	± 3 years	65
± 1.5 years	± 3 years	69
± 1.5 years	± 4 years	73
± 2 years	± 5 years	77
± 2 years	± 7 years	81

Of the 81 cases, 62 could be matched to controls using the defined criteria (Table 2.8). Only one control could be matched per case because few control animals died in old age. The 62 matched pairs formed the basis of the analysis. The mean ages at death for cases and matched controls were 20.0 ± 5.5 and 19.9 ± 5.5 years respectively. Twenty-eight (45%) of the matched controls were feral as were 32 (52%) of the cases; the remainder were laboratory reared.

There was no significant difference in animal weights between case and control groups. The mean weight at the time of death was 6.2 ± 1.8 kg for cases (range 2.9 - 10.4) and 6.5 ± 2.4 for controls (range 3.2 - 15.8).

Statistical methods

Paired comparisons of the continuous data were made using the Wilcoxon test and comparisons of proportions were made using the McNemar test. Four possible risk factors for endometriosis were considered: oestradiol implant, hysterotomy, laparoscopy, and number of offspring. Odds ratios and 95% confidence intervals were estimated from the matched cases and controls using conditional logistic

regression analysis (see below). The risk factors were considered both as binary variables (yes/no), and where relevant, they were grouped into three categories, defined to make clinical sense and to be as equal as possible in size. The categories used were: hysterotomies: 0, ≥ 1 ; oestradiol implants 0, 1-3, >3 ; laparoscopies 0, 1-3, >3 and offspring: 0, 1-7, >7 .

Conditional logistic regression

Conditional logistic regression analysis was conducted on matched cases and controls with the program EGRET (epidemiological graphics, estimation, and testing, Epidemiological Resources Inc., Seattle, Washington 1989). This model links a binomial proportion r/n , where $0 \leq r \leq n$, with a set of covariate values, usually presented as yes/no data.

To determine which variables should enter the model, crude odds ratios for each factor were first estimated. Odds ratios adjusted for the confounding effects of other factors were then estimated using a step-wise analysis to build the regression model. At each step, the factor with the lowest P value (according to the likelihood ratio test) entered the model. The cut-off point for inclusion was a P value less than 0.05. Any factor that changed the odds ratio associated with a statistically significant factor by at least 10% also entered the model as a confounder, even if it was not itself significantly associated with endometriosis. The level of statistical significance determined whether the variable entered the model as a binary (*i.e.* yes/no) or categorical variable.

2.3 RESULTS

Prevalence of endometriosis in the colony

The prevalence of endometriosis was 20% (81/399) in animals that died aged ≥ 4 years old and 29% (81/284) in those that died aged ≥ 10 years old (no animal < 10 years old was found to have endometriosis). The relative risk for endometriosis in the colony in relation to increasing age and the proportion of animals affected in each age group are shown in Figure 2.9. Age was a significant risk factor for endometriosis with a mean difference in age at death between animals with and without disease of 7.3 years (95% CI 5.5 - 9.1).

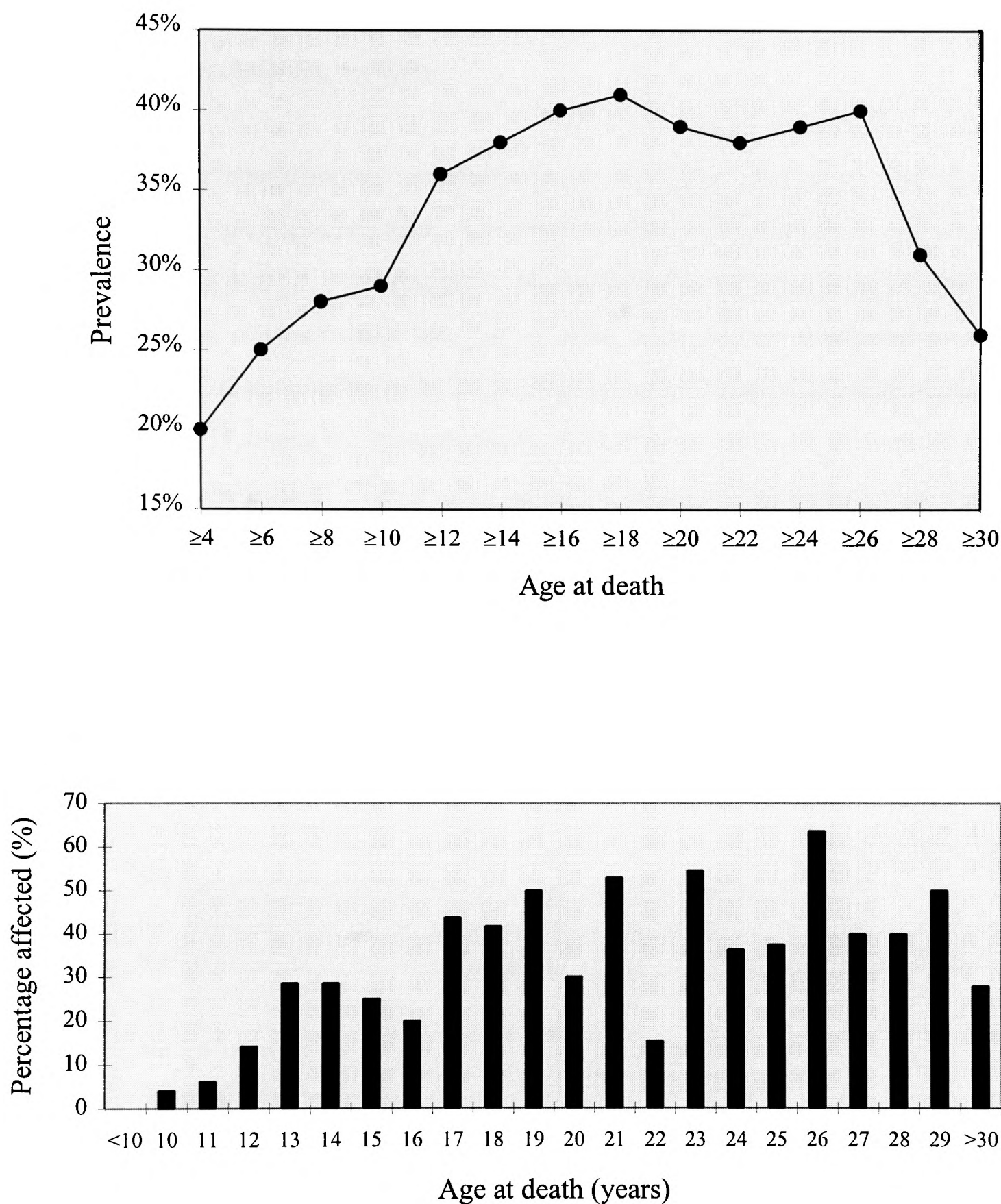


Figure 2.9. The prevalence of endometriosis in the colony and its relationship to increasing age (top) and the proportion of animals affected in each age group.

Analysis of all cases and controls

The original group of 81 animals identified with endometriosis at necropsy differed significantly from the group of 318 unaffected controls. The fundamental reason for this difference was the disparity in age distribution. Many of the control animals (45%) were in the 4 - 10 year old group and therefore had a very low chance of

developing endometriosis (Figure 2.9). Furthermore, the chance of having offspring was lower because of this age difference. These factors were important in deciding on the criteria for matching controls.

However, for completeness, comparisons of the entire case group and the entire control group are presented here. The mean number of laparoscopies per animal for the case group was 5.5 ± 8.1 (range 0 - 46) compared to 2.8 ± 5.4 (range 0 - 44) in the control group; 67% of cases had one or more laparoscopies compared to 46% of controls. The mean number of hysterotomies per animal was 0.58 ± 0.92 (range 0 - 4) and 0.19 ± 0.57 (range 0 - 3) respectively; 37% of cases and 12% of controls had one or more hysterotomies. The mean number of oestradiol treatments was 1.9 ± 3.2 (range 0 - 21) for cases and 1.1 ± 4.6 (range 0 - 46) for controls; 46% and 16% had one or more oestradiol treatments respectively. The mean number of offspring per animal in the two groups was 4.3 ± 3.1 for cases compared to 2.7 ± 3.3 for controls; 89% of cases and 63% of controls had one or more offspring.

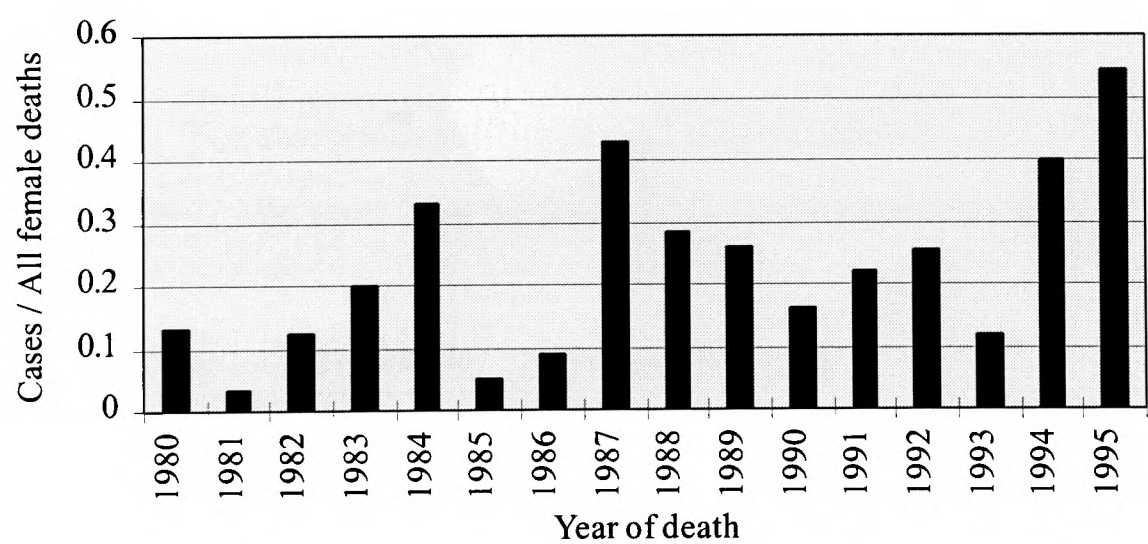


Figure 2.10. Cases of endometriosis at necropsy as a proportion of the total number of deaths in female animals aged ≥ 4 years between 1980 and 1995.

The rate of endometriosis found in the colony varied over time. More specifically, the number of cases of endometriosis found at autopsy as a proportion of the total number of female autopsies varied (Figure 2.10). This could have been attributed to variability in the mean age at death each year, had it not been found to remain fairly constant over the years (Figure 2.11).

Analysis of matched case and control groups

The potential risk factors of interest had very similar prevalences in the 62 matched cases as in the full set of 81 cases. The respective rates were: oestradiol implants 46% and 50%; hysterotomy 37% and 35%; laparoscopy 67% and 68%, and offspring 89% and 86%. This indicates that bias was not introduced by using this smaller set of cases in the analysis.

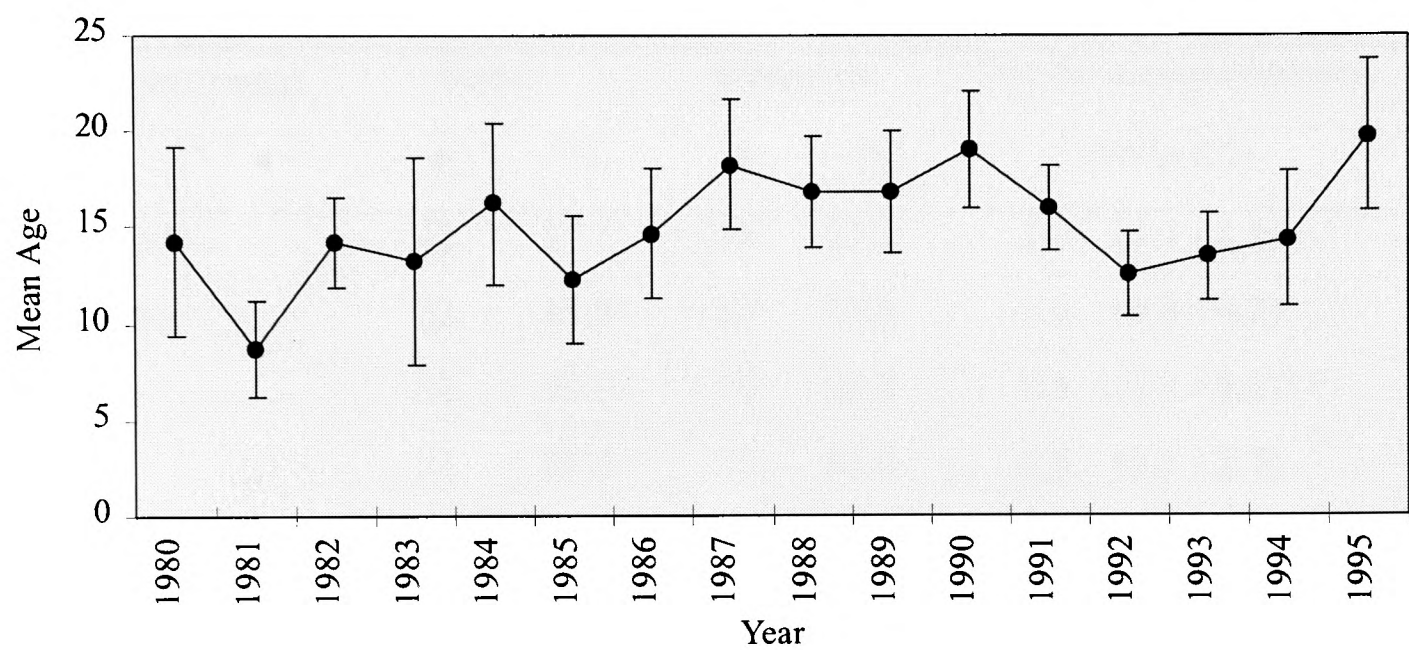


Figure 2.11. The mean age at death for all female animals aged ≥ 4 years between 1980 and 1995. The error bars represent 95% confidence intervals.

Risk factors for endometriosis: univariate analysis

Many of the cases and matched controls received at least one exposure to laparoscopy, hysterotomy, oestradiol treatment or offspring; these are summarised in Table 2.12. The odds ratios were calculated using the McNemar test, which takes the form of a 2 x 2 table, for example in calculating the odds ratio for oestradiol treatments $\geq$ one:

		Controls Exposed	
		Yes	No
Cases Exposed	No	10	21
	Yes	6	25

The odds ratio is the number of cases that were exposed, when the matched control was not ($n = 25$), over the number of matched controls that were exposed, when the case was not ($n = 10$). For oestradiol treatments the odds ratio was therefore 25/10 or

2.500. This gives a Chi-Square value of 5.600 and $P = 0.0180$, indicating that there is a 1.8% probability that these numbers occurred by chance. The results of the McNemar tests for all of the factors are presented in Table 2.12. Many of the monkeys were exposed to the procedures in combination, and these results are presented in Table 2.13.

Table 2.12. Comparison of the number of laparoscopies, hysterotomies, oestradiol implants and offspring for each of the 62 cases and matched controls showing the crude odds ratio (OR).

Exposure to $\geq$ one:	Cases		Controls		OR for endometriosis	
	n	%	n	%	(95% CI)	
Laparoscopy	42	67.7	33	53.2	2.0	(0.9 - 4.5)
Hysterotomy	22	35.5	14	22.6	1.9	(0.8 - 4.2)
Oestradiol	31	50.0	16	25.8	2.5	(1.2 - 5.2)**
Offspring	53	85.5	55	88.7	0.8	(0.3 - 2.7)

CI = confidence interval
** $P = 0.018$

Table 2.13. Table showing the proportions of cases and matched controls having each procedure or combinations of procedures.

Combinations of procedures	Cases		Controls	
	n	%	n	%
No treatments/procedures	12	19.4	19	30.6
Laparoscopy alone	4	6.5	17	27.4
Hysterotomy alone	2	3.2	6	9.7
Oestradiol alone	6	9.6	3	4.8
Laparoscopy + hysterotomy	13	21.0	4	6.5
Laparoscopy + oestradiol	18	29.0	9	14.5
Hysterotomy + oestradiol	0	0	1	1.6
All treatments/procedures	7	11.3	3	4.8

The median number of laparoscopies performed on cases and matched controls was 2 (range 0 - 46) and 1 (range 0 - 29) respectively ($P = 0.007$). The median number of

hysterotomies performed on cases and matched controls was 0 (range 0 - 3) and 0 (range 0 - 3) respectively ($P = 0.67$). The median number of oestradiol implants received by cases was 0.5 (range 0 - 21) and 0 (range 0 - 25) by matched controls ($P = 0.01$) The median number of offspring was 4 (range 0 - 10) and 4 (range 0 - 15) for cases and matched controls respectively ($P = 0.14$). The number of offspring per animal is presented in Figure 2.14.

The median length of time between the last laparoscopy and autopsy was 5.7 years (range 0 - 15.4) for cases and 7.1 years (range 0.1 - 15.1) for matched controls ($P = 0.8$). The median length of time between last pregnancy and death was 7.1 years (range 0.1 - 15.5) for cases and 3.4 years (range 0.1 - 20.2) for matched controls ($P = 0.003$).

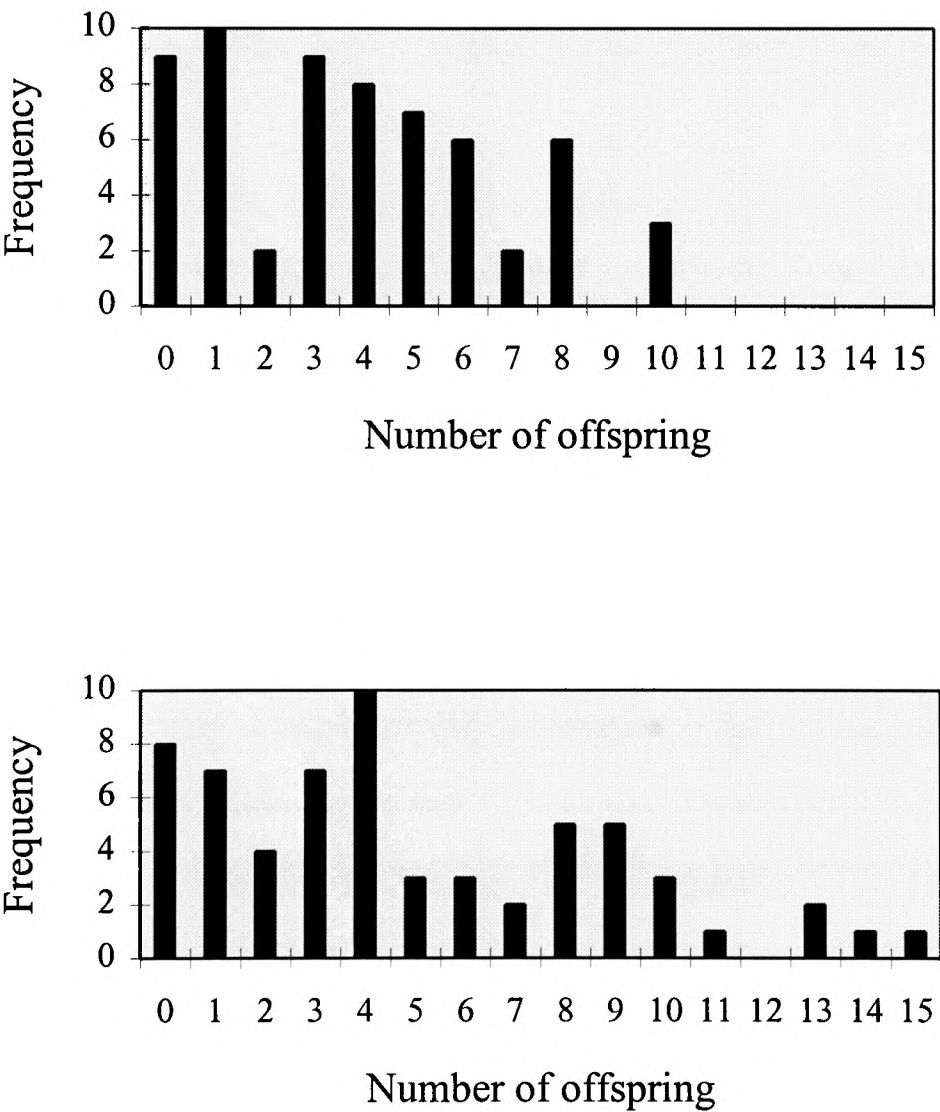


Figure 2.14. The number of offspring per animal for the 62 cases (top) and age-matched controls (bottom).

Risk factors for endometriosis: logistic regression analysis

The results of the regression analysis are presented in Table 2.15. Oestradiol treatments, in two groups 1-3 and >3 treatments, entered the regression model first as the most significant factor from the univariate analysis ($P = 0.018$, Model 1). The variable, hysterotomy, was itself a significant factor and also increased the odds ratio of >3 oestradiol treatments by 53% (Model 2). The variable, offspring, was not itself a significant factor; however, it increased the odds ratio of hysterotomy by 29%, and the odds ratio of >3 oestradiol treatments by 11% and it was therefore included as a confounding variable (Model 3). The variable, offspring, always had a negative correlation coefficient, indicating a protective but not significant trend. Model 3 was the final model ($P < 0.001$) and the results are presented in Table 2.16. The increased risk associated with oestradiol implants was apparent with more than three implants (OR 9.7, 95% CI 2.5 to 37.2). After adjusting for oestradiol implants and offspring, hysterotomy was also associated with a considerably increased risk of endometriosis (OR 5.8, 95% CI 1.6 to 20.2).

Laparoscopy did not enter the final model. It was not a significant factor and did not alter the odds ratio of a significant factor by more than 10% (OR of hysterotomy decreased by 5.3%, and OR of >3 oestradiol treatments decreased by 3.6%, Model 4).

Even when the number of offspring were divided into groups of 1-7 and >7, these were not significant variables, however, they did act in combination as confounding variables and increased the odds ratio of hysterotomy by 21%. The model that included offspring as two variables (Model 7) was not significant ($P = 0.230$) and the variable when divided had less of an effect on the odds ratios (21% increase in groups compared to 29% as a single variable) and hence offspring remained a single variable in the final model (Table 2.16).

Familial relationships between animals

There was a high degree of inter-relatedness in the colony, although there was almost no in-breeding as this was WRPRC policy. The animals studied were related both between and within the case and matched control groups. Twenty-six (42%) cases and 13 (21%) matched controls were related to at least one other animal in their respective groups ($P = 0.013$). Seventeen (27%) cases were related to at least one

Table 2.15. Multivariate analysis of seven binary risk factors and their interactions in a group of 62 endometriosis cases and 62 matched controls.

Model	Number of parameters	E ₂ treatments 1-3		E ₂ treatments >3		Hysterotomy ≥1		Offspring ≥1		Offspring 1-7		Offspring >7		Laparoscopies ≥1			P-value of model
		OR	P	OR	P	OR	P	OR	P	OR	P	OR	P	OR	P	P	
1	2	1.47	0.424	4.08	0.006												0.009
2	3	1.45	0.477	8.75	0.001	4.45	0.009										0.003
3	4	1.57	0.404	9.68	<.001	5.76	0.006	0.37	0.23								<0.001
4	5	1.54	0.433	9.33	0.001	5.45	0.011	0.39	0.27					1.14	0.787		0.002
5	4	1.18	0.753	3.41	0.020					0.97	0.97	0.36	0.24				0.226
6	3	1.39	0.510	3.81	0.010									1.76	0.195		0.188
7	5	1.30	0.641	8.27	0.002	5.38	0.010			0.43	0.30	0.20	0.11				0.230
8	4	1.39	0.527	8.11	0.002	4.10	0.017							1.29	0.578		0.577

E₂= oestradiol treatments
OR = odds ratio
P = P-value

animal in the matched control group. The sum coefficient of relatedness between the two groups was 3.5; within the case group, the coefficient was 4.375 and within the matched control group it was 1.6875 (the coefficient of relatedness is 0.25 for mother-daughter or full sibling relationships and 0.125 for half siblings etc.). The family groups identified and subsequent work conducted on the genetics of endometriosis in the colony are presented in detail in Chapter Three.

Table 2.16. Final conditional logistic regression model ($P < 0.001$).

Term	Odds Ratio [†] (OR)	95% CI for OR	P-value	Coefficient	Standard Error
Oestradiol Implants:					
1 - 3	1.6	0.5 - 4.5	0.40	0.45	0.54
> 3	9.7	2.5 - 37.2	<0.001	2.27	0.69
Hysterotomy:					
≥ 1	5.8	1.6 - 20.2	0.006	1.75	0.64
Offspring:					
≥ 1	0.4	0.1 - 1.8	0.23	-0.99	0.82

[†] the odds ratios are adjusted for all other factors shown

2.4 DISCUSSION

As outlined in the introduction, spontaneous endometriosis in the rhesus monkey is a valuable model of the human disease because it occurs spontaneously in this species and its clinical manifestations are similar to those in women. The colony of rhesus macaques at the WRPRC provided the ideal opportunity to investigate both the environmental and genetic aspects of endometriosis because: a) detailed autopsy records had been kept on all animals, b) detailed records of animals' life histories, including exposures to surgical and hormonal treatments, had been kept, and c) records of each animal's parents and other ancestors were available. Furthermore, the high prevalence of the disease in the colony (29% of animals that died aged ≥ 10 years old) meant that sample sizes were significant. The observation that 78% of the affected animals had moderate-severe disease, and the availability of age-matched controls without the disease, also strengthened the suitability of the colony for this investigation.

Although it is known that endometriosis occurs with a high prevalence in captive colonies of *Macaca mulatta*, neither the exact prevalence nor the reason for the preponderance of the disease in captivity are known. This study was therefore embarked upon to determine both the prevalence of the disease and the potential risk factors in the development of the disease. The aim was to gain a better understanding of the disease in the rhesus monkey and to draw parallels between the findings in this species and the disease in women.

Prevalence of endometriosis in the colony

The frequency of endometriosis observed was 20% in female animals that died aged four years or older in the 15 year period studied. The most striking observation was the difference in age distribution between cases and controls. The mean age at death for cases was 21 versus 13 years in the entire control group. No rhesus monkey under 10 years old had endometriosis at autopsy, so a more accurate representation of the prevalence is 29% in animals that died over this age. The prevalence peaked at 40% of animals which died age 18 or older but tails off with increasing age. This may be due to decreased oestradiol concentrations in macaques nearing the end of their reproductive lives, although monkeys often do not reach a menopause. Alternatively,

if genetic factors are involved, endometriosis may be more likely to develop before a certain age. The finding of a prevalence of 20-29% is consistent with the findings in a captive baboon colony, where the prevalence was 25% (D'Hooghe *et al.*, 1991), and a cynomolgus monkey colony, where the prevalence was 22% (Ami *et al.*, 1993). These findings indicate the strong influence of age on the development of endometriosis. It is interesting to note that the rhesus macaque has a menarche at 2-3 years of age, yet no endometriosis was observed until the age of 10. It is also interesting to compare this result to the aetiology of the disease in women: the age of 10 years old in the rhesus macaque is approximately equivalent to 24 years in women (given that the average life span in the rhesus monkey is 29). The fact that no animal under 10 had the disease is contrary to the findings in women, where cases of endometriosis in girls as young as 12-13 years have been reported (Durinzi & DeLeon, 1993, Emmert *et al.*, 1998, Hanson & Khong, 1996). The incidence of endometriosis in teenagers is however extremely low at about 0.07% per year (Houston *et al.*, 1988). By far the majority of women presenting with endometriosis are in the 30-45 year age group (Hadfield *et al.*, 1996, Houston *et al.*, 1988, Sangi-Haghpeykar & Poindexter, 1995), which is more congruous with the observations made in the rhesus macaque. Figure 2.17 (adapted from Houston *et al.*, 1988) illustrates the relationship between the incidence of endometriosis and age in women.

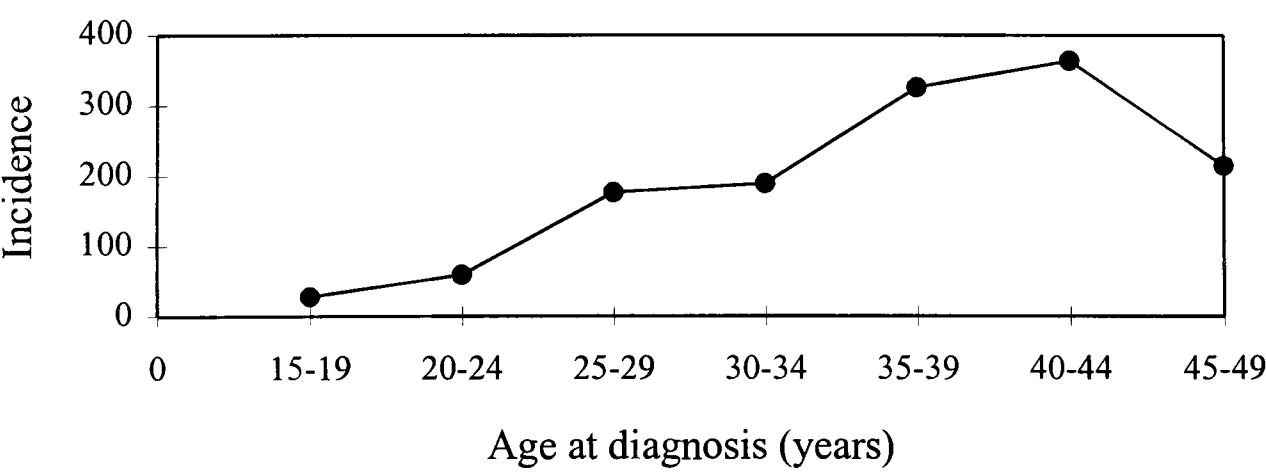


Figure 2.17. Incidence rates for surgically visualised and/or histologically confirmed pelvic endometriosis for females aged 15-49 years. Incidence rates given are cases per 100,000 person years (Houston *et al.*, 1988).

It can be concluded that age is a major risk factor for endometriosis in the rhesus macaque and that this finding is supported by existing data indicating age as an important risk factor in women.

The fact that age was such a significant risk factor meant that subsequent analyses to look at the potential risk factors of hysterotomy, oestradiol treatment, laparoscopy and offspring had to be carefully controlled to avoid bias. The way that the subsequent analysis was conducted, with cases and controls matched for age at death and year of death, obscures the fact that age was a major factor in the development of the disease.

Risk factors for endometriosis

Hysterotomy and treatment with ovarian steroids had previously been identified as possible risk factors in two small studies (Bertens *et al.*, 1982, McCann & Myers, 1970). Both of these studies involved very small numbers of animals (9 and 21 respectively) and did not include comparisons with matched controls. This study is the first analysis of risk factors for endometriosis using a large group of affected rhesus macaques and matched controls without the disease. Because the procedures and treatments were more often given in combination than independently, logistic regression analysis was used to estimate the independent effect of each procedure on the development of endometriosis.

Hysterotomy was associated with a significantly increased risk of endometriosis with an adjusted odds ratio of 5.8 ($P = 0.006$). This observation provides further evidence for the theory that endometriosis arises as a result of the exposure of the peritoneal cavity to endometrial cells. However, exposure to one or more hysterotomies did not invariably result in endometriosis: 9.7% of the matched controls had a hysterotomy and did not develop the disease. Hysterotomy is not considered to be a risk factor for endometriosis in women. In most women caesarean sections are performed at or near term pregnancy, and the risk of subsequent pelvic endometriosis is low perhaps because the hormonal environment does not favour the development of the disease. Breast feeding following pregnancy, and its associated hypo-oestrogenic state may further reduce the opportunity for viable endometriotic implants. In comparison, in this study hysterotomies were conducted at a variety of gestational ages (range 4 - 26 weeks, normal gestation approximately 23 weeks) and, as the hysterotomies were

conducted to facilitate foetal experiments, there may have been no subsequent prolonged lactation period. Nevertheless, there are case reports (Chatterjee, 1980, Kale *et al.*, 1971, Kennedy *et al.*, 1988) of endometriosis in caesarean section scars which implies a potential relationship. This line of investigation should be pursued in any future epidemiological studies into endometriosis.

The relationship between ovarian steroids and the development of an endometriotic lesion has already been well established in both human and non-human primates and endometriosis is considered to be an oestrogen dependent disease. Thus, it is known that oestradiol is not a prerequisite for the initial implantation of ectopic endometrial tissue in the rhesus monkey (Dizerega *et al.*, 1980), but ovarian steroids are required to sustain the viability of the implants. In this study, having more than three oestradiol implants was the strongest risk factor for endometriosis in the rhesus macaque, with an adjusted odds ratio of 9.7 ($P < 0.001$). Despite this strong relationship, exposure to one or more oestradiol implants did not always lead to endometriosis: 26% of controls were exposed but did not develop the disease. This may indicate the involvement of a protective mechanism, possibly genetic, in these animals.

Of the 31 animals with endometriosis that had oestradiol implants, only 7 (23%) also had a hysterotomy. This finding raises the question as to the source of endometrial tissue in the remaining 24 animals. The currently favoured theory in humans is that retrograde menstruation into the peritoneal cavity may be the source of the tissue. This may also be the case in non-human primates as it has been observed that retrograde menstruation occurs in 70% of baboons without disease and 92% of those with endometriosis (D'Hooghe *et al.*, 1994). Another possibility is that an increased concentration of oestradiol may itself cause endometriosis rather than just enhancing its growth and proliferation. The fact that endometriotic tissue has been observed in men undergoing oestrogen therapy as treatment for prostate cancer provides further evidence for this argument (Schrodt *et al.*, 1980). Recently, an inverse relationship in oestradiol plasma levels and natural killer cell activity in women with endometriosis has been observed (Provinciali *et al.*, 1995) which may give clues to the underlying mechanisms behind these observations.

Laparoscopy was not a significant risk factor, although D'Hooghe *et al.* (1992) in a study of baboons has suggested that repetitive laparoscopies could facilitate the development of endometriosis. The length of time between last laparoscopy and autopsy was not significantly different between cases and matched controls either.

The effect of pregnancy on endometriosis

The number of offspring was not a significant factor in the development of endometriosis. One or more offspring (or pregnancies) was however included in the final conditional logistic regression model as a confounding variable, that increased the odds ratio of hysterotomy by over 50%. This is hardly surprising, as the two variables are highly correlated. Because of this correlation, and because the majority of animals had at least one offspring, it was difficult to determine accurately the effect of this variable on disease development. Pregnancy is generally thought to cause endometriosis to regress, or disappear altogether, and it has been shown that increasing parity is associated with a decreased risk of disease in women ($P < 0.001$) (Candiani *et al.*, 1991). This is consistent with the finding of a negative correlation coefficient for the variable offspring. A significant difference in the length of time between last pregnancy and the diagnosis of endometriosis (*i.e.* autopsy) was also observed, with a median of 7 years for cases and 3 years for controls ($P = 0.003$). This is consistent with studies in women. It has been reported that in women where six years or more have elapsed since their last pregnancy there was a 2.9 fold increased risk of endometriosis ($P = 0.01$) (Sangi-Haghpeykar & Poindexter, 1995). Furthermore, those women had a 5.9 times increased risk of developing more severe forms of endometriosis. Other studies have shown that in a group of women undergoing tubal sterilisation, with a mean of 7 years since last childbirth, the prevalence of endometriosis was 6%, compared to 20% in a group with a mean of 10 years since last childbirth and 25% in a group of women at hysterectomy with a mean of 18 years since childbirth (Moen, 1995).

The presence of an inverse relationship between endometriosis and offspring and a positive relationship with time since last pregnancy may be due to amenorrhoea during pregnancy and therefore reduced risk of retrograde menstruation. Moreover, the extended period of amenorrhoea during lactation may further reduce the chance for endometriosis to develop.

Conclusion

Of the animals with endometriosis, 20% had not been exposed to either of the two major risk factors identified in this study (*i.e.* hysterotomy or oestradiol implants). If only these animals are considered, the prevalence of spontaneous endometriosis in the colony is still 11% in animals aged 10 or older. It is unclear why these animals developed endometriosis given that the prevalence in non-human primates in the wild is low (D'Hooghe *et al.*, 1996, DiGiacomo, 1977). It is conceivable that captivity itself has somehow made the development of the disease more likely. In the baboon, the prevalence of spontaneous endometriosis increases with the duration of captivity (D'Hooghe *et al.*, 1996), possibly because of increased stress or increased opportunity for retrograde menstruation as these animals had also been exposed to fewer pregnancies in captivity. Another important reason in the WRPRC colony may have been the conditions under which the animals were kept, namely a controlled environment with light and temperature maintained at a constant level throughout the seasons. Free-ranging animals and animals housed in semi-natural conditions exhibit reduced ovulatory frequency and increased variability in menstrual cycle during the summer months. In comparison, animals housed in a controlled environment, do not exhibit a seasonal effect upon the length of the menstrual cycle or number of ovulatory cycles (Hutz *et al.*, 1985). Both of these factors may contribute to the increased occurrence of endometriosis in captive rhesus monkeys. Finally, there is the possibility that genetic factors may be important in the development of endometriosis in this colony and this will be explored in greater detail in the following chapter.

There are inevitable problems with analysing autopsy records retrospectively; however, the fact that the same pathologist conducted the majority of the autopsies means that any variability should have been minimised. It is unclear why the rate of observation of endometriosis at autopsy is so variable (Figure 2.10) when the mean age at death has remained fairly constant. Given that the same pathologist conducted these autopsies, this is unlikely to be the cause of the variation. The most likely explanation is the variability in exposure to risk factors. For example, in 1977 a large number of animals were exposed to oestradiol implants, and 10 years later there was a significant increase in the observation of endometriosis at autopsy. Endometriosis has

not been a specific research interest of the WRPRC until recently which accounts for the lack of additional, clinical observations from the many laparoscopies conducted.

The large degree of inter-relatedness in the colony, as well as providing potential for the investigation of the genetic basis of endometriosis, may have introduced bias into this study. However, as the degree of relatedness between the case and control groups is not significantly different to the relatedness within the case group this is unlikely. Besides, the two risk factors identified in the colony represent strong associations which are unlikely to have arisen as a result of this slight bias.

Summary

This is the first study using large numbers and matched controls to analyse risk factors for the development of spontaneous endometriosis in the rhesus monkey. This study has shown that hysterotomy and oestradiol implants significantly increase the risk of developing the disease, but that laparoscopy has no significant effect. The length of time since last pregnancy has also been identified as a risk factor for the disease. These findings confirm observations already made of women with endometriosis, suggesting that the rhesus macaque provides an excellent model for the disease, and give some insight into the aetiology of the disease in women.

Chapter Three

Does endometriosis have a familial component in the rhesus macaque?

3.1 INTRODUCTION

As discussed in Chapter One, there is evidence that endometriosis may have a genetic basis in women. There have, however, been no studies investigating the genetic contribution to the development of spontaneous endometriosis in the non-human primate. In the study presented in Chapter Two of this thesis, which investigated risk factors for endometriosis in the rhesus macaque, there was also some evidence of familial clustering of the disease. In this chapter, these findings and further investigations on this topic will be presented.

The colony at the WRPRC provides an ideal opportunity to investigate the genetics of any disease, primarily because relationships between animals are almost always defined and can be traced back to the founding (or feral) members of the colony. In all cases the mother is known, and in most the identity of the father is also known. In addition, the environment to which the animals have been exposed is controlled and therefore can be taken into account in a genetic analysis.

The aim of this work was firstly to determine which animals at the WRPRC with endometriosis were related and to define all pedigrees with two or more affected animals. The second aim was to create a LINKAGE format file, containing details of all offspring of the ancestors of affected animals, to conduct segregation analysis. To enhance the power of the analysis, more affected animals were identified by scanning a selected group using MRI. The final aim was to explore the potential of the colony as a resource for linkage analysis. To determine whether linkage analysis could be conducted, it was first necessary to assess the availability of blood and/or tissue for DNA extraction from these animals and the feasibility of using human microsatellite markers in the rhesus macaque. To date only a few polymorphic markers have been

discovered in this species and a linkage mapping marker set is unavailable. In this chapter the results of the above studies will be presented and the techniques of MRI and segregation analysis will be introduced.

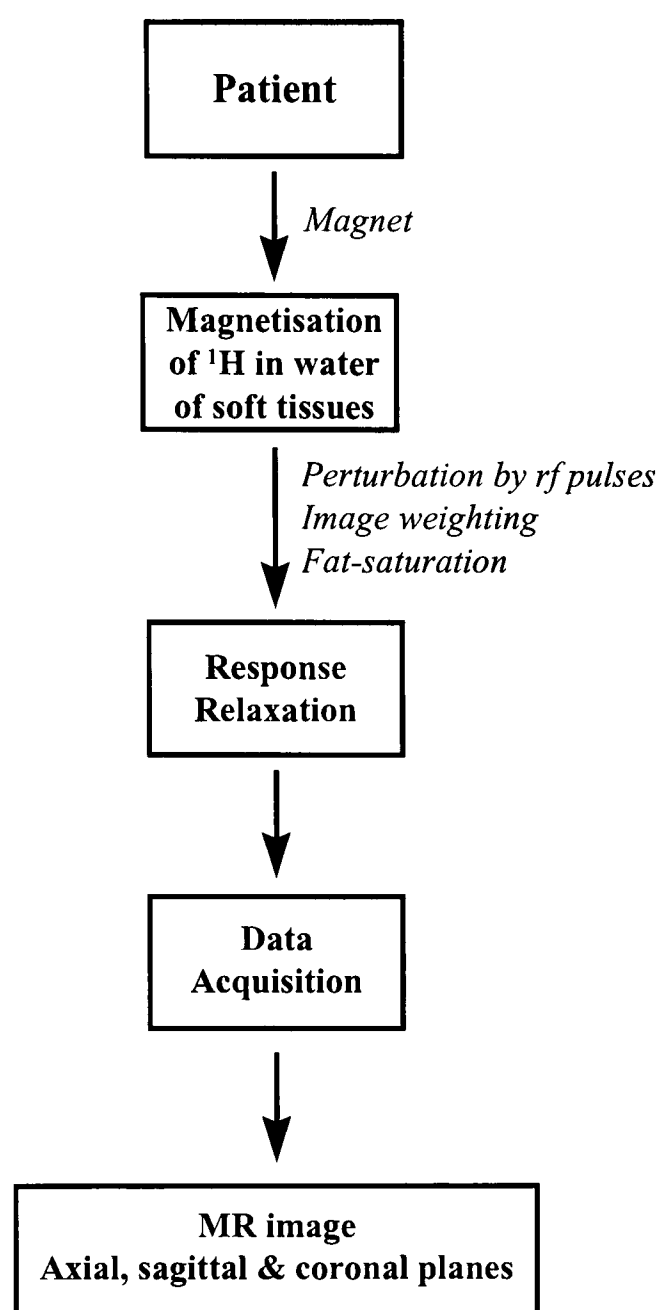
3.2 MAGNETIC RESONANCE IMAGING IN A RHESUS MACAQUE PEDIGREE TO DIAGNOSE ENDOMETRIOSIS

Introduction

Basic principles of MRI

MR imaging utilises the fact that there is a large amount of water in soft tissues and that the hydrogen nucleus, ^1H , has a large magnetic moment. In a strong external magnetic field the hydrogen nuclei align preferentially with the field, creating a bulk magnetisation. When the nuclei are exposed to a short radio frequency (rf) pulse the bulk magnetisation is rotated away from the external field direction (the z axis) into the xy plane. Once the rf pulse is switched off the bulk magnetisation that is perpendicular to the external field begins to circle or precess around this field giving rise to an oscillating signal in a detector coil. This signal is made up of many signals at different frequencies because each ^1H nucleus precesses at a frequency that depends on its exact environment. To separate each of these signals, the signal from the detector is transformed mathematically from an intensity vs. time to an intensity vs. frequency spectrum using a Fourier Transform. The two aspects of the resulting spectrum that are important in MRI are the frequency and the intensity of each peak in the spectrum. In MRI, an image is created by plotting the intensity of the water peak at different points in the area being scanned. The image is revealed by the variation in the intensity of the water signal just as a photographic image is revealed by differences in the intensity of reflected light. Magnetic field gradients allow the intensity of the water signal to be measured at different locations in the sample necessary for constructing the image.

Figure 3.1. A flow diagram showing the steps in acquiring a MR image.



It is also necessary to obtain information about the characteristics of the tissue which is achieved by altering the contrast of the image. Contrast is a result of the intensity of the water signal being low in some parts of the body, such as bone, and high in other parts, such as soft tissues. If the water signal was high throughout the area being scanned, the image would have poor contrast and would not be very informative. In such cases, the contrast can be improved by increasing the difference in the water signal across the sample by using T_1 or T_2 weighted images.

T_1 is the longitudinal relaxation time and measures the time taken for the bulk magnetisation to return to its equilibrium position along the z axis. T_2 is the transverse relaxation time and measures the time taken for the signal to decay in the xy plane. T_2 relaxation processes are characterised by both the return of the bulk magnetisation to the z axis and by the dephasing of the signal in the xy plane. Contrast in an MR image can be obtained by exploiting differences in the T_1 and T_2 for water in different tissues. For example, water in fat has a T_1 of approximately 280 ms and a T_2 of only

85 ms. In comparison, water in muscle has a much higher T_1 of 820 ms and a lower T_2 of only 45 ms. In the case of endometriotic cysts, the presence of methaemoglobin causes the T_2 of surrounding water to be greatly reduced due to the proximity of the unpaired electrons of this molecule. By manipulating the time between consecutive scans or the time between the rf pulse and the signal acquisition, T_1 and T_2 weighted images can be obtained.

Another technique that can improve contrast is fat saturation MR imaging which is especially useful in the pelvis as there is a large amount of adipose tissue in this region. Fat-saturation pulse sequences distinguish between blood and lipid within ovarian cysts and allow more accurate detection of small endometriotic implants. In one study, using conventional MRI without fat-saturation, only 50% of endometriotic implants < 30 mm in diameter were detected; the use of fat-saturation techniques improved the detection rate to 82% (Takahashi *et al.*, 1994). The steps in acquiring an MR image are summarised in Figure 3.1.

Image interpretation

Figure 3.4 (IIIa-c) illustrates the different signal intensities observed for an endometriotic cyst in T_1 and T_2 weighted images and a fat-saturated T_1 weighted image. The cyst appears as a high-intensity (bright) signal in the T_1 weighted image (IIIb) due to the short T_1 of water in the cyst. The cyst has a lower intensity, resulting in a shaded appearance, in the T_2 weighted image (IIIc), due to the short T_2 of water in the cyst. The low T_1 and T_2 of water in endometriotic cysts results from the presence of paramagnetic methaemoglobin. The fat-saturated T_1 weighted image is also included in Figure 3.4 (IIIa) to show how contrast is improved by this technique.

Adenomyosis can also be reliably diagnosed using MRI. T_2 weighted images in patients with diffuse adenomyosis show poorly defined thickening of the junctional zone between endometrium and myometrium. MRI enables more accurate differentiation between leiomyomas and adenomyosis, the margins of the former being well defined compared to adenomyosis. MRI has been found accurately to differentiate myomas from adenomyosis in 99% of patients (Mayo-Smith & Lee, 1995).

Advantages and disadvantages of MRI

The main advantage of MRI is that it is a non-invasive procedure. The technique is also safe as it does not use ionising radiation. MRI offers reliable detection of endometriotic cysts and also enables the diagnosis of adenomyosis which cannot be made at laparoscopy.

The major disadvantage of MRI is the failure of the technique to detect small endometriotic implants in comparison to laparoscopy. Interpretation of images may vary from person to person thus affecting accuracy and reliability. MRI is also relatively expensive, access is currently limited and image quality may be reduced due to patient movement, peristalsis and respiration.

Accuracy of MRI for endometriosis diagnosis

There have been several studies evaluating the accuracy of MR imaging for the diagnosis of endometriosis in patient groups which have been examined by MRI scan and laparoscopy. The introduction of fat-saturation techniques for endometriosis diagnosis by MRI in about 1994 has greatly increased the potential for detection of small endometriotic implants. However, even prior to the common use of these techniques, endometriomas could be reliably detected. In a study involving 64 patients who were found to have a total of 86 endometriotic cysts at laparoscopy, 77 (90%) were correctly diagnosed by conventional MR imaging techniques (Togashi *et al.*, 1991). Nine (10%) of the endometriotic cysts were misdiagnosed, although all were identified as cysts. In addition five adnexal masses out of 268 non-endometriotic pelvic masses (2%) were misdiagnosed as endometriotic cysts at MRI. This gives a 90% sensitivity and 98% specificity of MRI for endometriotic cysts using non-fat-saturated techniques.

The introduction of fat-saturation has increased the accuracy of detecting smaller endometriotic lesions; however, lesions smaller than 1cm in diameter are still at the limits of detection for this technique. In a study comparing fat-saturation with conventional MR imaging it was found that all lesions over 3 cm in diameter were detected with both techniques. All lesions between 1-3 cm were detected using fat-saturation techniques compared to 88% using conventional MRI. For lesions less than 1 cm, 49% and 25% were detected respectively (Takahashi *et al.*, 1994). A more

recent study by the same group, using fat-saturated MRI, has identified that all endometriomas more than 1 cm in diameter but only 66% <1 cm could be detected. Furthermore, they have demonstrated that endometriotic implants between 0.5 - 1 cm could always be detected, but only 50% of implants <0.5 cm (Takahashi *et al.*, 1996).

It can be concluded that MRI provides reliable diagnosis of endometriotic cysts and implants which are more than 1cm in diameter. Detection of any lesion or cyst smaller than this is variable.

MRI diagnosis of endometriosis in non-human primates

MR imaging has been successfully used to diagnose endometriosis in the pigtailed macaque (*Macaca nemestrina*) (Waterton *et al.*, 1992). Widespread endometriosis was identified in one animal by MRI and confirmed at autopsy and histologically; adenomyosis was also observed at both MRI and autopsy. Another animal was diagnosed with adenomyosis by MRI and treated with an anti-oestrogen (Waterton *et al.*, 1993). The use of MRI in non-human primates to diagnose endometriosis presents little practical difficulty, the only major difference being the need for sedation.

Methods

Identification of families for analysis

As presented in Chapter Two, a total of 81 animals in the colony were identified with endometriosis at autopsy. Using a computer program (Seekin, S. Sholl) all inter-relationships between these 81 affected animals were identified and twelve discrete families with two or more affected individuals were distinguished. In total, 33/81 (41%) were related to at least one other affected animal. Most of the affected animals were related as half-siblings sharing the same father. Other relationships included half-siblings (same mother), mother-daughter, full-siblings, half-aunt-niece (where aunt and parent are half-siblings). The types of relationships that were observed are illustrated in Figure 3.2. There were 27 half-sibling pairs (25 pairs sharing the same father and 2 pairs sharing the same mother), 5 mother-daughter pairs, 3 half-aunt-niece pairs and one full-sibling pair.

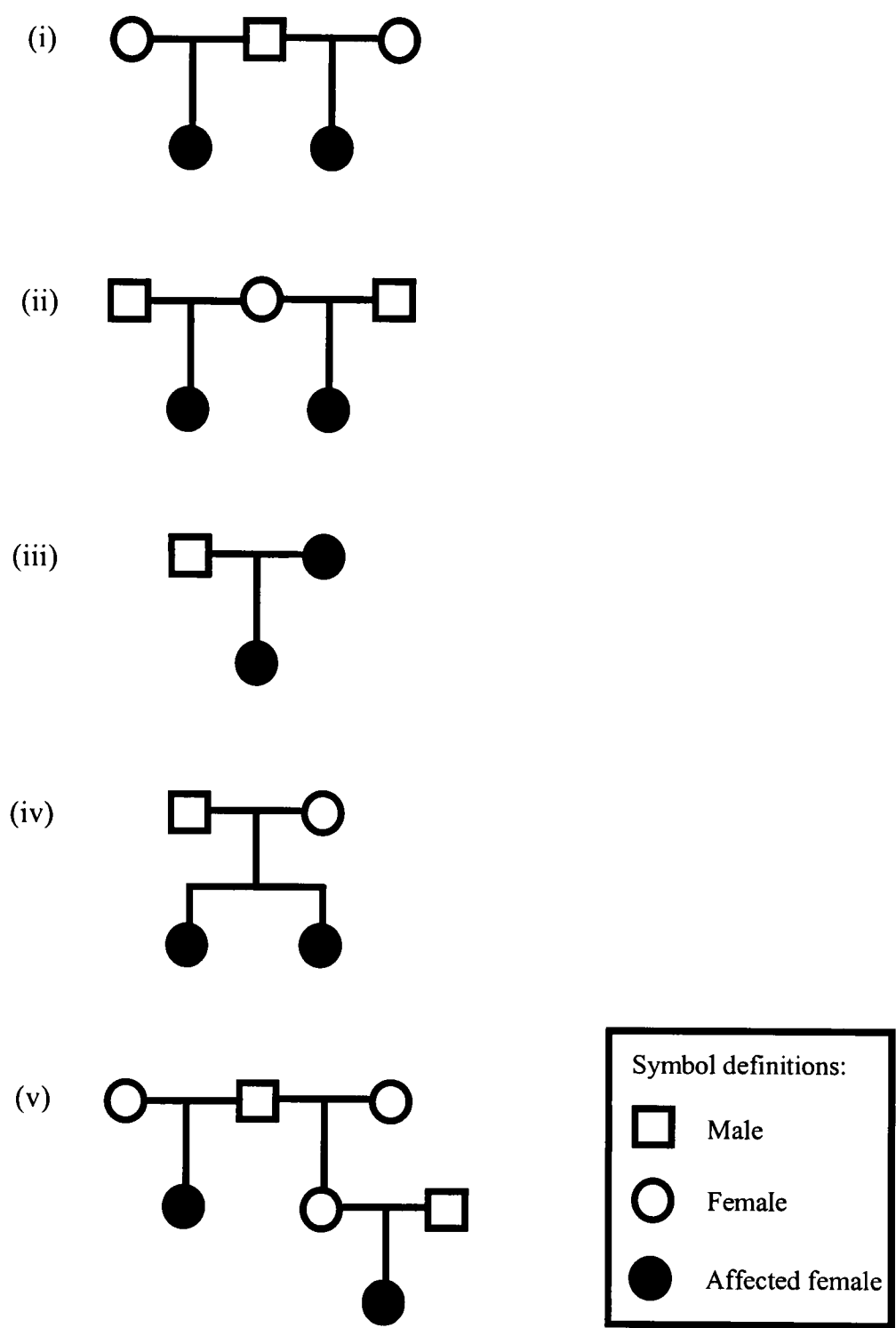


Figure 3.2. Family tree diagrams showing the types of relationships observed between rhesus macaques with endometriosis. The types of relationship were half-siblings with the same father (i), or mother (ii); mother-daughter (iii); full siblings (iv) and half-aunt-niece (v).

Identification of animals for MRI

To increase the number of animals with known disease status, and thus the power of segregation analysis, MRI was conducted on a selected group of animals. The first criterion for MRI selection was age. In Chapter Two, data were presented showing that no animal <10 years old had endometriosis. It was therefore decided that only animals ≥ 9 years old should be scanned to increase the chance of finding new,

affected animals. The second criterion for inclusion was the presence of at least one relative with endometriosis. Identification of all of the female macaques ≥ 9 years old with at least one affected relative was achieved by working backwards from each female with endometriosis. The individual's parents, grandparents, great-grandparents etc. were identified until the feral or founding ancestors were reached. All living, female descendants ≥ 9 years old of these ancestors were then classified as candidates for an MRI scan. In total, 166 females met the criteria for inclusion. Some of these animals were unavailable for MRI due to participation in WRPRC research protocols that did not allow them to be sedated for scanning.

MR imaging

Animals were sedated using ketamine HCl (10mg/kg administered intra-muscularly), and xylazine (0.6mg/kg intra-muscularly) was given for additional muscle relaxation prior to scanning. MRI images were obtained using a GE 1.0 Tesla Signa Advantage. Images of the pelvis and lower abdomen were obtained with animals in the supine position. The animals were scanned for approximately 30 minutes and then given Yohimbine (0.06 mg/kg intra-venously) to reverse the effects of xylazine. T_2 -weighted images were acquired in the coronal, sagittal and axial planes. T_1 -weighted images, both with and without fat suppression, were acquired in the axial plane.

Results

Cohort of macaques MRI scanned

Of the 166 animals meeting the chosen criteria for MRI scan only 113 (68%) were scanned. The mean age of the animals scanned was 17.6 (SD 5.0; range 10 - 27) years; the age distribution is shown in Figure 3.3. The mean number of offspring for these animals was 3.30 (SD 2.9; range 0 - 12). Some of the scanned animals had previously been exposed to the risk factors identified in Chapter Two: 36/113 (32%) were exposed to one or more hysterotomies, and 14 (12%) to more than three oestradiol implants. Four (3.5%) of the animals scanned had been exposed to both of these known risk factors.

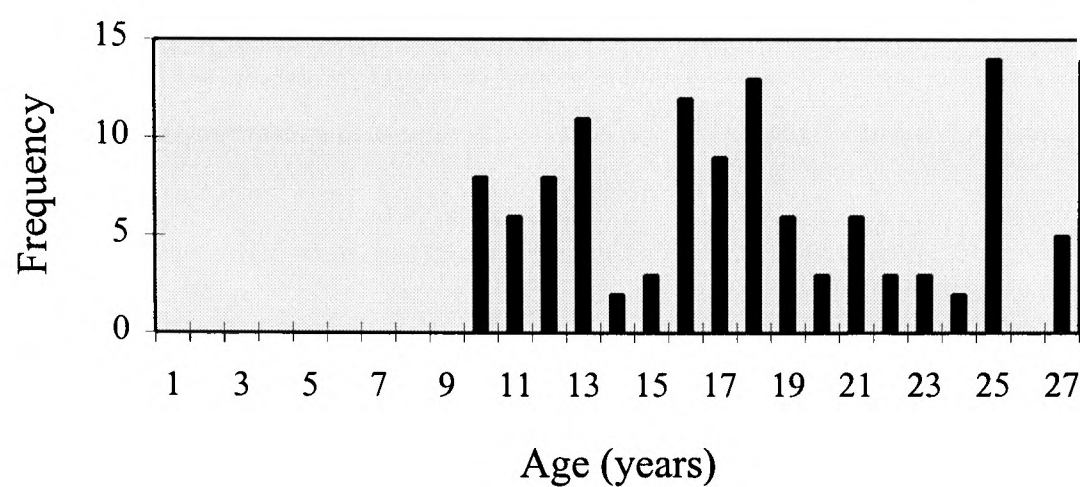


Figure 3.3. The age distribution of the 113 animals MRI scanned.

MR images

A selection of the MR images obtained are shown in Figure 3.4 to illustrate the quality and interpretation of the test. The images were interpreted by Stephen Golding at the Department of Radiology, John Radcliffe Hospital.

Results of MR imaging

Unequivocal endometriosis, with lesions >1cm in diameter, was observed in 8/113 animals (7%). Five of these animals (4% overall) had lesions >3cm in diameter. The lesions were observed around the uterus, on the ovary, in the pouch of Douglas and on the broad ligament. The mean age of the animals with unequivocal disease was 18.6 years (SD 3.3; range 15 - 25). Three of the affected animals had had one hysterotomy and none had >3 oestradiol treatments.

In addition, equivocal endometriosis, *i.e.* lesions <1cm in diameter, was noted in 14 animals, bringing the total number of animals with endometriosis or suspected disease to 22/113 (19%). The mean age of this group was 19 years (SD 4.1; range 13 - 27). Six animals had been exposed to ≥1 hysterotomies compared to 3 which had been exposed to >3 oestradiol treatments. None had been exposed to both known risk factors.

One animal had a normal pelvis on MRI but six months later a large endometriotic cyst was found at laparoscopy. This animal was deemed to be affected for the analysis.

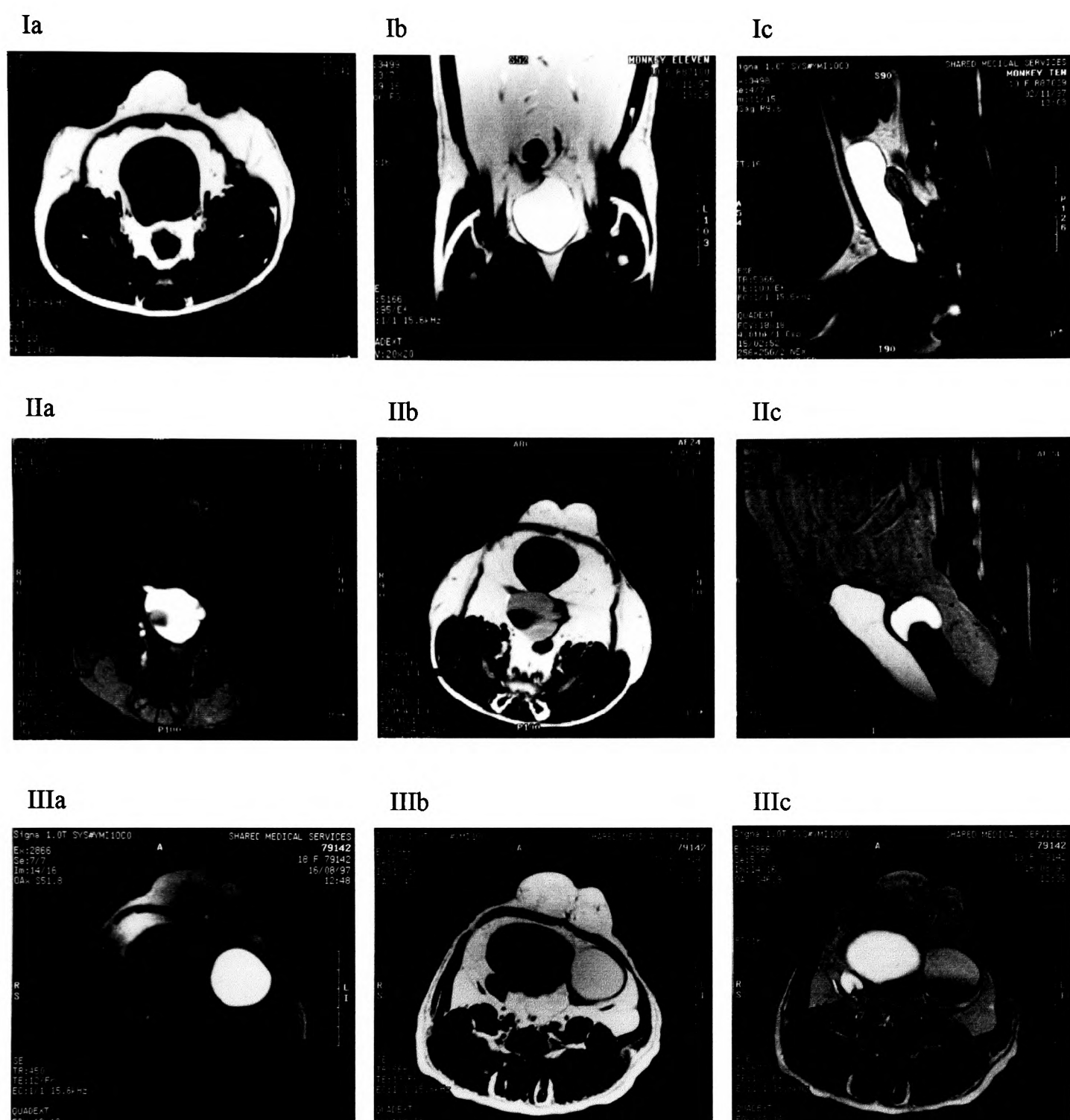


Figure 3.4. MR images showing:

- I. The appearance of a normal rhesus abdomen and pelvis in a T_1 -weighted image in the axial plane (Ia), a T_2 -weighted image in the coronal plane (Ib) and a T_2 weighted image in the sagittal plane (Ic).
- II. The appearance of endometriosis around the uterus in a T_1 -weighted image with fat-presaturation in the axial plane (IIa), a T_1 -weighted image in the axial plane (IIb) and a T_2 -weighted image in the sagittal plane (IIc).
- III. The appearance of ovarian endometriosis in a T_1 -weighted image with fat-presaturation in the axial plane (IIIa), a T_1 -weighted image in the axial plane (IIIb) and a T_2 -weighted image in the axial plane (IIIc).

Family groups identified

The new data from the MRI scans combined with the existing autopsy data allowed twelve family groups with two or more individuals of known disease status to be identified. The pedigrees, shown in Figure 3.5 (I - XII) only define relationships between affected individuals. For practical purposes, those family members of unknown or unaffected status are not always illustrated. After inclusion of the MRI data, there were 2 full sibling-pairs, 11 mother-daughter pairs and 64 half-sibling pairs within the group of affected animals.

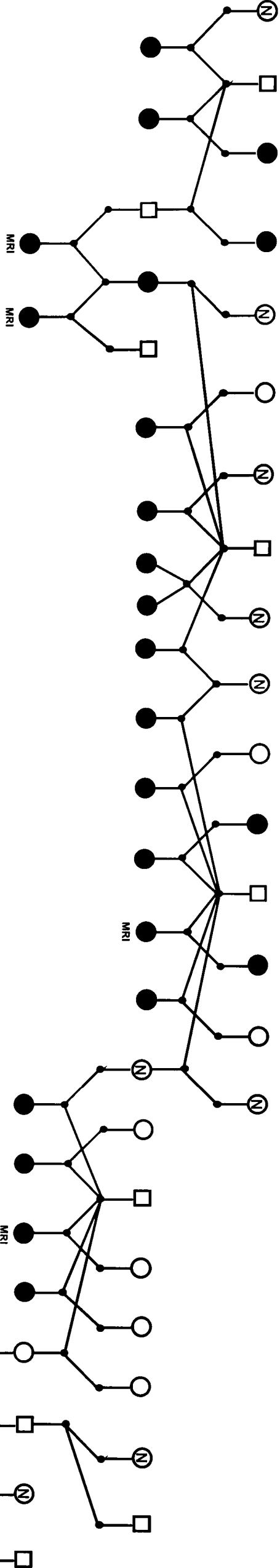
Following pages: Figure 3.5. Pedigrees I to XII illustrate all families with two or more animals with known disease status, determined either at autopsy or by MRI scan. The pedigrees are more extensive than shown; for clarity only relationships between affected animals are shown. Animals whose status was determined by MRI are labelled as such.

Discussion

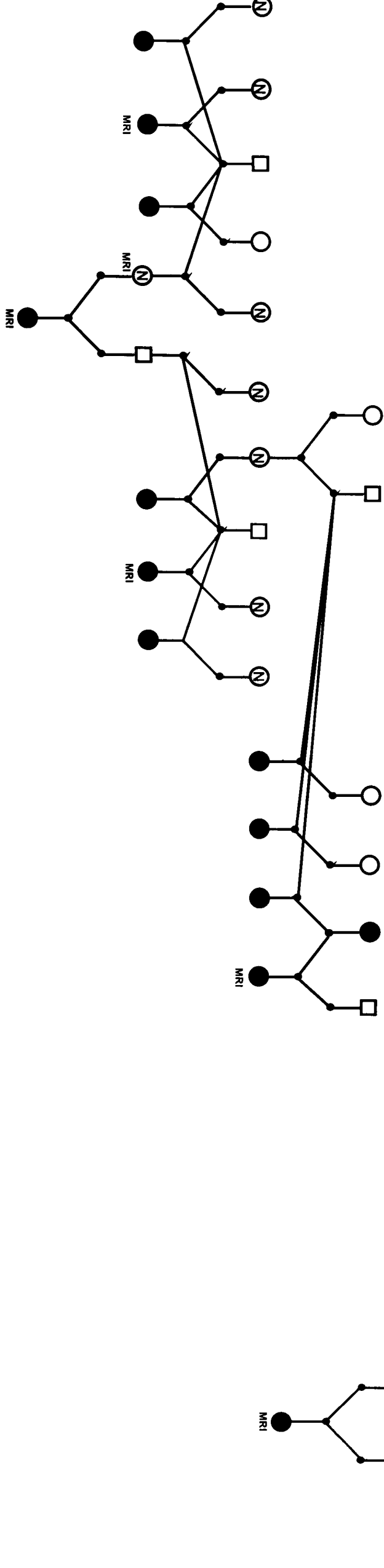
Whilst there is considerable evidence that familial clustering of endometriosis occurs in humans (Kennedy *et al.*, 1995, Moen & Magnus, 1993, Simpson *et al.*, 1980) no studies have been conducted to investigate this aspect of endometriosis in the non-human primate. The detailed records held at the WRPRC provided the ideal opportunity for such an investigation to be conducted. Over the 15 year study period autopsies had been conducted on 399 female rhesus macaques aged over 4 years, and 81 had endometriosis at the time of autopsy. Of the animals with endometriosis, 41% were related to at least one other animal with the disease. MRI was conducted on a group of the living descendants of these animals to increase the sample size and the statistical power. MRI has been used successfully in the past to diagnose both endometriosis and adenomyosis in non-human primates (Waterton *et al.*, 1993, Waterton *et al.*, 1992).

In this study, MRI has been used successfully for the first time to diagnose endometriosis in large numbers of rhesus macaques. The accuracy of this test was not compared to the diagnostic reference standard, *i.e.* laparoscopy, because of cost and the animals involvement in other experiments. However, over time, as these animals die and are autopsied, confirmation of disease status will become possible. One

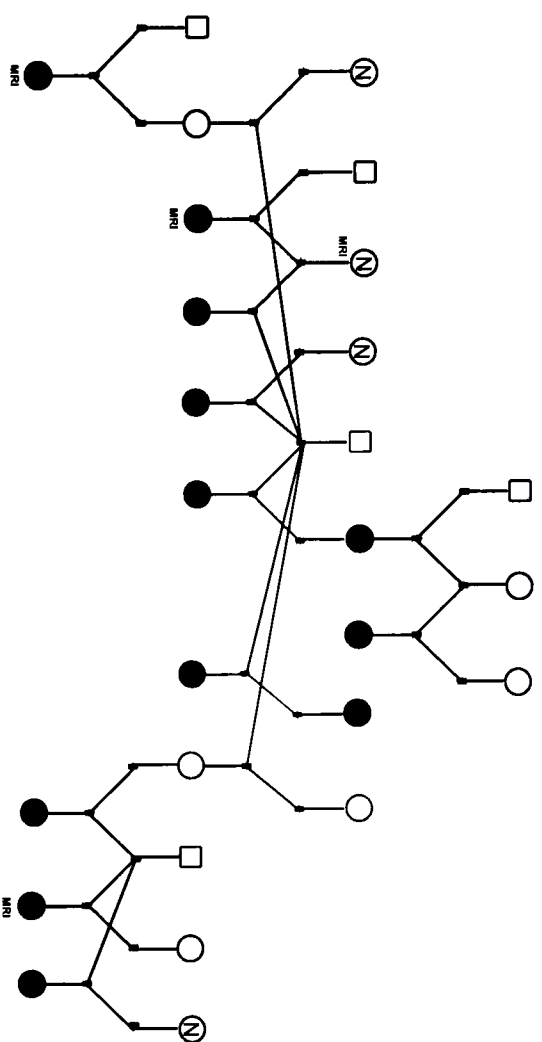
Pedigree I



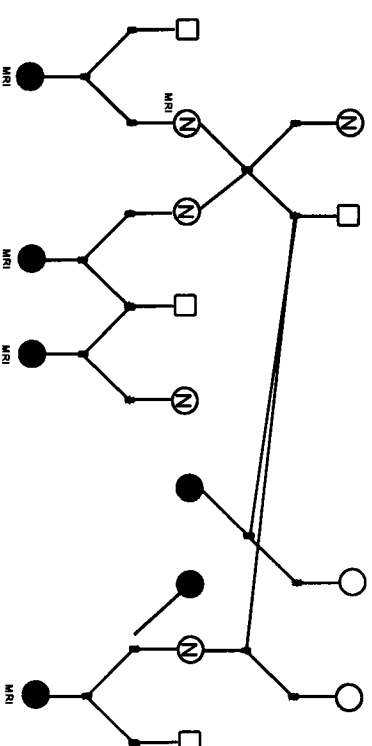
Pedigree II



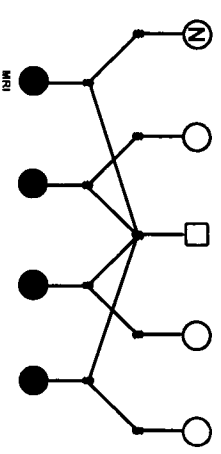
Pedigree III



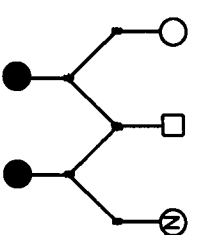
Pedigree IV



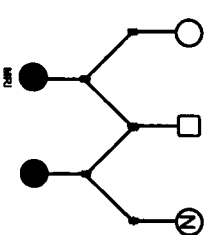
Pedigree V



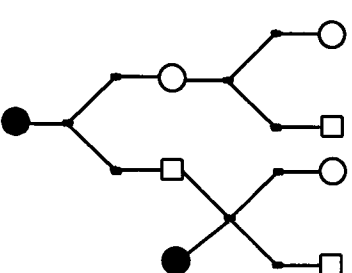
Pedigree VI



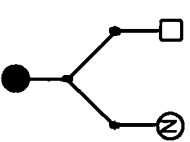
Pedigree VII



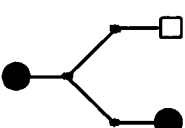
Pedigree VIII



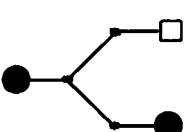
Pedigree IX



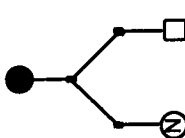
Pedigree X



Pedigree XI



Pedigree XII



animal had a normal pelvis at MRI and 6 months later a large endometriotic cyst was found at laparoscopy. This may indicate the shortcomings of MRI as a diagnostic tool for this disease. However, MRI has been shown to be very accurate for the diagnosis of cysts over 1cm in diameter (Takahashi *et al.*, 1994) and it is more likely that disease in this animal developed after the MRI scan. This also illustrates the dangers of defining an animal as unaffected by radiological criteria as it may go on to develop disease at a later stage.

The definition of animals scanned as affected or unaffected was problematic. Although animals with lesions or cysts >1cm in diameter were unequivocally affected, those with endometriosis <1cm were less easily categorised. Hence, the affected animals were divided into two groups based on the size of the implants and in the 113 animals scanned the prevalence of endometriosis ranged from 7% (implants >1cm) to 19% (all endometriosis).

The macaques that were scanned were selected to increase the probability of finding additional, affected animals. The criteria for selection were age ≥ 10 years (as no animal under this age was found to have endometriosis at autopsy) and the presence of at least one affected relative. If endometriosis has a familial tendency in this species, a significantly higher prevalence of disease in the group scanned compared to the overall colony prevalence would be expected. However, the overall colony prevalence in animals ≥ 10 years was actually higher at 29%. This may in part be due to the fact that the mean age of the group MRI scanned was lower at 18 years compared to the mean age of 21 years for those affected at autopsy. Furthermore, the group of animals selected for MRI had been less exposed to the two known risk factors compared to those affected at autopsy, with 32% exposed to ≥ 1 hysterotomy and 14% exposed to >3 oestradiol treatments in the MRI group compared to 37% and 27% respectively in the autopsy group.

From the MRI data it can be concluded that because of the exposure to known risk factors, the prevalence of disease in the MRI group provides evidence neither for nor against a familial component in this species. This study has shown, however, that MRI can be effectively used to diagnose endometriosis in the rhesus macaque and that

it provides a valuable tool for defining the disease status of a group of animals for subsequent genetic analysis.

3.3 SEGREGATION ANALYSIS

Introduction

Mendel's laws form the basis of all complex analyses to determine the mode of inheritance of a trait or disease. The first law states that alleles at a single locus segregate with equal probabilities and the second law states that alleles at different loci assort independently of each other. This second law of course does not hold true for alleles in linkage; a phenomenon exploited in genetic linkage analysis. Mendel observed the segregation of alleles in constructed matings and the ratios of offspring produced. Thus, in a simple $Aa \times Aa$ mating, the expected Mendelian ratio of offspring is $\frac{1}{4}$ AA, $\frac{1}{2}$ Aa and $\frac{1}{4}$ aa. In segregation analysis, the goal is to test for compatibility with expected Mendelian ratios. In this type of analysis, models of inheritance are tested against pedigree data to determine the one which best explains the data.

For autosomal dominant traits, the segregation ratio (number of affected offspring over total number of offspring) can be calculated directly from the pedigree data because the trait is dominant and therefore all heterozygotes express it. If the trait is segregating in a genetic manner then the segregation ratio should not be significantly different from expected Mendelian ratios. For example, if families with one affected parent for a rare, autosomal dominant trait are collected then 50% of offspring would be expected to exhibit the disease phenotype.

Segregation ratios can also be calculated directly for X-linked traits. For an X-linked recessive disease no father to son transmission will occur, and for an X-linked dominant disease an affected father will always pass the disease gene on to his daughters. It is important not to confuse X-linked traits with sex-limited traits. A disease gene may be inherited autosomally but, due to physiological or anatomical reasons, only be expressed in one sex. For example, endometriosis may be an autosomal trait that is only expressed phenotypically in women.

For recessive traits, it is not possible to calculate the segregation ratio through an affected parent, because for a rare disease, most children will be unaffected. The solution is to look at segregation in siblings by identifying an affected proband and the proportion of affected siblings. For heterozygous parents (Aa), the expected ratio of affected offspring (aa) would be $\frac{1}{4}$ and this can be compared to the observed proportion in the sample. However, the method of ascertainment can introduce bias which must be corrected for. If we compare the analysis for an autosomal dominant disease, where an affected parent is identified, many of the children will be unaffected and often all children in one family will be unaffected. By looking at only an affected person's siblings, no families such as this will be identified, resulting in a disproportionately high number of affected individuals. There are a number of methods which may be used to overcome ascertainment bias.

Truncate (or complete) ascertainment applies to situations in which all families with at least one affected individual are equally likely to enter the study. Single ascertainment is when the probability of finding more than one affected individual (or proband) per family is very low and hence the chance of entering the study is proportional to the number of affected individuals in the family. Incomplete, multiple ascertainment occurs most frequently and is intermediate between truncate and single ascertainment.

The most commonly used methods for each of these situations are outlined below, using the following notation:

s = total number of offspring in family

r = total number of affected offspring in a family

n = total number of families studied

p = the probability that an offspring is affected = segregation ratio

$q = 1 - p$ = the probability of being unaffected

$R = \sum r$ = the total number of affected offspring across all families

$T = \sum s$ = the total number of offspring across all families

The direct method

This method is used with truncate ascertainment. The fact that q^s is the probability that a family of size s produces no affected offspring means that $1 - q^s$ is the probability of that family being included in the study. The proportion of affected offspring in a family, given that the family has at least one affected offspring and has therefore been included in the study, is given by $p(1 - q^s)$. The total number of affected offspring, r , in a family is therefore $sp(1 - q^s)$. To estimate the segregation ratio by this method, the maximum likelihood estimate for the value of p which satisfies $R = \sum[sp(1 - q^s)]$ is determined.

The proband method

The proband method is used with single ascertainment. In this method, the segregation ratio is estimated as:

$$\begin{aligned} p &= \Sigma(r - 1) / \Sigma(s - 1) \\ &= (R - n) / (T - n) \end{aligned}$$

The general proband method and the singles method

Both the general proband method and the singles method are used with incomplete multiple ascertainment. When this has been the method of ascertainment a proper analysis requires use of computer programs to conduct a more complex analysis. In this case the segregation ratio is estimated as:

$$p = (R - P_1) / (T - P_1)$$

where P_1 is the number of families with only one proband and the variance estimated as:

$$\text{var}(p) = \frac{(R - P_1)(T - R)}{(T - P_1)^3} + \frac{2P_2(T - R)^2}{(T - P_1)^4}$$

where P_2 is the number of families having exactly two probands. Because $q = 1 - p$, the expression for variance can be rewritten as:

$$\text{var}(p) = \frac{pq}{T - P_1} + \frac{2P_2q^2}{(T - P_1)^2}$$

The results of a segregation analysis must be interpreted with caution. Firstly, misclassification bias may occur if the phenotype is incorrectly defined. For example, with endometriosis there is difficulty classifying an individual as ‘unaffected’ because the age at onset can vary considerably. Furthermore, other variables such as time since last pregnancy and number of pregnancies may reduce the chance of developing the disease. Traits may also exhibit reduced penetrance or variable expression which can produce deviations from the expected Mendelian ratio. Secondly, as discussed in detail in the previous chapter, environmental risk factors may act as confounders. In Chapter 2, hysterotomy and oestradiol implants (>3) were identified as risk factors in the rhesus macaque, and these must be taken into consideration in the analysis. Finally, the incorrect conclusion may be reached if the sample size is too small and there is insufficient power to reject the model (type II error) and conversely even if the model is correct it may be rejected in favour of another model (type I error). The presence of a statistically proven Mendelian pattern of inheritance does not provide conclusive evidence for a genetic component, nor does the absence provide conclusive evidence against a genetic component.

Despite the problems of segregation analysis, particularly in complex diseases such as endometriosis, the results may provide some evidence that a disease is genetic, encouraging confirmation of the results by further studies and ultimately by linkage analysis.

Methods

Creation of M-link format file

A database in LINKAGE format (Terwilliger & Ott, 1994) suitable for use with the program M-link (Ott, 1989, Weeks *et al.*, 1990, Cottingham *et al.*, 1993) was created using Microsoft Excel software. The database took the form shown in Table 3.6. A key to the codes used is included in Table 3.7. Animals were included if they were related to affected animals in the 12 originally defined pedigrees containing 2 or more affected animals. In practice, this entailed working backwards to feral or founding

ancestors and then including all descendants of these primary ancestors in the database. A total of 1,807 animals were included. S-link was used to conduct simulations of linkage analysis (Ott, 1989, Weeks *et al.*, 1990, Cottingham *et al.*, 1993). Statistical comparisons of proportions were made using Fisher's exact test.

1	2	3	4	5	6	7	8	9	10	11	12	13
Pedigree	Animal	Dam	Sire	Sex	Disease Status	DNA Available	MRI Given	MRI Disease Status	Age at MRI	Age at diagnosis	Age at death	Hermaphrodite
1	r78002	rh1573	rh038z	2	2	2	0	-9	-9	-9	17	0
1	r78051	rh1519	rh1606	2	1	1	0	-9	-9	-9	4	0
1	r78064	rh-r40	rh1445	2	1	2	0	-9	-9	-9	14	0
1	r78080	rh0526	rh1756	2	0	1	0	-9	-9	-9	-9	1

14	15	16	17	18	19	20	21	22	23
Stage of disease	Ovaries affected	Date of Death	Age at death/or current age	Origin	Current Status	Laparoscopy	C/S	Hormone E2	Hormone Other
3	2	06/04/95	17	1	1	2	3	1	0
0	0	-9	4	0	0	-9	0	0	-9
0	0	-9	14	0	0	-9	0	0	-9
0	0	01/06/78	0	1	1	0	0	0	0

24	25	26	27
Ovariectomy	Ovariectomy age	Blood Available	Tissue Available
0	-9	YES	YES
0	-9	NO	NO
0	-9	NO	YES
0	-9	NO	NO

Table 3.6. The 27 fields in the M-Link compatible Microsoft Excel database created to conduct segregation analysis on the pedigree of 1,807 animals. The column numbers and headings are shown along with four animal records included as examples. The codes used in the database are shown in Table

3.7.

Column		Description and/or code used
1	Pedigree	Pedigree number
2	Animal	ID of rhesus macaque
3	Dam	ID of mother of animal
4	Sire	ID of father of animal
5	Sex	1 = male, 2 = female
6	Hermaphrodite	0 = no, 1 = yes
7	Disease status	0 = unknown, 1 = unaffected, 2 = affected
8	DNA available	DNA available for genotyping, 1 = no, 2 = yes
9	MRI given	0 = no, 1 = yes
10	MRI disease status	.-9 = unknown, 1 = unaffected, 2 = affected
11	Stage of disease	0 = unknown/not applicable, 1 = mild, 2 = moderate, 3 = severe
12	Ovaries affected	0 = unknown, 1 = no, 2 = yes
13	Date of death	Date of death, -9 = unknown
14	Age	Age at death/Current age
15	Age at diagnosis	Age at which laparoscopic/surgical diagnosis made, -9 = unknown/not applicable
16	Age at death	Age at death for animals diagnosed at autopsy
17	Age at MRI	Age at which MRI scan performed
18	Origin	0 = unknown, 1 = non-feral, 2 = feral
19	Current status	0 = unknown, 1 = dead, 2 = alive
20	Laparoscopy	.-9 = unknown, 0 = none, x = number of procedures
21	Hysterotomy	.-9 = unknown, 0 = none, x = number of procedures
22	E ₂ implant	.-9 = unknown, 0 = none, x = number of procedures
23	Hormone other	.-9 = unknown, 0 = none, x = number of procedures
24	Ovariectomy	.-9 = unknown, 0 = no, 1 = yes
25	Ovariectomy age	Age at ovariectomy
26	Blood	Blood sample available
27	Tissue	Tissue available

Table 3.7. Key to the codes used in the Microsoft Excel database in M-Link compatible format.

Offspring	Status of mother		
a) All animals:	Unknown	Unaffected	Affected
Unknown	599	254	103
Unaffected	105	107	35
Affected	28	29	11
SR ¹	21.1%	21.3%	27.5%
SR ²	3.8%	7.4%	7.7%
b) Unaffecteds ≥15 years only:			
Unknown	778	216	125
Unaffected	42	29	13
Affected	36	21	11
SR ¹	46.2%	42.0%	45.8%
SR ²	4.2%	7.9%	7.4%

Table 3.8. Analysis of all affected individuals in the colony including a) all unaffected animals and b) only those ≥15 years old at the time disease was determined. Segregation ratios (SR) are expressed as percentages; SR¹ is the ratio of affected/affected + unaffected animals and SR² is the ratio of affected/affected + unaffected + unknown animals.

Results

In the first series of analyses, segregation ratios were calculated for all animals in the pedigree. Initially, all unaffected animals were included in the analysis and then only unaffected animals ≥15 years old. This discrimination was chosen based on the observation that the chance of a colony animal developing endometriosis increases with age, reaching a plateau at approximately 15 years (as illustrated in Figure 2.9, Chapter Two). However, the cut off age of 15 is arbitrary, and it is potentially misleading to define an animal of any age as unaffected. The results of this analysis are presented in Table 3.8.

For any disease with a genetic basis, an affected mother is expected to produce affected offspring more often than an unaffected mother. However, as shown in Table 3.8, the proportion of affected offspring born to affected mothers does not differ significantly, using Fisher’s exact test, from the proportion born to unaffected

mothers. For all animals of known disease status, females without endometriosis had 29/136 (21.3%) affected offspring compared to females with endometriosis who had 11/40 (27.5%) affected offspring. The same analysis conducted using only unaffected animals ≥ 15 years old similarly does not show a significant difference, with 21/50 (42.0%) and 11/24 (45.8%) offspring affected respectively.

Furthermore, the prevalence of endometriosis amongst animals born to an affected mother would be expected to be significantly higher than the overall prevalence in the colony if the disease had a genetic basis. The prevalence of endometriosis in the whole colony, for animals autopsied at ≥ 15 years, was 68/176 (39%; 95% CI 31-46%). This figure was not significantly different from the prevalence amongst offspring of affected mothers which was 45.8%.

As males do not develop endometriosis and there is no known male phenotype, the above analysis cannot be conducted in the same way for fathers. An alternative method is to look at the offspring born to parents with one or more daughter with endometriosis. Altogether 57 females and 32 males had at least one affected daughter. Unfortunately, the phenotype of many of the daughters is unknown. For example, as shown in Table 3.9, the 32 males with ≥ 1 affected daughter had 524 daughters in total and the phenotype was unknown in 70% (365/524). There were a number of reasons for the preponderance of unphenotyped animals, including 1) animals were autopsied aged < 4 years and therefore not included in the study; 2) animals left the colony before death; 3) animals were still alive but not ≥ 9 years and therefore not eligible for the MRI study, and 4) although eligible for MRI, some animals could not be scanned because of their involvement in other studies.

The lack of data made it difficult to determine the true segregation ratio. It may not be representative to include animals for whom the phenotype is unknown in a ratio calculation. Crude ratios have, therefore, been calculated with three different denominators: 1) offspring of all categories, 2) unaffected + affected offspring and 3) unaffected ≥ 15 years old + affected offspring. As shown in Table 3.9, the segregation ratio for females with at least one affected daughter could range from 41% to 87% and the segregation ratio for males with at least one affected daughter from 11% to 52%. These crude segregation ratios may be misleading because many of the

offspring are half siblings, *i.e.* the families are not independent, and the majority of mothers (68%) had only one daughter of known status.

Status of Daughter	Parent	
	Father (n = 32)	Mother (n = 57)
Unknown	365	69
Unaffected	100	22
Unaffected (≥15 years)	55	9
Affected	59	62
SR (all offspring)	11.3%	40.5%
SR (unaffected + affected)	37.1%	73.8%
SR (unaffected ≥15 years + affected)	51.8%	87.3%

Table 3.9. Analysis of offspring from those parents with at least one affected daughter, showing the proportion of offspring in each status group. Crude segregation ratios (SR) are shown expressed as a percentages and the denominator used is given in parentheses.

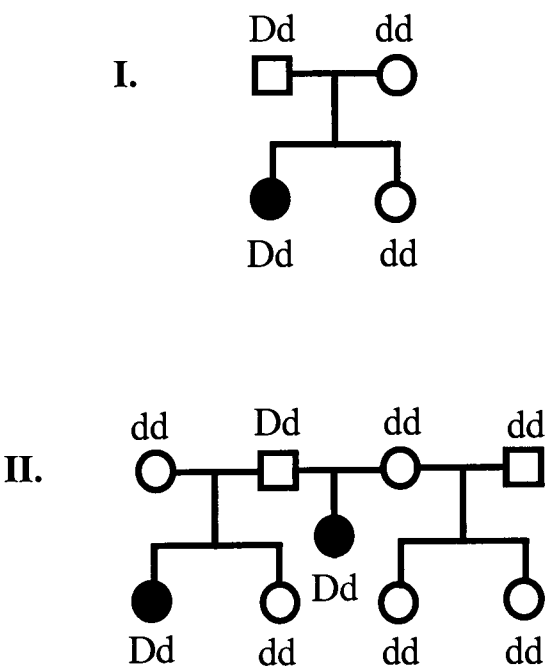


Figure 3.10. Diagram showing the expected proportions of affected offspring for an autosomal dominant disease where ‘Dd’ represents the disease genotype. For full siblings (I) 50% of offspring born to a disease carrying parent would be expected to be affected compared to (II) 25% of half siblings of an affected individual.

One would expect 50% of the female siblings (and 25% of the female half-siblings) of affected animals to have endometriosis, if the disease is autosomal dominant (Figure 3.10). However, for mothers with at least one affected offspring, there were only 27 siblings of known status resulting in a small sample size. Of these siblings, the proportion affected was 5/27 (19%) and for animals ≥ 15 years old the proportion was 5/14 (36%). The sample size was higher for fathers with at least one affected offspring, with 27/127 (21%) and 27/82 (33%) affected respectively. The prevalence of endometriosis in the whole colony, for animals autopsied at ≥ 15 years old, was 68/176 (39%), not significantly different from the disease prevalence in families where ≥ 1 animal is affected.

If the sample is limited to those animals who were not exposed to the known risk factors of >3 oestradiol treatments or ≥ 1 hysterotomy, only 12 half-sibling pairs exist.

Discussion

The results of the segregation analysis are inconclusive. Although the raw data and family tree diagrams suggest that familial clustering does occur, they may be misleading because not all the unaffected animals are shown. The main shortcoming is that disease status is defined for only a small proportion of animals, resulting in small sample sizes. In addition, defining animals as unaffected is problematic due to the fact that an animal may develop endometriosis at any age. The exposure of many animals to risk factors which were found to increase significantly the probability of developing endometriosis in Chapter Two and the resulting high prevalence of endometriosis in the colony, which may mask any genetic effects, also reduced the power of the analysis. The predominance of half-sibling pairs adds another layer of complexity and segregation analysis programs, which are primarily concerned with human pedigrees, are not designed for use with extensive animal pedigrees. It is therefore difficult to draw any clear conclusions from these data.

Nevertheless, although not statistically significant, there was a slight increase in the number of affected offspring born to affected mothers compared to unaffected mothers. For an autosomal dominant disease, we would expect 25% of the half-siblings of an affected animal to have the disease. However, with a colony prevalence of 39% in animals autopsied at age 15 or older, it is impossible to detect a change in

the prevalence due to genetic effects. Indeed, a decrease was observed in the prevalence for half-siblings of animals with disease, relative to the prevalence in the entire colony.

Ideally, the animals that were exposed to the two known risk factors should be removed from the analysis. Unfortunately, so many animals were exposed to either or both of these risk factors that there would only have been 12 half-sibling pairs remaining.

In women, the relative risk of endometriosis is 6-9 times higher if a first degree relative is affected. It is reasonable to extrapolate and conclude that half-siblings would therefore be at approximately 3-4 times increased risk. Due to the complexities of the data it has not been possible to discover whether such an increased risk occurs for half-siblings of rhesus macaques with endometriosis.

3.4 STUDIES ON THE FEASIBILITY OF LINKAGE ANALYSIS

Introduction

The extended pedigrees of rhesus macaques with defined disease status at the WRPRC are a potentially valuable resource for conducting linkage analysis, for both endometriosis and other diseases. Before such a study could be initiated a number of practicalities would need to be addressed such as whether blood and/or tissue for DNA extraction is available from deceased animals; whether DNA can be extracted from archival tissue, and the lack of a microsatellite marker set for this species. The work presented in this section assesses the feasibility of conducting linkage analysis for endometriosis in the WRPRC colony.

Methods

The first aim was to determine whether it was feasible to use for PCR DNA fragments extracted from tissue that has been stored in formalin for long periods of time. DNA was extracted from formalin-fixed rhesus macaque liver tissue using Qiagen™ tissue extraction kits. DNA extracted from this tissue was PCR amplified using primer pair D9S318 (purchased from Genentech, USA), known to amplify a 275-340 bp fragment of the rhesus genome (Dr L.A. Knapp, personal communication). Briefly, 100 ng of

DNA was amplified in a 50 µl reaction volume with 200 µM of each dNTP, 4 mM MgCl₂, 1x Q-solution™ (Qiagen™), 0.5 µM forward and reverse primers and 1 unit of Taq polymerase (Qiagen™). The reaction mixture was overlaid with mineral oil (Sigma) and then subjected to an initial denaturation temperature of 94 °C then 45 cycles of denaturation at 94 °C for 30 sec, annealing at 48 °C for 30 sec and extension at 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. The PCR product was visualised under UV light on a 1.5% agarose gel containing 1 µg/µl ethidium bromide.

To assess the feasibility of developing a microsatellite marker set for the rhesus macaque, high molecular weight, genomic DNA was extracted using phenol/chloroform from 5ml EDTA anti-coagulated blood samples of 10 unrelated female rhesus macaques from the University of Oxford colony. The rhesus genomic DNA was used to determine which primer pairs for polymorphic, human dinucleotide markers would amplify a product from the rhesus genome.

PCR was conducted in a 15 µl reaction volume containing 60 ng DNA, 250 µM of each of each dNTP, 0.6 units AmpliTaq Gold™ (Perkin Elmer), a 1x PCR buffer, 250 µM MgCl₂ and 0.33 µM forward and reverse primers. The forward primers were labelled with one of the fluorescent dyes FAM (6-carboxyfluorescein), HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein) or TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein). Panels of primers were grouped according to the expected size of product. The PCR reaction was overlaid with mineral oil and subjected to the thermal cycling profile shown in Table 3.11.

Step	Temperature	Time	Cycles
Activation of AmpliTaq	94 °C	12 min	
Melt	94 °C	15 sec	
Anneal	55 °C	15 sec	10
Extend	72 °C	30 sec	
Melt	89 °C	15 sec	
Anneal	55 °C	15 sec	20
Extend	72 °C	30 sec	
Final extension	72 °C	10 min	

Table 3.11. Thermal cycling profile for PCR using primer pairs for human polymorphic markers and rhesus macaque genomic DNA.

PCR samples were mixed with a gel-loading cocktail containing deionized formamide, blue dextran and size standards labelled with TAMRA (N,N,N',N'-tetramethyl-6-methyl carboxyrhodamine) (GeneScan-500, Perkin Elmer). Samples were loaded onto a 0.2 mm thick, 36 cm long, 6% polyacrylamide sequencing gel and run on an ABI prism 377 automated sequencer (Perkin Elmer). Results were analysed using Genescan™ and Genotyper™ software (Perkin Elmer).

Results

Genotyping

DNA was successfully extracted from rhesus macaque formalin-fixed liver tissue and a PCR product obtained using the primers known to work in this species. Either tissue and/or blood for DNA extraction would be available from an estimated 692/1807 (38%) of the animals. Of the animals with known disease status, DNA was available from an estimated 319/430 (74%).

Examples of the Genotyper™ profiles obtained with rhesus macaque genomic DNA are shown in Figure 3.12. The standard methods for human DNA worked well in this species and this figure illustrates the clarity of the results.

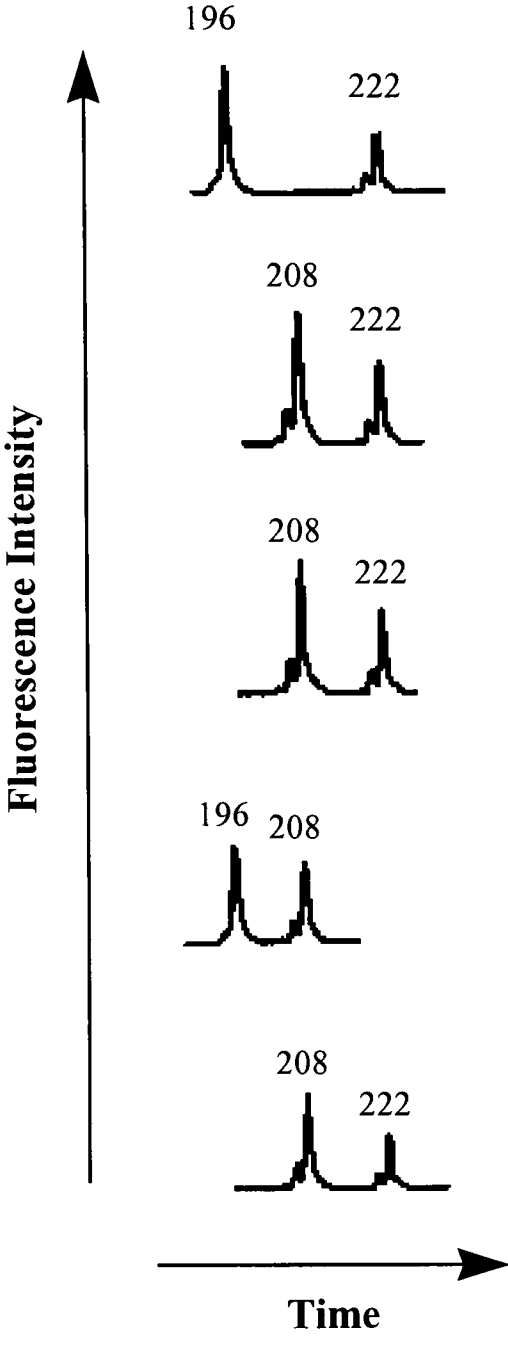


Figure 3.12. PCR products arising from amplification of rhesus genomic DNA samples with primers for polymorphic, human dinucleotide markers. Sequential lines show unrelated animals and the numbers are the approximate product sizes in base pairs. This figure illustrates the clarity of the results obtained using this method and the excellent signal-to-noise ratio.

Of the 333 human markers examined, 92 (28%) successfully amplified a product. The position of some of these markers on a human chromosome map is illustrated in Figure 3.13. Coverage of some chromosomes is better than others, presumably illustrating homology between the rhesus and human genome.

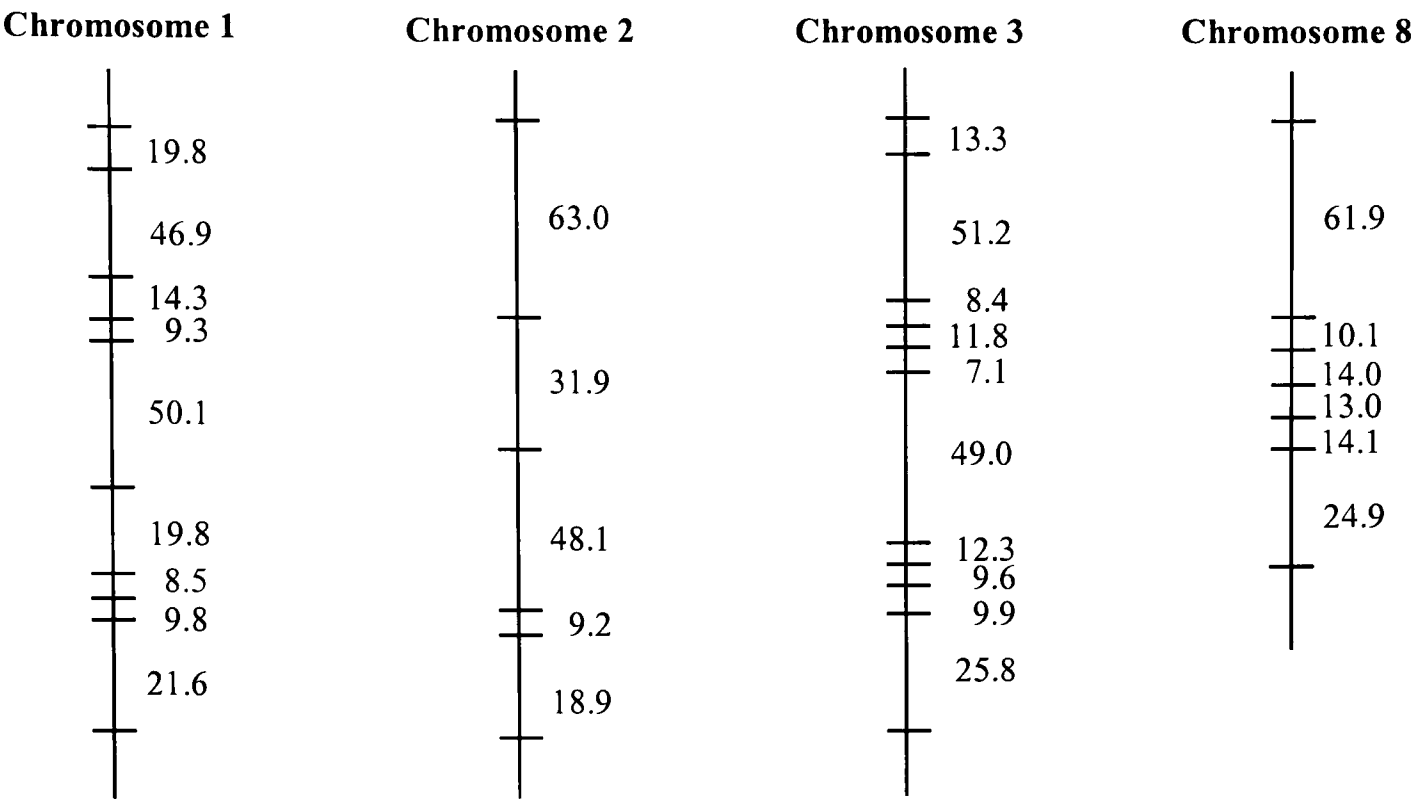


Figure 3.13. Illustration of the relative positions of some of the primers for polymorphic, human dinucleotide markers which successfully amplified a PCR product from the rhesus macaque genome for human chromosomes 1, 2, 3 and 8. Distances between markers are given in centimorgans (cM).

Discussion

Despite the availability of large colonies of rhesus macaques, with archival tissue and detailed records on disease history, longevity etc., surprisingly little research has been conducted to develop a linkage marker set in this species. Most publications only report a small number of newly identified microsatellite markers, often in non-human primate species other than the rhesus macaque (Gray & Jeffreys, 1991, Inoue & Takenaka, 1993, Jurka, 1995, Krawczak *et al.*, 1993, Smith, 1982, Stone *et al.*, 1993). Human markers have also been tested in the baboon and over 280 microsatellite markers identified for use in linkage studies in this species (Morin *et al.*, 1998).

The preliminary studies that have been conducted, using oligonucleotide primers for human microsatellite markers, on the rhesus macaque genome identified 92 potentially new microsatellite markers in this species. This study demonstrated that, if further work were carried out, this would be a suitable method for developing a genome-wide marker set in this species. Further investigations would need to be conducted to determine which regions are polymorphic, to screen larger numbers of human markers and to optimise conditions. However, from this preliminary work it

appears that the Taq polymerase and PCR conditions used are robust and optimisation is unnecessary for many of the primer pairs. All products amplified revealed high fluorescence intensities indicating the presence of ample labelled PCR product. If this strategy is adopted to develop a rhesus macaque microsatellite marker set, it is likely that some chromosomes would be better covered than others, according to the degree of genetic homology between *Macaca mulatta* and *Homo sapiens*.

3.5 CONCLUSION & SUMMARY

In this chapter, it has been demonstrated that MRI can be successfully used in the rhesus macaque to diagnose endometriosis and that the technique presents no practical difficulty in this species. The results of the segregation analysis are inconclusive, however, over time, as more animals are autopsied or diagnosed by other means, there may be sufficient data to confirm or disprove a genetic basis to endometriosis in the colony. The preliminary studies into the feasibility of genetic linkage analysis have shown that it would be possible to develop a linkage mapping set in this species and that there is genetic material available from animals in the colony.

Chapter Four

Familial endometriosis

4.1 INTRODUCTION

In view of the evidence that endometriosis is a complex multifactorial trait, OXEGENE (the Oxford Endometriosis Gene Study) was established, the objective being to collect affected pedigrees and conduct a genome-wide search for endometriosis susceptibility loci. Linkage analysis has been successfully used in the past to learn more about the aetiology of other complex traits, such as insulin dependent diabetes mellitus (Davies *et al.*, 1994). The method is particularly attractive for endometriosis, as so little is known about the mechanisms underlying the aetiology of the disease.

In this chapter, the methods used to recruit families for the OXEGENE Study are presented, including use of the World Wide Web (WWW) and MRI to identify study participants. Large numbers of women with documented endometriosis have been identified and a database established which allows unique observations to be made. For example, comparisons of disease severity, age at symptom onset and delay in diagnosis are presented.

This work is presented as separate, self-contained studies in each of four sections. Section 4.2 details the collection of affected families and an analysis of their characteristics, including a comparison of different disease severity groups. Section 4.3 details the use of MRI to recruit families for the study and to assess the relative risk of disease in first-degree relatives of affected women. Section 4.4 contains an analysis of the delay in the diagnosis of endometriosis experienced by patients and Section 4.5 contains data on the twin pairs recruited.

4.2 COLLECTION OF AFFECTED FAMILIES

Introduction

The OXEGENE Study was established to identify and collect blood samples from suitable families with the aim of conducting a genome-wide search for endometriosis susceptibility genes. Families with two or more affected individuals were invited to participate but affected sister pairs were particularly sought to conduct affected sib-pair analysis. This section describes the types of families that were recruited and their characteristics, and discusses the different recruitment methods used.

Methods

Families with endometriosis were recruited using:

- 1) Advertisements in the newsletters of the Endometriosis Association (USA), the Endometriosis Society (UK), and the Endometriosis Associations of Ireland, Canada, Australia, New Zealand, Germany, Norway and Sweden.
- 2) Referral to the study by British gynaecologists.
- 3) Identification of affected individuals through the records of the John Radcliffe Hospital.
- 4) Recruitment and collection by a large, international group of collaborating gynaecologists (Table 4.1).
- 5) Advertisement on the OXEGENE Study WWW page (www.medicine.ox.ac.uk/oxegene/oxegene.htm).

The patients identified were invited to participate in the study. They were sent a simple questionnaire and consent form to complete. The questionnaire requested the date of any gynaecological surgery. Consent was also obtained to allow access to the surgical records. Copies of the surgical records were obtained by writing to the relevant gynaecologist(s), and evidence of any histological confirmation was obtained, when available. The stage of endometriosis was assessed by Stephen Kennedy in accordance with the rAFS classification system and divided into Stage A (rAFS stage I-II or minimal-mild) and Stage B (rAFS stage III-IV or moderate-severe) disease. The separation of patients into these two disease severity groups was important because a) they may represent different phenotypic subsets; b) many women may have minimal disease at some stage of their reproductive lives, but

Centre	Collaborator
CALIFORNIA	Dr Adamson
MOSCOW	Prof. Adamyan
NEW YORK	Dr Batt
SWEDEN	Dr Bergqvist
OTTAWA	Dr Claman
MILAN	Dr Vercellini
CHICAGO	Prof. Dmowski
LONDON	Mr Edmonds / Miss Rose
AUCKLAND	Prof. Farquhar
MADRID	Dr Fernandez-Shaw
OMAHA	Dr Hilgers
BELGIUM	Prof. Koninckx
MEMPHIS	Dr Martin
NORWAY	Dr Moen
DUBLIN	Prof. O'Herlihy/Miss Wingfield
CONNECTICUT	Prof. Olive
UTAH	Dr Peterson
SAN ANTONIO	Prof. Schenken
HEIDELBERG	Dr Sillem
SYDNEY	Mr Driscoll
DETROIT	Dr Diamond
SOUTH AMERICA	Dr Correa
MELBOURNE	Prof. Healy

Centre	Collaborator
HONG KONG	Dr Loong
HONG KONG	Dr Stevenson
ABERDEEN	Mr Hamilton
OREGON	Dr Redwine / Ms Oberst
LOUISIANA	Dr Vernon
ATLANTA	Ms Laux / Dr Albee
STANFORD	Dr Giudice
SOUTH AFRICA	Prof. van der Spuy
MALAYSIA	Prof. Arumugam
INDIA	Dr Deenadayal
TOKYO, JAPAN	Dr Yoshinori
YOKOHAMA, JAPAN	Prof. Hiroshi
TURKEY	Dr Timur
MELBOURNE	Prof. Maher
FRANCE	Dr Canis
NETHERLANDS	Dr van der Linden
WALES	Prof. Shaw
TAIWAN	Dr Chang
INDIA	Dr Sr Lillian
NEW DELHI	Dr Mittal
SINGAPORE	Prof. Clement
PARIS, FRANCE	Dr Darai

Centre	Collaborator
HONG KONG	Dr Tang
SINGAPORE	Dr Loh
SYDNEY	Dr Fay
ISTANBUL	Dr Oral
JAPAN	Dr Masumi Inui
SYDNEY	Prof. Fraser
MEXICO	Prof. Livas
PUERTO RICO	Dr Flores
FRANKFURT	Prof. Starzinski-Powitz
ISTANBUL	Dr Attar
LONDON	Miss Davies
TENNESSEE	Dr Lin
MANCHESTER	Mr Seif
INDONESIA	Dr M. Darmasetiawan
ECUADOR	Dr Rodriguez
IRELAND	Mr O'Connor
TORONTO	Dr Lie
CANADA	Prof. Tulandi
SINGAPORE	Prof. TambyRaja

Table 4.1. OXEGENE study collaborators involved in the recruitment of patients for the study and collection of blood samples from their region and/or country.

clinically there is little doubt that a woman with stage B disease is affected, and c) in a genetic linkage analysis, including women with only stage B disease should increase the power to detect linkage. Women were excluded from the study if the operative records could not be obtained or if there was any doubt about the diagnosis. Blood samples (60 mL, EDTA anti-coagulated) were collected from extended families or from affected sister-pairs and their parents, and stored at -80 °C for subsequent genomic DNA isolation. The study had the approval of the Central Oxford Research Ethics Committee (COREC, No. 93.151). Comparisons of means were made using an unpaired *t*-test and of proportions using Fisher's exact test, unless otherwise stated.

Results

Participants in the OXEGENE study

Recruitment for the study is ongoing. Data on those patients recruited by the Oxford group between 1993 and 1998 are presented here as being relevant to this thesis, and those recruited by the international collaborators are excluded. Over the defined time period a total of 944 families with endometriosis, containing 1,367 individuals, entered the OXEGENE study. The majority of the women were residents of the UK (38%) and the USA (50%). The remaining participants were from Canada, Australia, New Zealand, Ireland, Germany, Italy, Mexico, Argentina, Bermuda, Chile, The Netherlands, Hong Kong, Israel, The Philippines, Slovenia, Sweden, Thailand, Denmark, The Channel Islands, Brazil and South Africa.

The operative records were obtained from 648 women and the findings are summarised in Table 4.2. The mean age ($\pm$ SD) of women with confirmed endometriosis was 40.5 ± 10 (range 22-84). Histological confirmation was available in 26 (11%) of the 235 women with stage A disease, and in 144 (45%) of the 323 women with stage B disease.

Disease status	N	%
Stage A	235	36
Stage B	323	50
Endometriosis confirmed [†]	34	5
No endometriosis	56	9
Totals	648	100

Table 4.2. Disease severity in women who entered the OXEGENE study. Stage A is rAFS minimal-mild disease and stage B is moderate-severe disease. [†]Insufficient information to determine disease stage.

Procedure	≥ 1 Procedure	≥ 2 Procedures	Range	Mean (± SD)
Laparoscopy	392 (70%)	137 (25%)	0 - 6	1.1 (± 1.0)
Laparotomy	160 (29%)	29 (5%)	0 - 9	0.4 (± 0.7)
Hysterectomy	128 (23%)	--	0 - 1	0.2 (± 0.4)

Table 4.3. Exposure to surgical procedures in the 558 women with endometriosis classified as either stage A (minimal-mild) or stage B (moderate-severe).

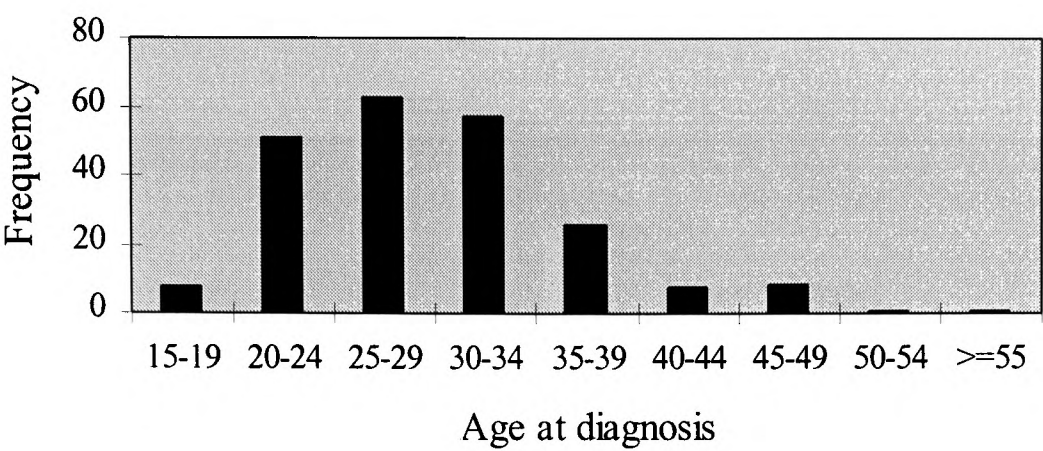
The exposure to surgical procedures for the 558 women classified with either stage A or B disease is presented in Table 4.3.

Characteristics of stage A versus stage B endometriosis

Women were grouped according to the stage of their disease for the purpose of making comparisons. The mean age at diagnosis was 29.4 ± 7.1 for the 235 women with stage A disease. In comparison, the mean age at diagnosis was 32.5 ± 7.7 ($P < 0.001$) for the 323 women with stage B disease.

The distributions of age at diagnosis for each disease stage are illustrated in Figure 4.4. Significantly more women ($P = 0.0003$) in the 20-24 year age group were diagnosed with Stage A (22%) rather than Stage B (11%) disease. There was no significant difference between stage A and B disease for the other age groups.

I. Stage A



II. Stage B

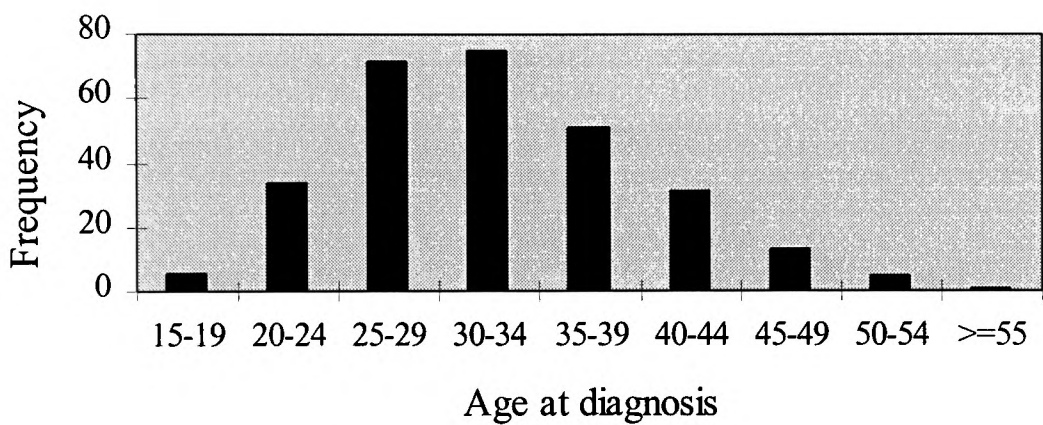
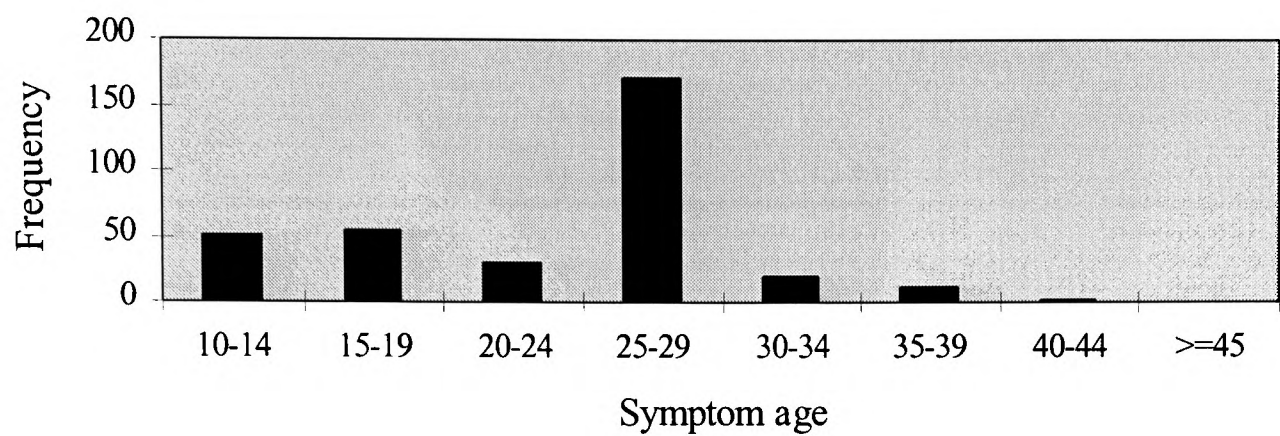


Figure 4.4. Comparison of the age at diagnosis distributions for stage A and B endometriosis.

The mean age at the onset of symptoms was 21.3 ± 7.8 for women with stage A disease and 22.5 ± 8.8 for stage B disease ($P = 0.12$). The distributions are illustrated in Figure 4.5. For both disease severity groups, the proportion of women who reported symptom onset between 25-29 years was significantly higher than the proportion of women in any other age group ($P < 0.0001$).

I. Stage A



II. Stage B

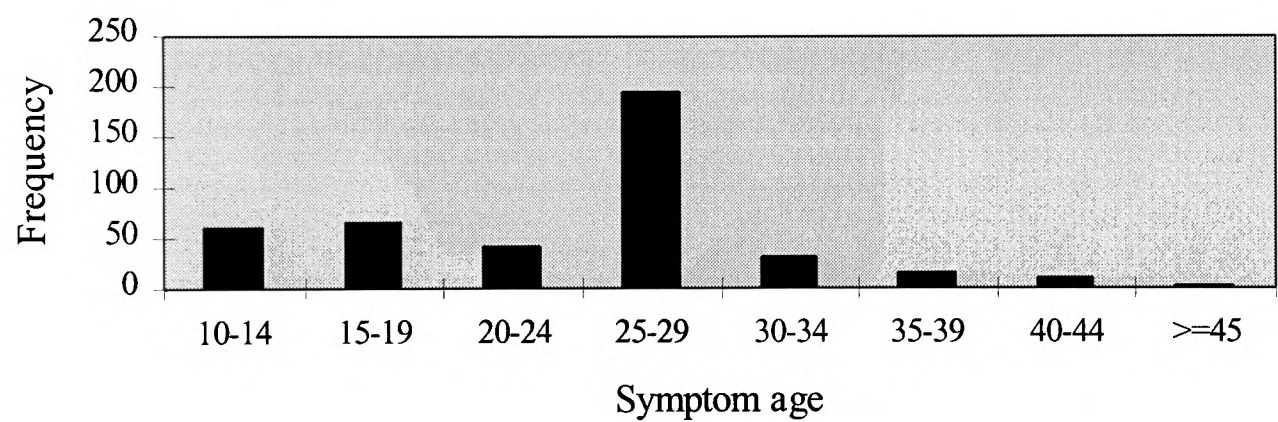


Figure 4.5. Comparison of distribution for age at symptom onset for stage A and B endometriosis.

The exposure to the surgical procedures laparoscopy, laparotomy and hysterectomy was compared for stage A and B disease, and these data are shown in Table 4.6. There was a significant difference, between stage A and B disease, in the proportion of women exposed to more than one laparoscopy, laparotomy or hysterectomy.

There was no significant difference in the proportion of women who reported infertility between the two groups. For stage A disease, 54 (23%) women failed to answer the question or did not know their fertility status, but of the remaining 181 women, 86 (48%) reported infertility. In comparison, for stage B disease, 63 (20%) failed to answer, and 143/260 (55%) reported infertility.

Procedure	Stage A (n = 235)	Stage B (n = 323)	P value
<i>Laparoscopy:</i>			
Mean ± SD	1.1 ± 0.7	1.0 ± 1.2	NS
Range	0 - 4	0 - 6	--
≥ 1	0.84	0.61	<0.0001
≥ 2	0.24	0.25	NS
<i>Laparotomy:</i>			
Mean ± SD	0.15 ± 0.5	0.52 ± 0.8	<0.0001 [†]
Range	0 - 3	0 - 9	--
≥ 1	0.12	0.41	<0.0001
≥ 2	0.03	0.08	0.0168
<i>Hysterectomy:</i>			
Mean ± SD	0.17	0.27	0.004 [†]
Range	0 - 1	0 - 1	--
Yes / No	0.17	0.27	0.0054

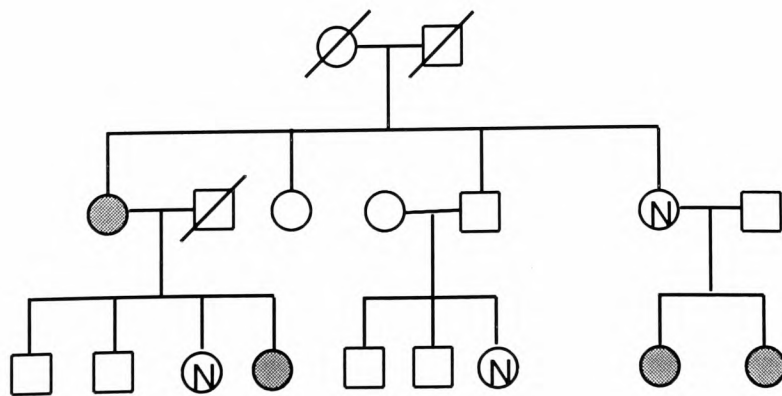
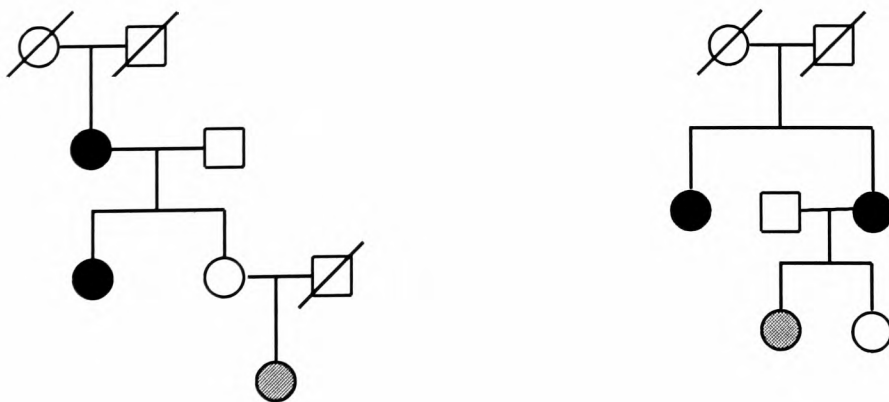
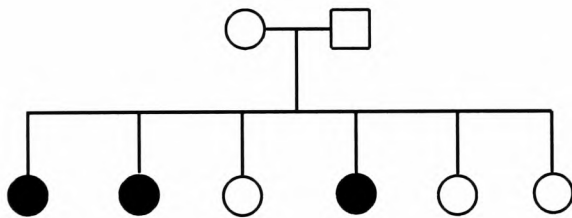
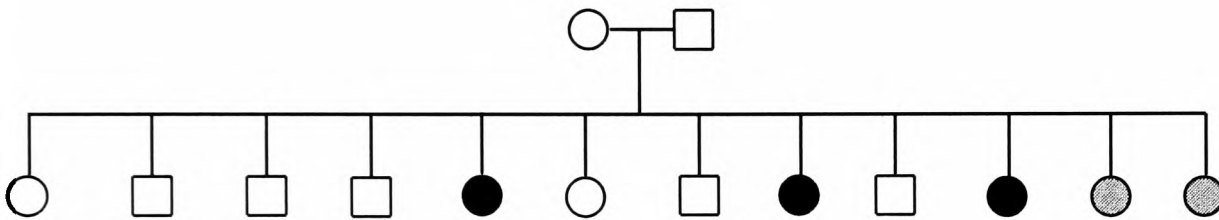
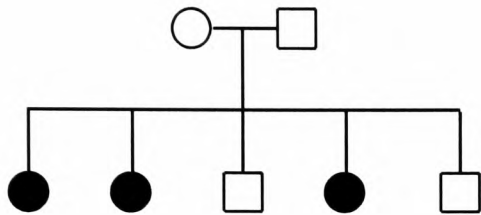
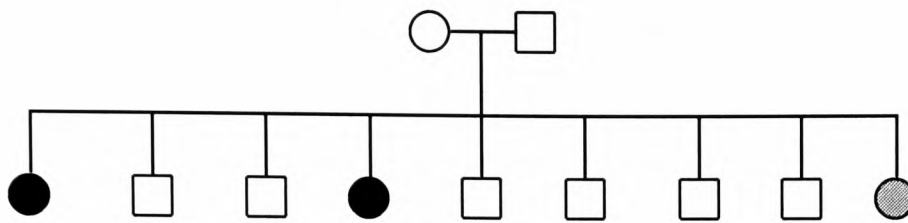
Table 4.6. Differences in the exposure to laparoscopy, laparotomy and hysterectomy according to severity of endometriosis. [†]Unpaired *t*-test with Welch correction.

The majority of women in both severity groups reported pain symptoms related to endometriosis: 213/224 (95%) in the stage A group (11 failed to answer) and 269/284 (95%) in the stage B group (39 failed to answer).

Familial endometriosis

Examples of some of the affected families identified are shown in Figure 4.7. In the first 100 families identified, 230 women with surgically confirmed endometriosis were identified. Within the first 100 families there were 56 affected sister pairs, 19 affected mother-daughter pairs, 18 extended families with three or more affected members, 5 families with 3 affected sisters, one family with 5 affected sisters and one pair of cousins (Kennedy *et al.*, 1995).

By the end of 1998, blood samples had been collected from a total of 83 sister pairs and 32 other relative pairs (e.g. mother-daughter, aunt-niece) identified by the Oxford group, and DNA had been extracted for genotyping. In addition, over 100 sister pairs had been collected by the international group of collaborators. The process of recruiting more affected families is ongoing.



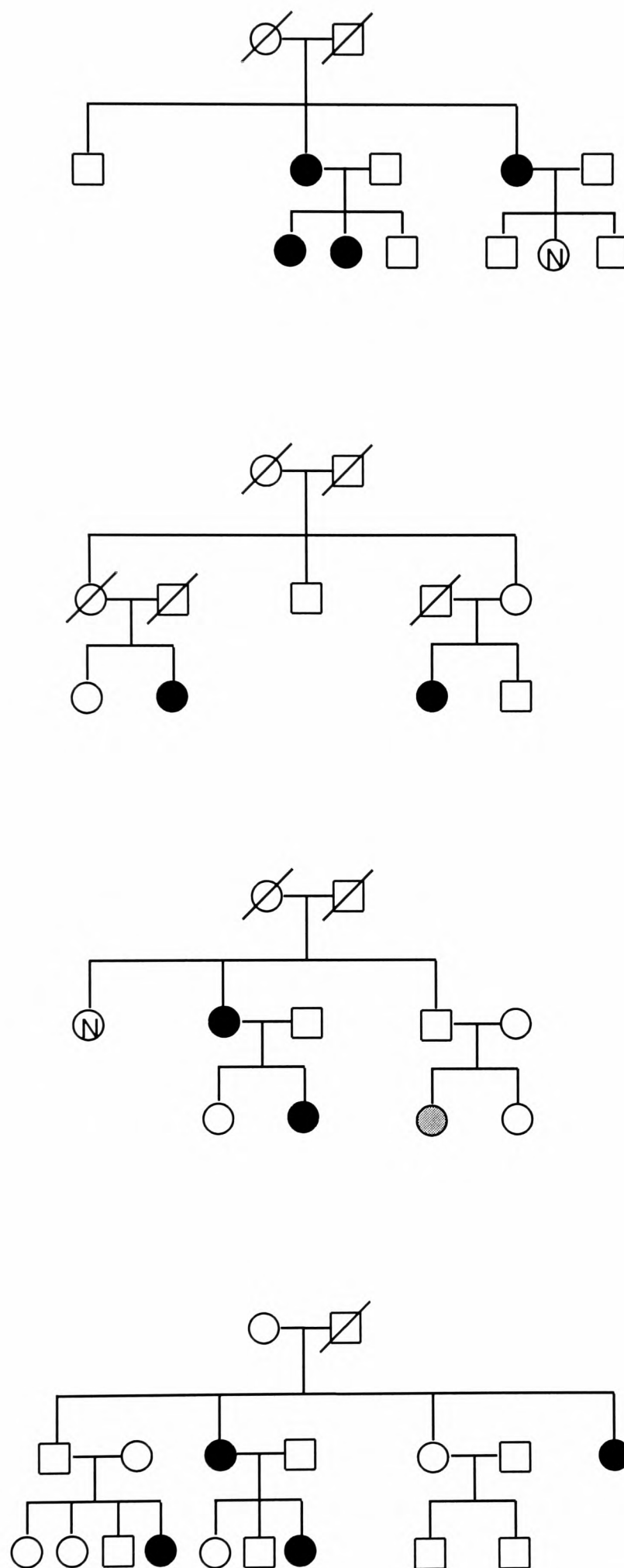


Figure 4.7. Examples of some of the families identified in the OXEGENE study.

● = stage B endometriosis ● = stage A endometriosis

Ⓝ = unaffected by endometriosis at surgery ○ = unknown disease status

Recruitment via the World Wide Web

Participants were also recruited using the novel method of advertising on the WWW. A web page was designed and published on the WWW in March 1997 with the dual aim of informing patients and potential collaborators of the study’s existence, and of recruiting women. Although recruitment is ongoing, to assess the effectiveness of this method of recruitment the data collected over the first 20 months (March 1997 to November 1998) have been analysed.

The page was accessed over 22,000 times in the 20 month period. Responses, either by electronic mail or completion of an electronic form, were received from approximately 745 individuals, of whom 294 (39%) were unsuitable for the study because they had no affected relatives or were themselves unaffected. 451 (61%) responders reported belonging to a family with two or more affected individuals. Of these, 296 (66%) completed the form on the web page and 155 (34%) responded by e-mail. The electronic form (Figure 4.8) asked for the relationship to the index case of any other relatives surgically diagnosed with endometriosis. These data are presented in Table 4.9. Index cases reported a mean number of 1.7 relatives with a surgical diagnosis of endometriosis.

Relationship to index case	Number of relatives
Mother	101 (20.5%)
Sister	126 (25.6%)
Daughter	8 (1.6%)
2° relatives - Mother’s side	126 (25.6%)
>2° relatives - Mother’s side	51 (10.4%)
2° relatives - Father’s side	50 (10.2%)
>2° relatives - Father’s side	30 (6.1%)

Table 4.9. The relationship to the index case of family members with a surgical diagnosis of endometriosis for 296 responses to the web page form. The category

OXEGENE Study

Please complete this form and then choose the 'Send Details' button if you would like to participate in the study.

Your name:

Address :

Telephone (optional):

Internet e-mail address:

Do you have surgically diagnosed endometriosis? (please mark the box which applies to you)

☐ YES

☐ NO

How many members of your family also have surgically diagnosed endometriosis?:

☐ None

☐ 1

☐ 2

☐ 3

☐ 4 or more

What relationship to you are these members of your family with endometriosis? (please check the box or boxes which apply)

Mother ☐

Sister(s) ☐

Daughter(s) ☐

Aunt(s): ☐ Mother's side of the family ☐ Father's side of the family

Cousin(s): ☐ Mother's side of the family ☐ Father's side of the family

Grandmother: ☐ Mother's side of the family ☐ Father's side of the family

Niece(s): ☐ Mother's side of the family ☐ Father's side of the family

Please include any other details here (optional):

Clear Form

Send Details

Figure 4.8. The format of the electronic form used for recruitment via the WWW.

second degree (2°) relatives includes aunts, grandmothers and granddaughters and higher than second degree (>2°) relatives includes first cousins.

At the time of analysis, the disease status in the index case had been confirmed in 67 (15%) of the 451 women recruited *via* the web. Of the 67 index cases who definitely had endometriosis, 24 had stage B disease (12 with histological confirmation) and 43 had stage A disease (7 with histological confirmation). In one woman there was no record of endometriosis from surgery and 5 (1%) were unsuitable as they had not had surgery.

Discussion

The data presented here confirm that the disease has a familial tendency and add weight to the growing body of evidence that endometriosis is a genetic disease (Coxhead & Thomas, 1993, Kennedy *et al.*, 1995, Moen & Magnus, 1993, Simpson *et al.*, 1980). The identification of a large number of families with endometriosis for the OXEGENE study has enabled the characteristics of a cohort of women with surgically confirmed disease to be examined.

The methods employed have been successful in recruiting women with surgically confirmed Stage A and B endometriosis. The use of the WWW to identify participants is a new method of recruitment and has not been assessed previously for effectiveness. Over a 20 month period, 451 suitable families were identified using this method; however, relatively few family members have been confirmed to have endometriosis. Only 67 individuals with documented endometriosis were identified which indicates that the WWW is a viable, but to some extent inefficient, method of recruitment.

The data generated through the form on the web-page were interesting, although they obviously must be interpreted with extreme caution because not all responders had confirmed endometriosis. The data suggested that the proportions of mothers and sisters affected (21% and 26% respectively) were similar, which is what one would expect for a dominant disease. Index cases reported that affected second degree relatives were more often on their mother's (26%) compared to their father's side (10%) ($P < 0.0001$). This result may indicate that women are more likely to put

forward relatives on their mother's side, either because they think of endometriosis as a female disease, which is inherited through the maternal side, or because they are more likely to have discussed their symptoms with members of that side of their family. Interestingly, if these data were representative of the true ratio of maternal to paternal relatives, *i.e.* 2.5 : 1, this would be consistent with the expected ratio of maternal to paternal second degree relatives of an affected index case for an X-linked, dominant disease. However, this is a highly biased sample of women predominantly without confirmed disease and it is therefore inappropriate to draw any conclusions.

For all women with surgically confirmed endometriosis, the mean age at diagnosis was significantly higher for women with Stage B disease. In comparison, the mean age at symptom onset was not different for Stage B compared to Stage A disease. This may be indicative of the progressive nature of endometriosis, in that Stage A disease may develop into Stage B disease if it is not diagnosed and treated early enough. Furthermore, significantly more women with Stage A disease were diagnosed in the 20-24 age group compared to Stage B, but there were no significant differences between the severity groups at other ages (Figure 4.4). Interestingly, for both disease stages, a significant majority of women reported that their symptoms began between the ages of 25-29 (Figure 4.5). This information may be of particular use in identifying those women at high risk of endometriosis, particularly in a primary care setting.

The majority of women in both disease severity groups reported pain symptoms and approximately half reported problems conceiving. The sample of women studied may be biased towards women with pain and/or infertility as women with these symptoms probably have a higher chance of being diagnosed. Further bias may have been introduced because some women are related and because the methods of recruitment may have favoured certain sectors of the population.

Many of the families collected here have been used for a genome wide search for susceptibility genes for endometriosis, the results of which are presented in Chapter Six.

4.3 MRI STUDIES

Introduction

MRI was used to facilitate the recruitment of families with endometriosis for the OXEGENE study, and also to calculate the prevalence of endometriosis in the first degree relatives of women with stage B disease. The prevalence in the relatives of affected women has been assessed in several studies (Coxhead & Thomas, 1993, Moen & Magnus, 1993, Simpson *et al.*, 1980). However, an accurate assessment of the risk in relatives has not been possible because these studies investigated the relatives of women with all stages of endometriosis, including mild forms, and because not all affected women have necessarily had a surgical diagnosis. It is likely that the relative risk of endometriosis in the first-degree relatives of women with stage B disease only is higher and, in one study, λ_R increased from 7.2 to 8.6 when probands with Stage A disease were removed from the analysis (Moen, personal communication). As discussed in the previous chapter, MRI is a non-invasive method of diagnosis that has high sensitivity and specificity for the detection of endometriotic cysts and implants >1cm in diameter (Takahashi *et al.*, 1994, Takahashi *et al.*, 1996).

In this study, females of reproductive age, who were relatives of women with stage B disease were invited for an MRI scan to determine their disease status.

Methods

Probands were identified through the records of the John Radcliffe Hospital and female relatives were invited to participate in the study by letter. A total of 29 women with surgically confirmed, Stage B endometriosis were identified and their 56 female relatives of reproductive age were invited to participate in the study. Of those invited, 47 (84%) agreed to have an MRI scan to determine their disease status. The study had the approval of the Central Oxford Research Ethics Committee (COREC). Before the MRI scan the participants were offered three options for the way their results could be given: a) they could choose not to learn the scan result, and request that the information not be given to their General Practitioner (GP); b) the information could be passed on to their GP only, or c) they could choose to learn the scan result and also have the information passed on to their GP. The women were also informed that they

were participating as volunteers not as potential patients. In other words, there was no obligation for Oxford to provide medical care in the event of pathology being identified unless the GP made a referral. MRI scans were all performed at the Oxford Magnetic Resonance Imaging department at the John Radcliffe Hospital using spin echo imaging with a 1.5 Tesla Signa system (GE Medical Systems). T_2 weighted images were obtained in the coronal, sagittal and axial planes. T_1 weighted images were obtained in the axial plane, both with and without fat presaturation. The MRI images were all interpreted by the same radiographer who was aware that the individuals were relatives of affected women.

Results

The 47 women scanned were related to the probands as sisters ($n = 33$), daughters ($n = 2$), nieces ($n = 5$) and first cousins ($n = 7$). The mean age of the women scanned was 33.5 (range 16 - 51) years, and 27/47 (57%) reported being free of symptoms. Based on the MRI scans, endometriosis was suspected in 15/47 (32%) women as either ovarian cysts ($n = 4$, 8.5%), peritoneal lesions ($n = 2$, 4%) or equivocal findings with lesions <1 cm in diameter ($n = 9$, 19%). Adenomyosis alone was present in 8/47 (17%) of the women. In four (9%) women, there was evidence of both endometriosis and adenomyosis. Of the 33 sisters studied, 5 (15%) had unequivocal evidence of endometriosis on MRI scan. However, the prevalence may be as high as 58% (19/33) if any evidence of endometriosis, including equivocal findings and adenomyosis, is included. The prevalence data in all relatives are presented in Table 4.10. Pedigree diagrams for two of the families are shown in Figure 4.11.

Three of the women who participated in the study subsequently had a laparoscopy. In two women, the surgical and MRI findings concurred: one woman had a normal pelvis and the other had endometriosis. In this symptomatic woman, whose sister had rAFS Stage III surgically diagnosed disease, MRI showed a 2.5 cm diameter left ovarian endometrioma. At laparoscopy, Stage III disease was confirmed with a leaking left ovarian endometrioma adherent to the ovarian fossa and small endometriotic foci on the right utero-sacral ligament and surface of the right ovary. In a third woman, no endometriosis was seen on MRI scan but laparoscopy revealed the presence of Stage I disease (Kennedy *et al.*, 1997, Kennedy *et al.*, 1998). MRI is acknowledged not to be useful in detecting minimal disease.

	1st degree relatives	≥ 2nd degree relatives
Any evidence of endometriosis and/or adenomyosis on MRI	19/35 (54%)	3/12 (25%)
Endometriosis excluding equivocal findings/adenomyosis	5/35 (14%)	1/12 (8%)

Table 4.10. Findings on MRI scan of 47 relatives of women with Stage B endometriosis.

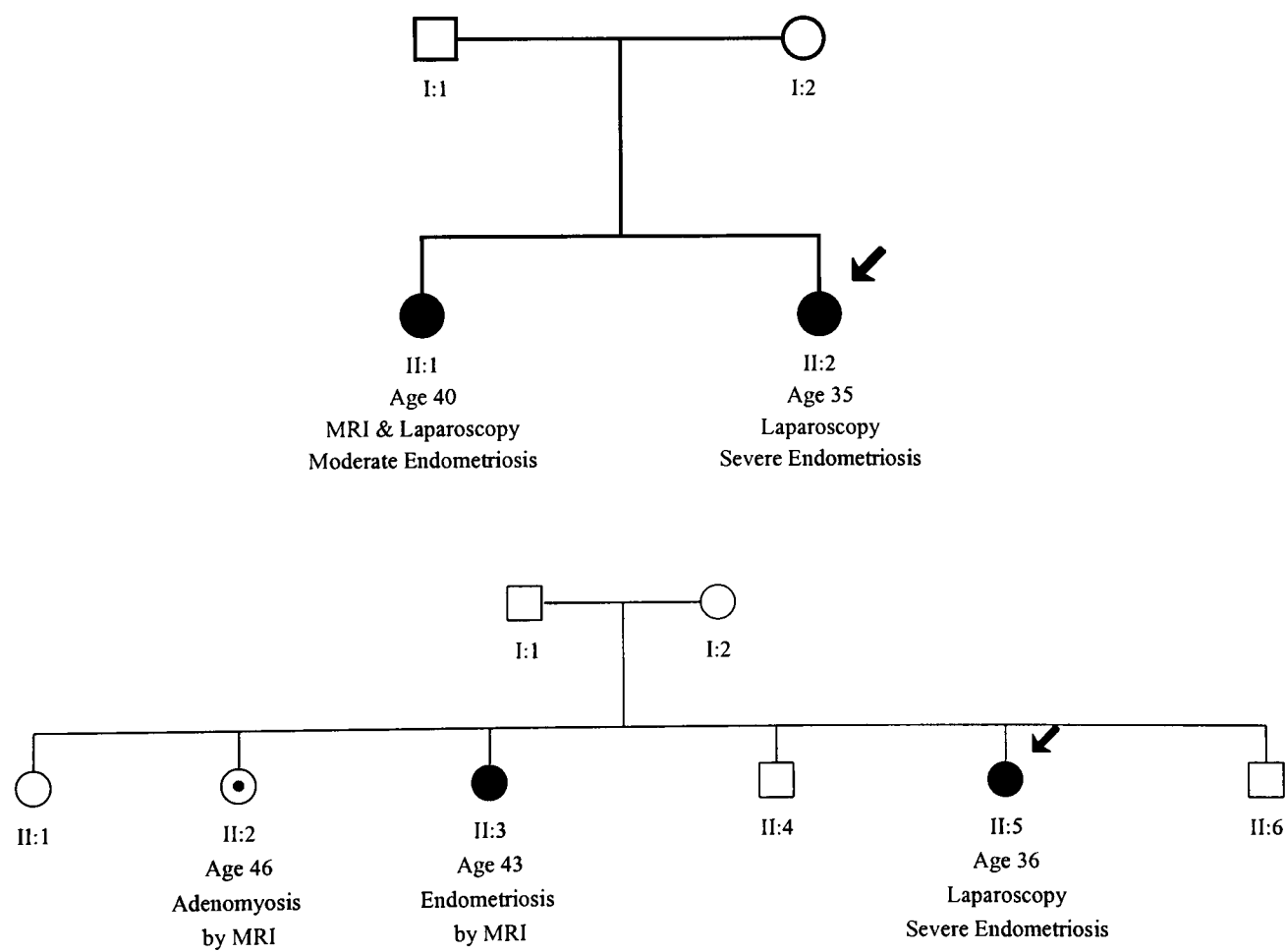


Figure 4.11. Pedigree structures for two of the families participating. Arrows indicate probands.

Discussion

This study illustrates the usefulness of MRI as a noninvasive method for screening women who may be at increased risk of developing endometriosis. Using this method, additional pedigrees were identified for use in linkage and affected sib-pair analysis. The informativeness of some pedigrees was also enhanced by assigning affected or unaffected status to additional family members.

In addition, a more accurate assessment of the risk of endometriosis in the relatives of women with endometriosis has been established and for the first time the risk in the relatives of women with Stage B disease has been estimated. Studies estimating the incidence of endometriosis have reported hugely varied figures, but if the population frequency were approximately 1%, then the relative risk in first degree relatives (λ_R) from these findings may be as high as 14 (95% CI 4.8 - 30.3), and the relative risk in the sisters of affected women (λ_S) may be as high as 15 (95% CI 5.1 - 31.9). These values are considerably higher than has been estimated by previous studies, which have put λ_S at between 6.1 - 11.6 (Lamb *et al.*, 1986, Moen & Magnus, 1993, Simpson *et al.*, 1980), although the 95% confidence intervals for λ_S from each of these studies are wide. In the present study, only the relatives of women with stage B disease were investigated and this may provide evidence that predisposing genetic factors play a more important role in advanced disease. In comparison, the other studies have investigated relatives of women with all stages of disease severity. The noninvasiveness of MRI has also enabled investigation of the majority of the female relatives of probands, whereas previous studies were limited to those women who had already obtained a surgical diagnosis.

Women who were symptomatic may have been more likely to participate in this study thereby biasing the results and giving a falsely high impression of the risk of disease in relatives. However, the fact that the participation rate was high at 84% of those invited and a high proportion of women (57%) reported being symptom free indicates that such bias should be minimal.

Determining the age at which women should be included in a study investigating relative risk is a problem for a disease such as endometriosis, that has varying age at onset. Factors that may affect disease progression, such as pregnancy and oral

contraceptive use, confound the difficulty. For example, a 20 year old may go on to develop disease later in life and labelling the individual as 'unaffected' at such a young age may be inaccurate. These considerations suggest that all estimates of relative risk may be lower than the true value. One of the participants had an MRI scan at which no endometriosis was seen, but subsequent laparoscopy revealed minimal endometriosis. The patient may have developed disease between the MRI scan and the laparoscopy but the discrepancy is more likely to be due to the limitations of MRI. Whilst MRI with fat presaturation has been shown to be sensitive for the detection of endometriotic cysts and implants >1 cm in diameter, the technique is relatively insensitive for lesions smaller than this. In one study, only 49% of lesions <1 cm in diameter were detected by MRI (Takahashi *et al.*, 1994). In another study, only 66% of endometriomas <1 cm were detected and 50% of endometriotic implants <0.5 cm in diameter (Takahashi *et al.*, 1996). Therefore, it is likely that the relative risk determined by MRI is a conservative estimate of the true value, given that the presence of minimal endometriosis may be overlooked using this technique.

The high overall prevalence of adenomyosis, at 26%, may suggest a relationship between endometriosis and adenomyosis and may also be indicative of a familial tendency to this condition, as suggested by previous studies (Arnold *et al.*, 1994, Arnold *et al.*, 1995). Because of the difficulty in diagnosing adenomyosis, the frequency of this condition in the general population is unknown. However, the prevalence amongst women undergoing hysterectomy has been estimated at 25% (Vercellini *et al.*, 1995).

In conclusion, this study has enabled identification of new pedigrees for genetic studies and the data suggest that the relative risk of endometriosis in the first degree relatives of women with stage B disease may be as high as 14.

4.4 DELAY IN THE DIAGNOSIS OF ENDOMETRIOSIS

Introduction

In unpublished reports, endometriosis self-help groups have highlighted the problem of the delay endometriosis sufferers experience before the disease is diagnosed. For example, in a 1993 survey of 2,102 members of the National Endometriosis Society of Great Britain the average delay between the onset of pain symptoms and a diagnosis of endometriosis was 6.8 years. A similar delay of 6.1 years was found in a survey of 748 women by the Australian Endometriosis Association. The Australian data showed a mean delay in reporting symptoms to a doctor of 1.7 years and a mean delay between the first report to a doctor and an eventual diagnosis of 4.4 years. The patient's age when symptoms were first reported to a doctor was related to the delay in diagnosis experienced: the average delay in diagnosis was 8.3 years for women aged 15 - 19 years compared to 1.3 years for those aged between 30 - 34 years. These data are consistent with the findings from a more recent survey of members of the American Endometriosis Association (Milwaukee, Wisconsin, USA). In 1998, the Endometriosis Association conducted a survey of 4,000 members, of whom 96% reported a diagnosis of endometriosis. The average delay in diagnosis experienced was 9.3 years, with a mean delay in reporting symptoms to a doctor of 4.7 years and a mean delay between the first report to a doctor and eventual diagnosis of 4.6 years. In total, 47% of Association members indicated that they had to see a doctor five or more times before being diagnosed.

Women with endometriosis often claim that they are adversely affected by this apparent delay in diagnosis. They believe that endometriosis may become progressively more severe and therefore more difficult to treat the longer it is left undiagnosed. They also believe that their general health is affected unnecessarily by such a delay (Kennedy, 1991).

In this study, data gathered *via* a postal survey of OXEGENE study participants with surgically confirmed endometriosis, from both the United Kingdom (UK) and the United States of America (USA) were analysed. The aim was to calculate the delay

between the onset of pain symptoms and the time of diagnosis in an initial cohort of study participants.

Methods

Women were recruited for the OXEGENE study as described earlier in this chapter (see Section 4.2). The women were asked to complete a postal questionnaire about their gynaecological history and the age at which they first experienced pain symptoms. Women were excluded from the study if the operative record was not obtained or if the diagnosis was uncertain; histological confirmation of endometriosis was also obtained if available. The severity of disease was assessed on the basis of the operative findings using the rAFS classification system, and women were divided, as before, into two groups: Stage A (rAFS Stage I - II, minimal - mild disease) and Stage B (rAFS Stage III - IV, moderate-severe disease). Statistical comparisons of means were made using an unpaired, two-tailed *t*-test and of proportions using Fisher's exact test.

Results

Questionnaires were sent initially to 337 women and 270 (80%) were returned. Endometriosis was confirmed in 218 (81%) of those who responded (UK = 134, USA = 84); the remaining 52 women were excluded from the study. The method of recruitment was through advertisements in the newsletters of Endometriosis Associations for 212 women (97%) and through the records of the John Radcliffe Hospital for 6 women (3%). The mean age ($\pm$ SD) at the onset of pain symptoms was 22.4 ± 8.9 (range 10 - 46) years; the mean age at diagnosis was 31.8 ± 8.2 (range 16 - 69) years and the mean delay in diagnosis was 9.4 ± 8.6 years (range 0 - 44) years. In total, 75 women had Stage A (UK = 48, USA = 27) and 143 women had Stage B disease (UK = 86, USA = 57) (Hadfield *et al.*, 1996).

There was no significant difference ($P = 0.47$) in the mean delay in diagnosis between women with Stage A (8.9 ± 8.6 years) and Stage B disease (9.7 ± 8.6 years).

Comparison of UK and USA cohorts

The mean age at the onset of symptoms was 24.4 ± 8.6 (range 10 - 46) years for women from the UK and 19.2 ± 8.4 (range 10 - 46) years for women from the USA, a

difference that was statistically significant ($P < 0.0001$). The mean age at diagnosis was 32.6 ± 8.0 (range 16 - 50) years for women from the UK and 30.9 ± 8.6 (range 16 - 69) years for women from the USA ($P = 0.15$).

The mean delay between the onset of symptoms and the diagnosis of endometriosis was 8.0 ± 7.9 (range 0-33) years for women from the UK and 11.7 ± 9.1 (range 0-44) years for women from the USA, a difference that was statistically significant ($P = 0.0014$). The delay in diagnosis increased relative to the age at which women were diagnosed (Table 4.12), and a statistically significant difference between the UK and USA women was present only in the 21-30 ($P = 0.0003$) and 41-50 ($P = 0.03$) age groups. A delay in diagnosis of 5 years or more was reported by 74 women (55%) from the UK and 61 women (73%) from the USA ($P = 0.01$) (Table 4.13).

Age at diagnosis (years)	Average delay in diagnosis (years)				<i>P</i>
	UK		USA		
	<i>n</i>	Mean ± SD	<i>n</i>	Mean ± SD	
≤ 20	6	4.8 ± 3.4	8	4.4 ± 2.4	0.77
21 - 30	59	5.5 ± 5.1	36	9.6 ± 5.4	0.0003
31 - 40	43	8.6 ± 7.8	30	12.3 ± 9.1	0.06
41 - 50	26	13.4 ± 11.1	8	23.0 ± 9.3	0.03
> 50	0	0	2	24.5 ± 27.6	--

Table 4.12. Delay in the diagnosis of endometriosis and the relationship with age at diagnosis.

Delay in diagnosis	UK		USA		<i>P</i>
(years)	(n = 134)		(n = 84)		
	<i>n</i>	%	<i>n</i>	%	
< 5	60	45	23	27	0.01
≥ 5	74	55	61	73	0.01
≥ 10	53	40	48	57	0.01
≥ 20	14	10	15	18	0.15

Table 4.13. Number of women from the USA and the UK with a delay in diagnosis of endometriosis, divided into groups depending on the length of delay.

Discussion

This was the first study to investigate the time difference between the onset of pain symptoms and diagnosis in a group of women with surgically confirmed endometriosis. The findings of unpublished surveys amongst the members of endometriosis self-help groups in Australia, the UK and the USA, which showed a symptom onset to diagnosis delay of 6-9 years, have been confirmed. However, in this study only women with surgically confirmed endometriosis were included, which is important because documented proof of the disease was unobtainable in approximately 20% of the women in this study who were members of self-help groups.

Considerable delay from the time of the initial occurrence of pain symptoms to the diagnosis of endometriosis was reported by women from both the USA and the UK, but the mean delay in diagnosis was significantly greater for the American women. This may in part be due to women in the USA reporting that their symptoms, on average, commenced approximately 5 years earlier.

There are a number of factors which could have contributed to the apparent delay in making a diagnosis. Firstly, there may be bias inherent in the methods used to recruit subjects for the study. Most of the subjects were recruited through endometriosis self-help groups and women that belong to these groups may be more likely to have had

negative experiences with the medical profession. In addition, women who made the effort to respond to an advertisement and subsequently completed a questionnaire may be more likely to have had bad experiences. Women may also have poor recollection of the precise date of the onset of their symptoms.

It is likely that many women do not report their symptoms to a medical practitioner immediately, which contributes to the delay in diagnosis. Indeed, data from the self-help groups suggest that this may be an important factor, particularly in the United States. Once the symptoms have been reported, the medical practitioner may have difficulty in distinguishing between other causes of pelvic pain such as pelvic inflammatory disease and irritable bowel syndrome, especially if obvious clinical signs of endometriosis are absent. Empirical treatments such as the combined oral contraceptive or non-steroidal anti-inflammatory drugs may be used instead of obtaining a definitive diagnosis, because of the cost and morbidity related to laparoscopy. Accessibility to health care is yet another factor which could affect the delay in diagnosis and could account for the longer delay observed for American women.

The fact that there was no difference in the delay in diagnosis between the two disease severity groups is not surprising because there is not a strong relationship between pain symptoms and the severity of endometriosis (Mahmood & Templeton, 1991). The delay in diagnosis for endometriosis is considerably longer than for other chronic diseases such as rheumatoid arthritis. In one study a median delay to diagnosis for rheumatoid arthritis of 36 weeks was reported (Chan *et al.*, 1994) whereas, the median delay to diagnosis for endometriosis was 7.5 years from these data. It is likely that the substantial delay between symptom onset and diagnosis decreases women's ability to cope with the symptoms of endometriosis.

4.5 ENDOMETRIOSIS IN TWIN SISTERS

Introduction

Data on the familial tendency of endometriosis and the increased prevalence of the disease in the relatives of affected women have provided evidence for a genetic basis, although concordance data from monozygotic (MZ) and dizygotic (DZ) twins would provide invaluable supportive data. However, the problems inherent in diagnosing endometriosis and the lack of large twin registers containing women in the 30-40 age group have in the past made it difficult to conduct such a study, and until recently only a collection of case reports has appeared in the literature (Moen, 1994). The study by Moen confirmed a genetic basis to endometriosis because for affected probands, 75% of the MZ twin sisters had endometriosis compared to 3.8% of other sisters ($P < 0.0005$). A very recent study, published after our data were obtained and analysed, has for the first time presented data on the occurrence of endometriosis in MZ and DZ twins from a large twin register in Australia (Treloar *et al.*, 1999). Treloar *et al.* (1999) surveyed over 3,000 twin sisters and found that the prevalence of concordance for surgically confirmed endometriosis amongst MZ twins was 2.1% (18/841) and 1.6% (3/485) for DZ twins.

Among sister pairs with endometriosis recruited for the OXEGENE study was a group of twin pairs and these data are presented here.

Methods

Women were recruited for the OXEGENE study as described earlier in this chapter (see Section 4.2). Twin sister pairs were never actively recruited for this study.

Zygoty determination

A standard questionnaire was revised and used to determine the zygoty of any twin pairs (Magnus *et al.*, 1983). The questionnaire (shown in Figure 4.14) was sent to both twin sisters. This method produces a correct classification in over 95% of cases, even if only one twin responds, and has been used and verified in large twin studies (Eisen *et al.*, 1989). Questions about ABO and Rhesus D blood group status, and whether any previous tests to determine zygoty had been carried out were also included.

TWIN QUESTIONNAIRE

1. As children, were you and your twin “as alike as two peas in a pod”, or of only ordinary family resemblance? (tick one)
- Alike as two peas in a pod

Of only ordinary family resemblance

Don’t know

☐

☐

☐

2. When you were children, how often did the following persons have difficulty in telling you and your twin sister apart? (tick one for each line)

	All of the time	Sometimes	Never	Don’t know	Does not apply
a) Parents					
b) Other brothers & sisters					
c) Grandparents					
d) Class-mates					
e) Teachers					
f) Strangers					

3. The following list describes characteristics that you and your twin sister may or may not have shared when you were children. For each characteristic listed, please consider your childhood years (before you were teenagers), and describe whether you and your twin sister were very similar, somewhat similar, or not similar (tick one for each line).

	Very similar	Somewhat similar	Not similar	Don’t know
a) Eye colour				
b) Hair colour				
c) Hair type				
d) Heights				
e) Weights				
f) Teeth				
g) Voices				
h) Muscular strength				
i) Manual skills/dexterity				
j) Temperaments				
k) Musical abilities				
l) Language abilities				

4. Do you yourself believe that you and your twin are identical (monozygotic) twins, or do you believe that you are fraternal (dizygotic) twins? (tick one)
- Identical (monozygotic)

Fraternal (dizygotic)

Don’t know

☐

☐

☐

5. Do you know your blood group? e.g. O+ or A-

If so please enter it here _____

6. Have you ever had any tests, such as written tests or blood tests, to determine whether you and your sister are identical (monozygotic) twins or fraternal (dizygotic) twins? If so please give details and the result, if known.
-

.....

Figure 4.14. The questionnaire sent to twin sisters for zygotity determination (adapted from Magnus, 1983).

The responses to different questions from the questionnaire were used in the analysis depending on whether questionnaires were received back from both twins of a pair (Table 4.15) or only one twin (Table 4.16) (Magnus *et al.*, 1983). Questions were scored +1 if the answer indicated similarity (for example, if the answer was ‘alike as two peas in a pod’ or if the answer indicated that characteristics were very similar). Questions were scored -1 if the answer indicated dissimilarity and zero if the answer was inconclusive. When questionnaires were received from both twins the mean of the scores was used. For each pair of twins a discriminant was calculated as:

$$b_1Q_1 + b_2Q_2 + b_3Q_3.....+ b_nQ_n + C$$

where $b_1, b_2, ... b_n$ are the coefficients, $Q_1, Q_2, ... Q_n$ are values scored for the questions and C is the constant value, as shown in Tables 4.15 and 4.16.

If the discriminant value was +2.50 or greater, this was taken as evidence that the twins were monozygotic. Likewise, if the value was -2.50 or less, this was taken as evidence for dizygosity. This method has been found to classify 98.3% of twin pairs correctly if both returned the questionnaire, and 96.1% if only one returned the questionnaire (Magnus *et al.*, 1983).

Question	Coefficient
Alike as two peas in a pod (Q1)	2.111
Mistaken by strangers (Q2f)	0.691
Similarity in eye colour (Q3a)	0.394
Similarity in manual skills/dexterity (Q3i)	0.366
Own opinion on zygosity (Q4)	0.481
Constant	-0.087

Table 4.15. Questions and coefficients of regression used in the analysis of questionnaires where both twins responded.

Question	Coefficient
Alike as two peas in a pod (Q1)	1.494
Mistaken by teachers (Q2e)	0.640
Mistaken by strangers (Q2f)	0.647
Similarity in voices (Q3g)	0.347
Similarity in muscular strength (Q3h)	-0.417
Similarity in manual skills/dexterity (Q3i)	0.458
Own opinion on zygoty (Q4)	0.417
Constant	0.007

Table 4.16. Questions and coefficients of regression used in the analysis of questionnaires where only one twin responded.

Results

There were 19 twin pairs amongst the first 627 responses to the OXEGENE study. Zygoty was determined (MZ=18, DZ=1) on the basis of both twins (n=11) or one twin (n=7) responding to the zygoty questionnaire, or from previous DNA testing (n=1). The discriminant score from the zygoty questionnaire ranged from 2.8 to 4.0 for MZ twins and was -3.2 for the DZ pair. In the seven pairs of MZ twins who both reported their ABO and rhesus blood group, full concordance was found.

Disease status was inadequately documented in 2 MZ pairs who were excluded. Of the remaining 16 MZ pairs, 9 were concordant for Stage B endometriosis. In 5 pairs, one twin had Stage A disease and the other had either Stage B (n = 4) or deeply infiltrating disease (n = 1). Two MZ pairs were discordant: both probands had Stage B disease but their twin sisters either had no disease at laparoscopy or were asymptomatic, having had no gynaecological surgery.

Of the 14 MZ pairs concordant for endometriosis, data were available on the age at symptom onset in 8 pairs. The mean difference between sisters was 2.4 years, which was not statistically significant ($P = 0.56$, paired t -test). Data on age at diagnosis were available in 13 pairs and a mean difference of 1.15 years was observed ($P = 0.29$).

Histological confirmation of disease was obtained in 22/30 women (73%) with a surgical diagnosis. Of the nine twin pairs concordant for Stage B endometriosis, 8/9 (89%) both had histological confirmation. Seventeen women (57%) reported that they had problems conceiving; 7 (23%) had not attempted to conceive and 2 (7%) failed to respond to this question. Both twins without endometriosis reported that they had had difficulty conceiving. Adenomyosis was present in three of the women although no twin-pairs were concordant for this finding. One MZ pair also had a non-twin sister with Stage A endometriosis.

The only DZ pair was concordant for endometriosis: one sister had Stage B disease and the other Stage A disease.

Discussion

Twin concordance studies for endometriosis would be invaluable to determine the relative effects of genetic and environmental factors. Unfortunately they have been difficult to conduct in this disease. Moen (1994) reported 75% concordance in 8 MZ pairs, and in this study there was 87% concordance in 16 MZ pairs. In total, 56% of MZ twin pairs were concordant for stage B endometriosis.

It is interesting that in the two discordant MZ pairs, both twins without endometriosis reported infertility. Whilst impossible to draw any meaningful conclusions from a sample size of two, this observation may indicate that a more complex relationship between endometriosis and infertility exists, that warrants further investigation.

The expected proportions of twin births are shown in Figure 4.17. Given that 627 female probands responded, approximately one MZ twin sister and one DZ twin sister would be expected. Hence, a disproportionately high number of MZ twin pairs (at approximately 18 times the expected number), and approximately the expected number of DZ pairs have been recruited.

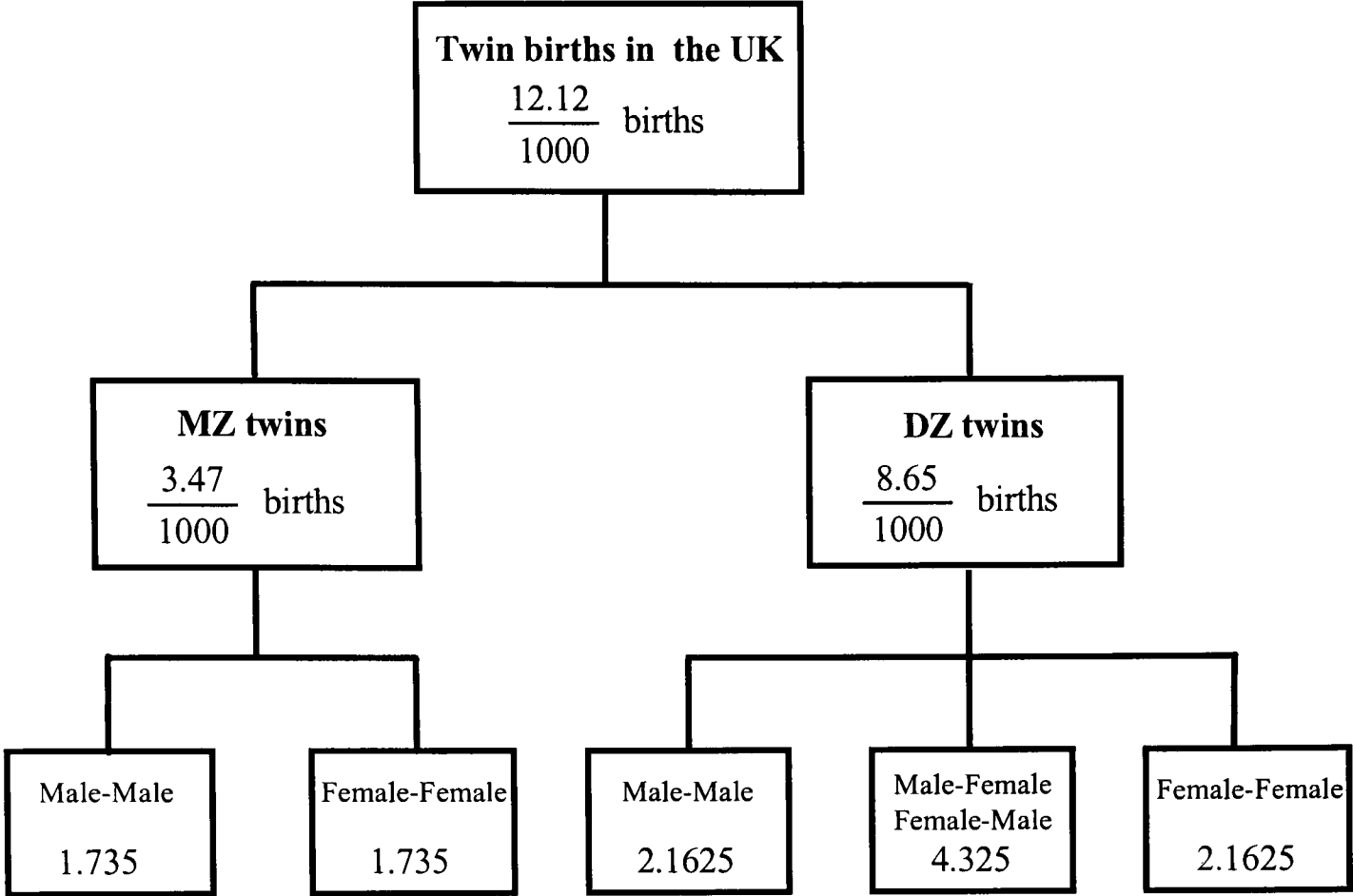


Figure 4.17. The expected ratios of twin births in the UK, illustrating that the ratio of female-female MZ twins to DZ twins should be approximately 1.735:2.1625 (*i.e.* 1:1.25) in an unbiased study.

There is inherent ascertainment bias in the method of data collection because sister-pairs with endometriosis were specifically recruited. Furthermore, it is likely that MZ twins with disease would be highly motivated to participate in a genetic study which may explain why comparatively few DZ pairs responded. It has been estimated that the proportion of twin types in an unbiased study should be approximately 46% MZ and 54% DZ (Lykken *et al.*, 1987, Lykken *et al.*, 1978). However, in most twin volunteer studies the proportion of MZ twins is higher than expected at about 62%. In comparison, of the 19 pairs of twins in our study only one set was DZ. The high proportion of MZ twins responding and the high concordance for endometriosis supports existing evidence suggesting that endometriosis may have a genetic basis. However, no firm conclusions can be drawn from these studies because of inherent recruitment bias.

Treloar *et al.* (1999) have recently reported that the prevalence of concordance for surgically confirmed endometriosis amongst MZ twins was 2.1% (18/841) and 1.6% (3/485) for DZ twins in a survey of 3,000 members of a twin register. These results agree with our findings, and provide further evidence for a genetic basis to endometriosis.

4.6 CONCLUSION

The studies presented here provide further evidence that endometriosis has a familial tendency and is likely to have a genetic basis. Pedigrees with multiple affected members have been identified, supporting previous observations of familial clustering. MRI studies have, for the first time, produced an accurate assessment of the relative risk of disease: 14 for the first-degree relatives of women with stage B endometriosis. A high degree of concordance of endometriosis amongst MZ twin pairs provides additional evidence for the role of genetic factors in disease development.

Data on the age at symptom onset suggest that the majority of women probably develop disease in the 25-29 year age group. Women with stage A disease tend to be diagnosed at an earlier age than women with stage B disease, implying that earlier diagnosis may be important in preventing disease progression. Finally, for the first time, the considerable delay in diagnosis experienced by endometriosis patients has been quantified at 9.4 years.

Whilst the primary purpose of collecting these families was to conduct linkage analysis, the results of which are presented in Chapter Six, jointly they provide a database of women with documented disease from which useful conclusions about disease aetiology have been drawn.

Chapter Five

Candidate gene association and linkage studies

5.1 INTRODUCTION

Several strategies can be adopted for the genetic dissection of a complex trait such as endometriosis. These include linkage analysis, allele-sharing methods such as sib-pair analysis, and association studies (Figure 5.1).

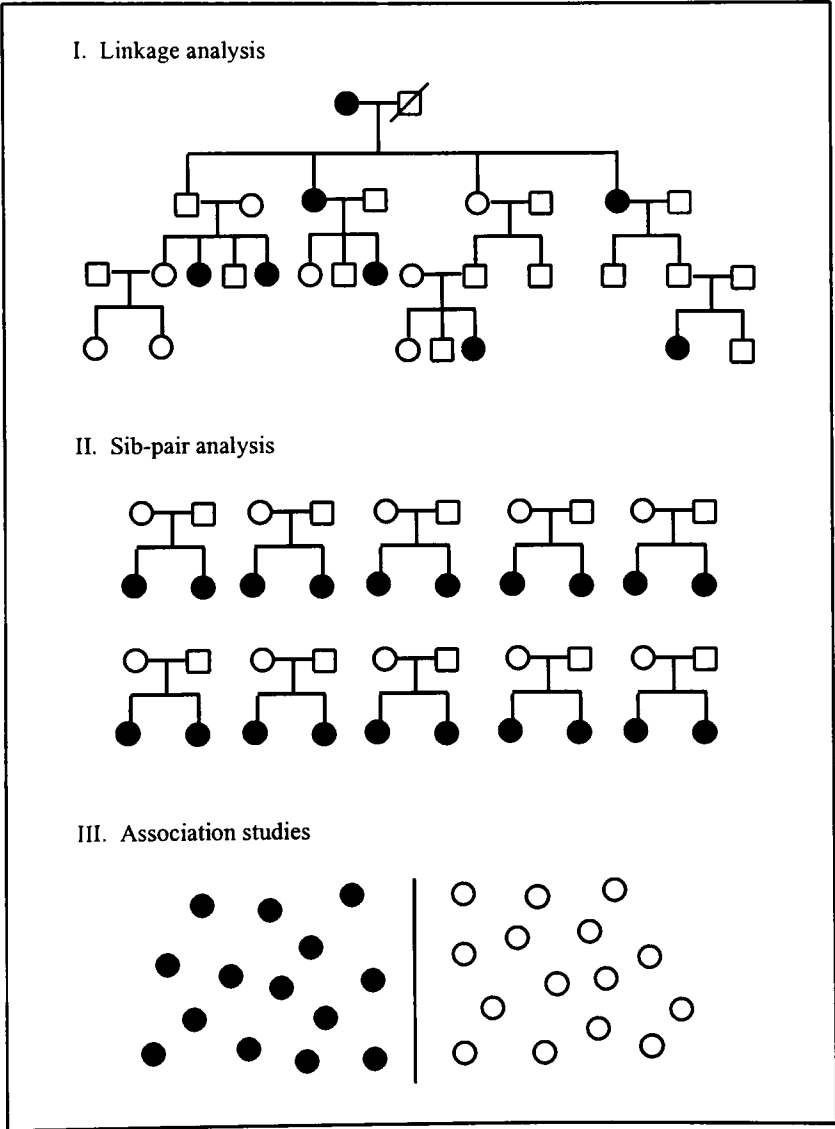


Figure 5.1. Three different strategies for the dissection of a complex genetic trait such as endometriosis (adapted from Lander & Schork, 1994).

Linkage analysis involves the recruitment of extended pedigrees with multiple affected members and has been commonly used for the investigation of simple Mendelian traits. However, the mode of inheritance must be known therefore applying this method to complex traits is problematic. Endometriosis is especially difficult for

a number of reasons: a) large multi-generational pedigrees are rare because the disease has not reliably been diagnosed until recently; b) endometriosis causes infertility; c) there is no known male phenotype; d) the age at onset is variable, and e) defining individuals as unaffected may be inaccurate. Further confounding the difficulty, minimal disease may not represent a true disease state (Koninckx, 1994) and the impact of environmental factors on disease aetiology is to a large extent unknown. Many of these problems also apply to affected sib-pair analysis; however, the two main advantages of this method are that the mode of inheritance does not need to be known and sister-pairs rather than large pedigrees can be recruited. These methods are discussed in more detail in the following chapter.

In contrast to linkage and sib-pair analysis, association studies are case-control studies and familial inheritance patterns are not considered. Association studies look for an increased frequency of a candidate allele within cases compared to controls and the data are analysed in a 2x2 contingency table, using Chi-square or Fisher's exact test. The serious difficulty in association studies is choosing an appropriate control group. For example, in endometriosis should the control group consist of known unaffecteds (*i.e.* women found to have a normal pelvis at hysterectomy) or of women drawn from the general population, who may or may not have, or go on to develop, the disease later.

A positive association between a disease and marker allele can arise for several reasons. Firstly, the marker allele may directly result in increased susceptibility for the disease. In this case, the same association would be observed in groups of affected individuals drawn from any population. Secondly, the marker allele may be in linkage disequilibrium with a disease-causing allele. At a population level, multiple, random recombination events occur over many generations along a chromosome, but they are less likely to occur between alleles spaced less than approximately 200 kilobases (kb) from each other. This may result in linkage disequilibrium and this phenomenon will vary according to a population's history. For example, it is more likely to occur in an isolated population than a mixed population, so that associations may be observed in one population, but not replicated in another more mixed population. Lastly, associations may be spurious findings caused by comparing case and control groups drawn from heterogeneous populations. Numerous false

associations have been reported in the past and can be avoided by performing studies within well-defined homogeneous, populations. They can also be avoided by collecting DNA from the parents of affected individuals and using the non-transmitted alleles as control alleles, and by testing any associations identified using the transmission disequilibrium test (TDT). The basis of the TDT is that a parent who is heterozygous, and carries a copy of the putative allele, is more likely to pass that allele on to an affected child than the other, non-associated allele. The test must be performed on a different population, as the TDT cannot be applied to the population in which the association was originally found, because of the already defined excess of the associated allele.

No candidate genes had been identified for endometriosis up until 1996. Since then associations have been reported between endometriosis and a polymorphism in the galactose-1-phosphate uridyl transferase gene (GALT) (Cramer *et al.*, 1996b) and a null mutation of the glutathione-S-transferase M1 gene (Baranov *et al.*, 1996, Baranova *et al.*, 1997a). A very recent study, published after the data presented here were obtained and analysed, has reported a relationship between endometriosis and N-acetyltransferase 2 (NAT2) polymorphisms and the glutathione-S-transferase T1 gene null mutation (Baranova *et al.*, 1999).

In this chapter, studies which attempted to replicate these associations are presented. The investigation of a new candidate, the cytochrome P450 1A1 MspI polymorphism, is also presented.

5.2 SUBJECTS STUDIED

Blood or tissue was obtained from the following five groups and DNA extracted for genotyping: A) male blood donors (n = 100), B) pre-menopausal women aged 40-50 with a normal pelvis at hysterectomy performed for benign gynaecological indications (n = 54), C) sporadic cases with histologically confirmed endometriosis (n = 101), D) unrelated women with Stage B endometriosis and a family history of the disease (n = 57), and E) mothers of women with Stage B endometriosis (n = 30).

Population Controls

Samples from males ($n = 100$) who had donated blood in the Oxford region were supplied anonymously by the National Blood Service (*Group A*).

Normal Controls

Pre-menopausal women ($n = 54$) aged between 40-50 with a normal pelvis at hysterectomy were identified through the records of the John Radcliffe Hospital, Oxford. Women from this age group were chosen instead of younger women to maximise the probability that they were unaffected by endometriosis. Choosing young, unaffected women as controls is inappropriate as they may go on to develop the disease in later life. The surgical records were checked to ensure a normal pelvis was found at hysterectomy. Women with adenomyosis were excluded, because the disease may be related to endometriosis, as were women who had a vaginal hysterectomy, because this surgical approach does not allow adequate enough inspection of the pelvic cavity to exclude endometriosis. Altogether, 35/54 (65%) women had uterine fibroids. The mean age ($\pm$ SE) of the women studied was 45.4 ± 0.4 (range 40 - 50 years). Normal tissue, from the endometrium or cervix, fixed in formalin and paraffin embedded, from these patients was provided anonymously from the archives of the Cellular Pathology Department at the John Radcliffe Hospital (*Group B*).

Sporadic Cases

Women with histologically confirmed endometriosis ($n = 101$) were identified from histopathology reports and samples of normal, formalin-fixed, paraffin embedded tissue, from the endometrium or cervix, were provided anonymously as for the normal controls (*Group C*). The surgical reports were checked, where possible, to determine the severity of endometriosis although all women studied had histologically proven disease. Altogether 37/101 (36.6%) women had Stage B disease and 17 (16.8%) had stage A disease. In two women (2%), the stage of disease could not be determined from the surgical record, and operative records were not checked in the remaining 45/101 (44.6%).

Familial Cases

Families with endometriosis, including sisters, mother-daughter pairs and other pedigrees were recruited for the OXEGENE study as described in Chapter Four. DNA from only one individual with Stage B disease from each family recruited in the UK was studied ($n = 57$). The mean current age of the women studied was 43.9 (range 27 - 64) years (*Group D*).

Mothers of affected women

Blood samples were obtained from the mothers of women with stage B endometriosis in Group D ($n = 30$). Two of the mothers (7%) also had endometriosis (*Group E*).

DNA extraction

Genomic DNA was extracted from 9 ml of EDTA anti-coagulated whole blood using phenol-chloroform (patient groups D and E) or the QIAmp™ (Qiagen) Blood Kit (group A) according to the manufacturer's instructions. For the phenol-chloroform extraction procedure, samples were frozen at $-80\text{ }^{\circ}\text{C}$ and stored until use. The samples were thawed at $37\text{ }^{\circ}\text{C}$ and mixed with 30 mL lysis buffer (0.9% NaCl, 0.2% Igepal CA-630). After centrifugation at 1000g for 15 mins, the supernatant was discarded and the pellet washed again in lysis buffer (30 mL). Following centrifugation, the pellet was resuspended in 3.5 mL extraction buffer (10mM Tris HCl, 0.1M disodium EDTA, 0.5% SDS, pH 8.0) containing 0.2 mg/mL RNase (DNase free, Boehringer Mannheim) and incubated for 1 hour at $37\text{ }^{\circ}\text{C}$. Proteinase K was added to give a final concentration of 0.36 mg/mL and the samples were incubated overnight at $37\text{ }^{\circ}\text{C}$. A volume of 5 mL of Tris-washed phenol (BDH) was added and the samples were mixed thoroughly, 2.5 mL of chloroform/isopropanol (24:1) was then added and the samples were mixed again. Following centrifugation at 1000g for 15 mins, the upper aqueous layer was removed and placed in a clean tube. Another 5 mL of chloroform/isopropanol was added, and after mixing and centrifugation the aqueous layer was transferred into a third clean tube. Ice-cold ethanol (20 mL) was added to precipitate DNA. The DNA was removed and resuspended in 500 μL of sterile water. Unless otherwise stated, reagents were purchased from Sigma.

For groups B and C, DNA was extracted from normal paraffin-embedded, formalin-fixed, tissue samples using the QIAmp tissue kit™ (Qiagen). Segments of tissue 5

mm³ were cut from histopathology section blocks and placed into microfuge tubes containing 1 mL of 70% ethanol. The samples were heated to 70 °C to melt the paraffin, and then the liquid was pipetted off and the process repeated. Following this, the manufacturer's protocol for DNA extraction from tissue samples was carried out. The QIAmp procedure relies on adsorption of DNA within the lysate onto an affinity silica membrane within a microfuge spin column. Once loaded the membrane is washed twice with an ethanol containing buffer to remove residual contaminants. The DNA is eluted from the column by adding water heated to 70 °C.

5.3 GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE

Introduction

Galactose-1-phosphate uridyl transferase (GALT) is one of three enzymes required to metabolise galactose (Figure 5.2). The GALT gene maps to the short arm of chromosome 9 (Sparkes *et al.*, 1980) and there is a high degree of GALT amino acid sequence conservation between *Escherichia coli*, yeast, mouse and human (Leslie *et al.*, 1992), indicating the important role of this enzyme.

- [1] Galactose + ATP → Galactose-1-P + ADP
- [2] Galactose-1-P + UDP-glucose → UDP-galactose + Glucose-1-P
- [3] UDP-galactose → UDP-glucose

Figure 5.2. The three steps in the metabolism of galactose to glucose, equation [1] is catalysed by the enzyme galactokinase, [2] by GALT and [3] by UDP-galactose-4-epimerase (Frey, 1996).

A number of gene sequence changes, resulting in altered amino acid sequences, have been identified in the human GALT gene (Reichardt *et al.*, 1992, Reichardt & Woo, 1991). In exon 6 of the gene, a single nucleotide substitution (CAG → CGG), causing an amino acid change from glutamine (Q) to arginine (R) at position 188, results in classical galactosaemia and accounts for 60-70% of all cases of this disease. Patients with the Q188R polymorphism lack GALT activity, and the protein crystal structure reveals that this region probably plays an important role in enzyme catalytic function

(Wedekind *et al.*, 1995). Galactosaemia is treated by excluding galactose from the diet but even so, the long term prognosis includes mental retardation and primary ovarian failure.

Another single nucleotide substitution that results in reduced GALT activity is an adenine to guanine transition (codon change AAC → GAC) in exon 10 of the gene, causing an asparagine (N) to aspartic acid (D) amino acid change at position 314 (Reichardt & Woo, 1991). This N314D polymorphism, which is common in the general population, results in partially reduced enzyme activity.

The polymorphism has been associated with endometriosis in a North American population (Cramer *et al.*, 1996b). Thirty-three women attending a fertility clinic with all stages of endometriosis were genotyped for the GALT polymorphism and their GALT enzyme activity was also assessed. In total, 10/33 (30%) women (including 6/11 (54%) with stage B disease), carried the N314D polymorphism. The frequency in all patients was significantly different from the control group, which consisted of 111 pre-menopausal, female controls of whom 15 (14%) carried the polymorphism ($P = 0.02$). The mean ($\pm$ SE) GALT activity in patients with the N314D polymorphism was 19.04 ± 2.18 (μ moles of hexose converted per hour per gram haemoglobin) compared to 24.6 ± 0.59 for patients without the polymorphism ($P = 0.0011$).

The observed relationship may arise because reduced GALT activity associated with N314D may be an aetiological factor in endometriosis or because the polymorphism is in linkage disequilibrium with a disease susceptibility allele. The GALT gene locus, has been mapped to 9p21 (Bricarelli *et al.*, 1981) a region where loss of heterozygosity at candidate ovarian tumour suppressor loci has been reported in endometriotic tissue (Jiang *et al.*, 1996). Jiang *et al.* demonstrated loss of heterozygosity on chromosome 9p in the endometriotic tissue of 8/40 (18%) cases. Another possible explanation is that maternal GALT deficiency could increase the risk of endometriosis in female offspring. This is supported by the fact that the female offspring of rats fed a high galactose diet had delayed vaginal opening and reduced oocyte number (Chen *et al.*, 1981). Two reports have also shown a relationship between maternal GALT deficiency and Müllerian aplasia/vaginal agenesis (Cramer

et al., 1996a, Cramer *et al.*, 1987), which may be relevant given the known association between endometriosis and Müllerian anomalies.

The frequency of the N314D polymorphism in the UK population is not known (Linda Tyfield, Molecular Genetics Unit, Southmead Hospital, Bristol, personal communication), however studies in the USA have revealed a frequency of 14% in a sample drawn from the general population of women of reproductive age in Boston (Cramer *et al.*, 1996a). The polymorphism had previously been associated with infertility, premature ovarian failure (Cramer *et al.*, 1996a, Cramer *et al.*, 1989) and ovarian cancer (Morland *et al.*, 1998).

The aim of this study was to determine whether the N314D polymorphism was associated with endometriosis in a UK population. Women with endometriosis and mothers of women with endometriosis were genotyped and compared to both a population frequency and a control group of women without endometriosis at hysterectomy. As the population frequency for the N314D polymorphism is unknown in the UK, the prevalence was estimated by genotyping male blood donors.

Methods

A 311bp fragment of exon 10 containing the polymorphic region of interest was amplified using PCR, as described by Lin *et al.*, 1994. Primers were synthesised by Gibco Life Technologies (5'-GGGTTTGGGAGTAGGTGCT; 3'-GGGCAACAGAA-GTATCAGGT). The 50 µL PCR reaction contained 0.1 µg of the DNA sample to be genotyped, 0.5 µg each primer, 1x PCR buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP and 1 unit Taq DNA polymerase (all purchased from Qiagen). Reactions were overlaid with mineral oil (Sigma) and subjected to an initial denaturation of 97 °C for 5 mins followed by 40 cycles of denaturation at 94 °C for 1 min., annealing at 57 °C for 10 s and elongation at 72 °C for 40 s, followed by a final elongation of 72 °C for 5 mins. Following PCR amplification, 7.5 µL of the reaction mixture was combined with 3 units of the restriction endonuclease Ava II, 1x buffer A (both purchased from Boehringer Mannheim), 2mM spermidine and 0.15 mg/mL bovine serum albumin (BSA) to give a final volume of 15 µL. The digestion mixture was then incubated for 3 hours at 37 °C. The entire 15 µL was loaded onto a single lane of a 4% NuSieve ® 3:1 agarose gel (FMC, Rockland, ME, USA) containing ethidium

bromide (0.1 µg/mL). The products were visualised under ultraviolet (UV) light. The region of exon 10 amplified contains one Ava II restriction site normally and two restriction sites if the polymorphism is present, thereby providing an internal control of enzyme digestion. The process of genotyping is illustrated in Figure 5.3 and an example gel is shown in Figure 5.4. The restriction fragment length polymorphism (RFLP) analysis was successful in 95/100 (95%) of male controls, 53/54 (98%) of controls with no endometriosis at hysterectomy, 91/101 (90%) of sporadic cases and 57/57 (100%) of familial cases. The PCR was successful less often when DNA was extracted from paraffin-embedded, formalin fixed archival tissue. As a result of the fixation in formalin, the extracted DNA fragment sizes are <600 bp, and so the likelihood of successful amplification is diminished. Statistical comparisons were made using Fisher's exact test. Power calculations were made based upon the observed proportions obtained by Cramer *et al.* (1996). To ensure 80% power, a sample size of 100 in both case and control groups was required.

Results

No individual tested was found to be homozygous for the N314D polymorphism. The estimated population frequency of heterozygosity for the polymorphism in the UK, determined by testing 95 male blood donors, was 13/95 (13.7%, 95 CI 7.5% - 22.3%).

For all cases with endometriosis (groups C and D), 22/148 (14.9%, 95% CI 9.6% - 21.6%) were heterozygous for the N314D polymorphism. In comparison, 6/53 (11.3%, 95% CI 4.3% - 23.1%) women with a normal pelvis at hysterectomy (group B) were heterozygous. The frequency in cases was not significantly different to the frequency in either the male blood donors or in women with a normal pelvis at hysterectomy ($P = 0.85$ and 0.65 respectively). Nor was there a significant difference in the frequency amongst the mothers of women with Stage B endometriosis (group E) compared to the control groups: 5/30 (17%) mothers were heterozygous for the polymorphism ($P = 0.76$ and 0.52 respectively).

Lastly, the cases were divided into groups of Stage B disease only and sporadic or familial cases only, but no statistically significant differences were observed in the

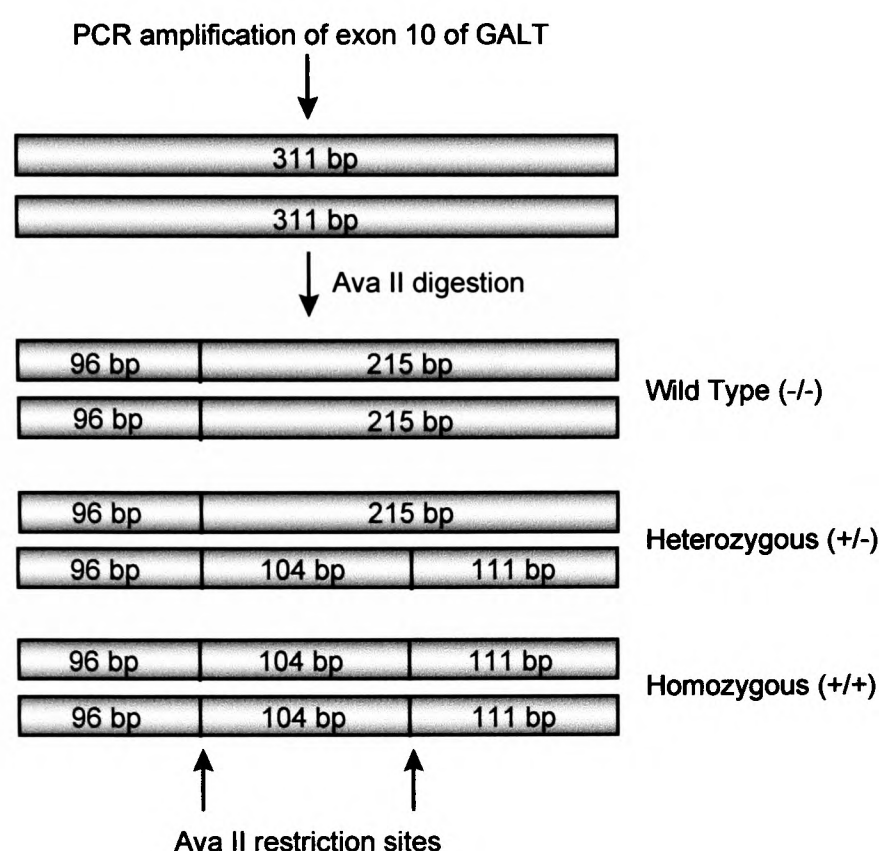


Figure 5.3. Diagram showing the products generated by PCR amplification of a 311bp fragment of exon 10 of the GALT gene followed by restriction with the enzyme AvaII.

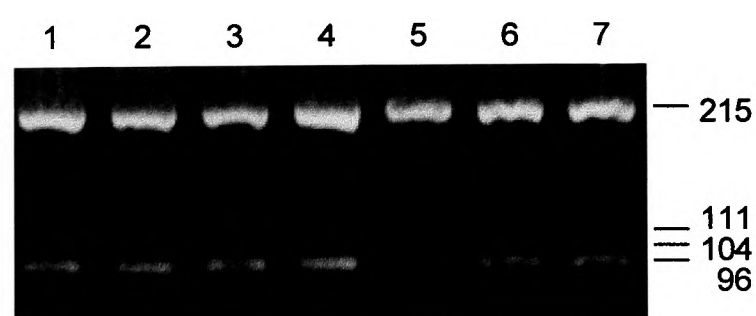


Figure 5.4. Gel electrophoresis of the digested PCR products stained with ethidium bromide showing individuals without the N314D polymorphism (lanes 1,4,5,6 & 7) and heterozygous for the polymorphism (lanes 2 & 3).

frequency of the polymorphism between any of these groups and the controls (Table 5.5).

Among the familial cases (group D), 28 women reported that they were infertile, of whom 8 (29%) carried the N314D polymorphism. Of the 18 women in group D who reported no problems conceiving only one (6%) carried the polymorphism. Eleven of the familial cases did not know or did not report their fertility status. Data on fertility status were not generally available for the sporadic cases, however 7 were known to have been investigated for infertility. Of these 3/7 (43%) were heterozygous for the N314D polymorphism. If all cases with known fertility problems are combined then 11/35 (31%) carried the polymorphism, significantly different than the frequency amongst both the male controls ($P = 0.04$) and women with a normal pelvis at hysterectomy ($P = 0.03$).

Subjects	<i>n</i>	N314D polymorphism present (%)	95% CI
Controls:			
Male controls	95	13 (13.7%)	7.5 - 22.3
Women with a normal pelvis at hysterectomy	53	6 (11.3%)	4.3 - 23.1
Cases:			
All cases [†]	148	22 (14.9%)	9.6 - 21.6
Stage B disease only	102	17 (16.7%)	10.0 - 25.4
Sporadic cases only	91	13 (14.3%)	7.8 - 23.2
Familial cases only	57	9 (15.8%)	7.5 - 27.9

Table 5.5. Prevalence of the N314D polymorphism of galactose-1-phosphate uridyl transferase in women with endometriosis and controls. [†]Sporadic + familial cases.

Discussion

In this study of UK patients, an association between the GALT N314D polymorphism and endometriosis was not observed, nor was there any evidence that mothers of women with disease were more likely to carry the polymorphism than the general population.

The findings contrast with those of Cramer *et al.* (1996b) who reported that 10/33 (30%) North American women with all stages of endometriosis, and 6/11 (54%)

women with Stage B disease, carried the N314D polymorphism compared to 15/111 (14%) pre-menopausal, female controls. There are several possible reasons for the observed differences. Firstly, these may represent actual genetic differences between the North American and UK populations. Secondly, the sample size in Cramer *et al.*'s study was small and the findings may represent a type I statistical error. Lastly, the cases studied were attending a fertility clinic and the polymorphism is already known to be associated with infertility (Cramer *et al.*, 1989) which may have introduced bias. The finding that 11/35 (31%) women with endometriosis and infertility were heterozygous for the N314D polymorphism in the present study, in combination with Cramer's findings, suggests that the relationship between GALT N314D, infertility and endometriosis requires further investigation.

Another group has since reported that they failed to reproduce Cramer's findings in a sample drawn from the UK population (Morland *et al.*, 1998). Morland *et al.* reported that 14/78 (18%) endometriosis cases carried the N314D polymorphism compared to 42/248 (17%) controls in the UK, data consistent with our findings. The frequency of the polymorphism in their controls was not significantly different to the control group of blood donors ($P = 0.51$) or women with a normal pelvis at hysterectomy ($P = 0.41$) in the present study.

In the investigation of cancer and other chronic diseases such as multiple sclerosis, the reproducibility of candidate loci association studies has been poor (Ebers & Dessa Sadvnick, 1994). The ability to replicate candidate gene studies to confirm associations, is particularly important in defining disease aetiology. The lessons learned from experience in other diseases should be taken into account in this new area of research into the causes of endometriosis.

In conclusion, an association between the GALT N314D polymorphism and endometriosis in the UK population has not been identified, although the study may have some limitations.

5.4 CYTOCHROME P450 1A1 (CYP1A1)

Introduction

Enzymes belonging to the cytochrome P-450 (CYP) and glutathione *S*-transferase (GST) families are involved in the two stage detoxification process of a number of pro-carcinogens (Figure 5.6). The genes for these enzymes are part of the aryl hydrocarbon (Ah) gene battery and are under Ah receptor control (Nebert & Gonzalez, 1987). The Ah receptor binds a number of different classes of chemicals, including halogenated aromatics such as dioxin, polycyclic aromatic hydrocarbons and plant metabolites, such as β -naphthoflavone, that induce transcription of the gene battery genes (Safe, 1995).

Dioxin, and the other compounds, act by binding to and activating the Ah receptor. The receptor-dioxin complex is translocated to the nucleus where it binds to DNA in the 5'-flanking region and activates transcription of CYP genes, such as CYP1A1 and CYP1A2, which code for phase I detoxification enzymes. These enzymes act by metabolising pro-carcinogens into reactive, carcinogenic intermediates that are subsequently catalysed with enzymes such as the glutathione-*S*-transferases (GSTs), in the phase II detoxification process (Figure 5.6).

Dioxin is an environmental pollutant produced by the combustion and manufacture of chlorinated carbon products such as PVC and is a by-product formed in the production of herbicides such as 2,4,5-trichlorophenoxy acetic acid, which was used extensively as a defoliant in the Vietnam war. Dioxin is a carcinogen and teratogen in rodents; it has numerous effects on reproductive health in humans and it may be a risk factor for endometriosis.

As presented in Chapter One, rhesus monkeys given 5-25 parts per trillion in their food over 4 years were at increased risk of developing the disease compared to unexposed controls (Rier *et al.*, 1993). There is also some evidence that dioxin levels may be higher in women with endometriosis compared to controls (Mayani *et al.*, 1997) and the incidence of endometriosis is high in Belgium, a country with one of the worst dioxin pollution records in the world (Koninckx *et al.*, 1994).

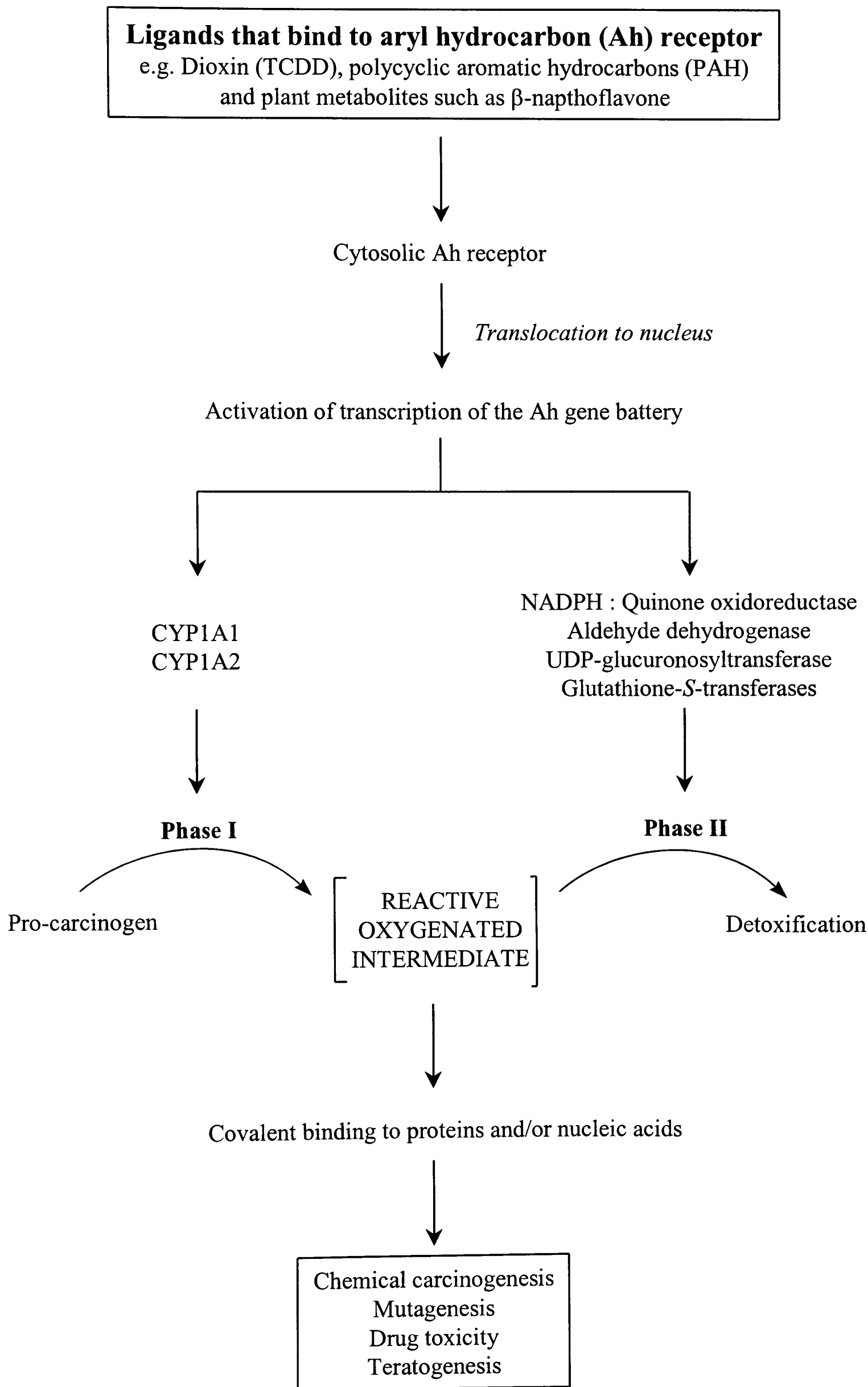


Figure 5.6. Schematic diagram of the aryl hydrocarbon receptor mediated phase I and II detoxification process.

The possible relationship between endometriosis and dioxin, and the known relationship between dioxin and activation of transcription of the Ah gene battery, led investigators to look at a possible relationship between one of these genes, GSTM1, and endometriosis. The GST enzymes are discussed in more detail in the following section (Section 5.5).

The enzyme CYP1A1 also has a polymorphism associated with an increased risk of tumorigenesis (García-Closas *et al.*, 1997, Guengerich, 1988, Kawajiri *et al.*, 1990, Sivaraman *et al.*, 1994, Xu *et al.*, 1996). This polymorphism is 264 bases downstream from the polyadenylation site at the 3' end of exon 7 of the CYP1A1 gene. The thymine to cytosine substitution introduces a new MspI restriction site, facilitating genotyping. This MspI polymorphism has been associated with an increased risk for smoking-induced lung cancer (Xu *et al.*, 1996). The gene for CYP1A1 has been mapped to 15q22-q24. In view of the previously reported association between endometriosis and GSTM1, it seemed logical to investigate the role of the CYP1A1 MspI polymorphism in endometriosis.

It has been demonstrated that high-inducibility of the CYP1A1 gene is related to the presence of the MspI polymorphism. The exact mechanism of this relationship is unknown although a number of possibilities have been put forward. For example, transcription may run through this polymorphism and the polymorphism may stabilise the resulting transcript. Alternatively, the polymorphism may be in linkage disequilibrium with important regulatory genes or the presence of the polymorphism may directly affect regulatory protein binding to this region, thus affecting enzyme inducibility (Petersen *et al.*, 1991). Evidence has implicated CYP1A1 in the metabolism of caffeine, which is inhibited by progesterone and 17 β -oestradiol (Eugster *et al.*, 1993). These mechanisms may be relevant to the aetiology of endometriosis.

The aim of this study was to determine whether the CYP1A1 MspI polymorphism was associated with endometriosis in a UK population. Women with endometriosis were genotyped and compared to both a population frequency and a control group of women without endometriosis at hysterectomy.

Methods

A 340 bp fragment of the 3'-end of CYP1A1 containing the polymorphic region of interest was amplified using PCR, as described previously (Sivaraman *et al.*, 1994). Primers were synthesised by Gibco Life Technologies (5'-CAGTGAAGAGG-TGTAGCCGCT; 3'-TAGGGAGTCTTGTCTCATGCCT). The 15 µL PCR reaction contained 0.1 µg of the DNA sample to be genotyped, 0.2 µg each primer, 1x PCR buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP and 0.3 units Taq DNA polymerase (all purchased from Qiagen).

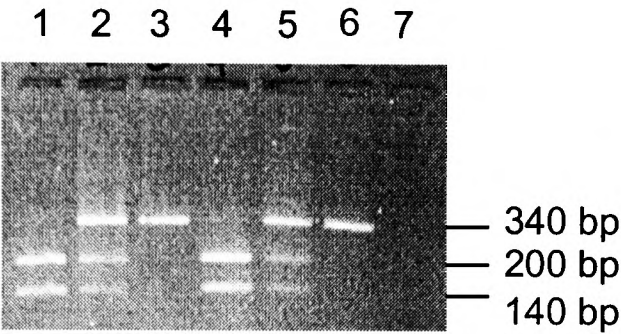


Figure 5.7. Agarose gel electrophoresis of the digested PCR products stained with ethidium bromide showing individuals homozygous for the MspI polymorphism (lanes 1 & 4), heterozygous for the polymorphism (lanes 2 & 5) and without the polymorphism (lanes 3 & 6). Lane 7 shows a negative PCR control.

Reactions were amplified using the following thermal profile: initial denaturation of 95 °C for 2 mins, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s, and a final elongation of 72 °C for 5 mins. PCR was conducted without mineral oil, using an ABI 9700 thermocycler (PE Applied Biosystems) with heated lid. Following PCR amplification, 3 µL of the reaction mixture was combined with 3 units of the restriction endonuclease MspI and 1x buffer L (both purchased from Boehringer Mannheim) to give a final volume of 15 µL. The digestion mixture was then incubated for 3 hours at 37 °C. The entire 15 µL reaction mixture was loaded onto a single lane of a 1.5% agarose gel (FMC, Rockland, ME, USA) containing ethidium bromide (0.1 µg/mL). The products were visualised under ultraviolet (UV) light as shown in Figure 5.7. Genotyping was successful in 98/100 (98%) male controls, 49/54 (91%) controls with no endometriosis at hysterectomy, 76/101 (75%) sporadic cases and 53/57 (93%) familial cases. As stated before, the reduced success rate in sporadic cases can be attributed to

poorer amplification during PCR, using DNA extracted from formalin-fixed tissue, which is <600 bp in length.

Results

The CYP1A1 MspI variant allele frequency in male blood donors was 0.10 which is comparable to the published frequency observed in cancer studies. An allele frequency of 0.09 was observed amongst American Caucasian controls in one study (Sivaraman *et al.*, 1994) and the allele frequency was also 0.09 in a control group drawn from the French population in another study (Lucas *et al.*, 1996).

Subjects	<i>n</i>	Variant allele frequency	CYP1A1 MspI (+/-) or (+/+) (%)	95% CI
Controls:				
Male controls	98	0.10	18 (18%)	9.8 - 25.6
Women with a normal pelvis at hysterectomy	49	0.14	12 (24%)	13.3 - 38.9
Cases:				
All cases [†]	129	0.11	27 (21%)	14.3 - 29.0
Moderate-severe disease only	98	0.11	21 (21%)	13.8 - 30.9
Sporadic cases only	76	0.10	14 (18%)	10.5 - 29.0
Familial cases only	53	0.12	13 (25%)	13.8 - 38.3

Table 5.8. Prevalence of the CYP1A1 MspI polymorphism in women with endometriosis and controls. Frequencies are given as both the variant allele frequency and the frequency of carrying either one (+/-) or two (+/+) alleles with the polymorphism. [†]Sporadic + familial cases.

The results are summarised in Table 5.8. No significant differences were found between the polymorphism frequency in sporadic cases compared to male controls or women with a normal pelvis at hysterectomy (*P* = 1.0 and 0.50 respectively). Likewise, no significant differences were observed between the frequency in familial cases and the two different control groups (*P* = 0.40 and 1.0 respectively).

Discussion

In this study no significant differences were observed between the frequency of the MspI CYP1A1 polymorphism in endometriosis patients compared to controls. As the differences between cases and controls were not significant, it is appropriate to discuss

the power of the experiment. The normal allele frequency is 0.10, and to detect an increase of 0.15 with >80% power, a sample size of 100 alleles (*i.e.* 50 patients) in case and control groups would be required.

The CYP1A1 MspI polymorphism has previously been associated with an increased risk for a number of different conditions. In a sample drawn from the US population, a significant association was observed between the MspI polymorphism and lung cancer (Xu *et al.*, 1996) with an odds ratio of 2.1 for combined heterozygotes and homozygotes. Another study, in a Japanese population, found evidence of an association between homozygosity for the polymorphism and colorectal cancer (Sivaraman *et al.*, 1994). A further association was found between homozygosity for the MspI polymorphism and oral squamous cell carcinoma in Japanese patients (Tanimoto *et al.*, 1999) with 15% of patients compared to 8% of controls homozygous for the MspI variant.

As illustrated in Figure 5.6, CYP1A1 is involved in the phase I detoxification system, and the glutathione-*S*-transferases are involved in the phase II detoxification system. Therefore, potentially, the presence of the CYP1A1 MspI polymorphism which may enhance the upregulation of transcription by compounds such as dioxin, in combination with the absence of a phase II enzyme, such as GSTM1, could result in an excess of reactive intermediate which may in turn be involved in disease aetiology. An analysis of the combination of these genotypes in cases and controls, to determine whether women with endometriosis were more likely to carry both the MspI polymorphism and the GSTM1 null mutation, was therefore conducted. These data are presented in the next section.

This approach is supported by findings in lung cancer. Benzo[a]pyrene diol-epoxide DNA adduct levels in the lung tissue of lung cancer patients were significantly higher in those patients who were both homozygous for the CYP1A1 MspI polymorphism and the GSTM1 null mutation, compared to patients with other genotype combinations (Rojas *et al.*, 1998). Other studies have found that Japanese patients with prostate cancer were at increased risk if they had variant CYP1A1 in combination with the GSTM1 null genotype (Murata *et al.*, 1998). Patients with both genotypes were also at further increased risk of acute lymphoblastic leukemia (odds

ratio = 3.3) compared to the risk of either genotype alone (Krajinovic *et al.*, 1999). A third study investigating lung cancer found that neither the GSTM1 null genotype nor the CYP1A1 MspI genotype alone conferred an increased risk for lung cancer; however, the presence of both genotypes was associated with increased risk (odds ratio = 2.0, 95% CI 1.0 - 3.4) (García-Closas *et al.*, 1997).

In conclusion, whilst there is no evidence from this study that the CYP1A1 MspI polymorphism alone plays a role in disease susceptibility, evidence from other diseases suggests that both the GSTM1 null mutation and CYP1A1 MspI variant may act together to increase the risk of disease and this possibility is explored in the following section.

5.5 GLUTATHIONE-*S*-TRANSFERASE *M1* AND *T1* NULL MUTATIONS

Introduction

The glutathione-*S*-transferases (GSTs) are a family of isoenzymes involved in the phase II detoxification system. These enzymes catalyse the conjugation of reactive hydrophobic and electrophilic compounds to reduced glutathione (a tripeptide of glutamine-cysteine-glycine) thereby deactivating their toxicity. Many of the known GST substrates are xenobiotic, and different classes of enzymes are specific for different substrates (Board *et al.*, 1990). Two of the human GST isoenzymes are polymorphic and are known to have gene deletions which result in a null allele, for which homozygosity confers a complete lack of enzyme activity. These are the μ -class enzyme glutathione-*S*-transferase M1 (GSTM1) and the θ -class enzyme glutathione-*S*-transferase T1 (GSTT1). The substrate specificity of these enzymes is still being elucidated, however they appear to be important in the detoxification of products of oxidative stress, for example lipid hydroperoxides, alkenals and DNA hydroperoxides, as well as potential carcinogens such as methyl halides and benzo[a]pyrene epoxides (Sarhanis *et al.*, 1996). The null mutations of these enzymes have been associated with cancer in a number of studies.

One such study observed a significant increase in the number of individuals with colon cancer who were null for the GSTM1 polymorphism compared to controls, with 110/196 (56%) GSTM1 null in the case group compared to 94/225 (42%) in the

control group (Zhong *et al.*, 1993). Moreover, 51/72 (71%) of those patients with tumour in the proximal colon were GSTM1 null. The same study failed to find a relationship between bladder cancer or breast cancer and GSTM1.

Another study found that the frequencies of GSTM1 and T1 null mutations were not significantly different in patients with epithelial ovarian cancer compared to controls (Sarhanis *et al.*, 1996). However, in patients with tumour showing p53 expression, 20/23 (87%) were GSTM1 null compared to 192/312 (62%) of controls. The authors hypothesised that GSTs may be important in the detoxification of the reactive oxygen species produced during the repair of damage to the ovarian surface epithelium caused by ovulation. The inability to repair such damage, due to the absence of these genes, may result in damage to other genes within the cell, including p53. Of note, there is evidence that loss of heterozygosity and alterations of chromosome 17 and the p53 tumour suppressor gene occur in endometriotic tissue (Bischoff *et al.*, 1997).

Other studies have demonstrated a relationship between the GSTM1 null mutation and chronic bronchitis in heavy smokers (Baranova *et al.*, 1997b) and prostate cancer in a Japanese population (Murata *et al.*, 1998). A null mutation in the gene coding for GSTM1 was first implicated in the aetiology of endometriosis in 1996 in a study investigating the correlation between this genotype and a number of diseases in Slavic populations (Baranov *et al.*, 1996). The authors observed a significant increase in the proportion of patients with the GSTM1 homozygous null (0/0) genotype and lung cancer (47/58, 81%), other tumour types (24/37, 65%), alcoholic cirrhosis (17/22, 77%), severe chronic bronchitis (39/53, 74%) and endometriosis (34/42, 81%) compared to controls (26/67 (39%) for north-western and 106/157 (68%) for central-eastern European Russia).

The same group then investigated the association between endometriosis and GSTM1 in a French population (Baranova *et al.*, 1997a). The authors found that 43/50 (86%) women with all stages of endometriosis had the 0/0 genotype, compared to 33/73 (46%) of a control group consisting of women admitted to hospital for termination of pregnancy ($P < 0.0001$). If only women with Stage B disease were included then 19/21 (90%) had the 0/0 genotype. A similar relationship was found in Slavic populations (Baranov *et al.*, 1996).

The gene for GSTM1 has been assigned to the short arm of chromosome 1, in region 1p13 (Zhong *et al.*, 1992). In view of the strong association found between endometriosis and the GSTM1 null mutation in the two studies described, it was decided to adopt a dual approach and endeavour both to replicate the association by genotyping groups of cases and controls, and also conduct linkage analysis in a set of affected sister-pair families using several microsatellite markers in this region. The relationship between the null mutation of GSTT1 and endometriosis was also investigated, and conveniently both GSTM1 and T1 could be genotyped in the same multiplex PCR reaction. The correlation between the CYP1A1 MspI polymorphism and the GSTM1 null mutation was also investigated, because, as described earlier, the simultaneous presence of both of these variants has been shown to increase the risk of cancer in several studies.

Methods

Multiplex PCR for genotyping of GSTM1 and GSTT1

A multiplex PCR was conducted to genotype for GSTM1 and GSTT1 simultaneously, as described by Arand *et al.*, 1996. In this method, primers for fragments of the GSTM1 and GSTT1 genes and primers for an omnipresent albumin gene control are placed into the same PCR reaction tube. When the study subject is null for the gene no PCR product is present, but the albumin gene fragment acts as a positive PCR control. Primers were synthesised by Gibco Life Technologies (Table 5.9). The 15 μ L PCR reaction contained 0.1 μ g of the DNA sample to be genotyped, 4.5 ng GSTM1 primers, 1.5 ng GSTT1 primers and 9 ng Albumin primers, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.3 units Taq DNA polymerase (all purchased from Qiagen).

Reactions were amplified using the following thermal profile: initial denaturation of 95 °C for 2 mins, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min and elongation at 72 °C for 1 min, followed by a final elongation of 72 °C for 5 mins. PCR was conducted without mineral oil, using an ABI 9700 thermocycler (PE Applied Biosystems) with heated lid. Following PCR amplification, the entire 15 μ L reaction mixture was loaded onto a single lane of a

1.5% agarose gel (FMC, Rockland, ME, USA) containing ethidium bromide (0.1 µg/mL). The products were visualised under ultraviolet (UV) light as shown in Figure 5.10.

Primer	Sequence	Product Size
GSTM1 forward	GAACTCCCTGAAAAGCTAAAGC	215 bp
GSTM1 reverse	GTTGGGCTCAAATATACGGTGG	
GSTT1 forward	TTCCTTACTGGTCCTCACATCTC	480 bp
GSTT1 reverse	TCACCGGATCATGGCCAGCA	
Albumin forward	GCCCTCTGCTAACAAGTCCTAC	350 bp
Albumin reverse	GCCCTAAAAAGAAAATCGCCAATC	

Table 5.9. Primer sequences used for multiplex PCR to genotype simultaneously for GSTM1 and T1 null mutations (as described by Arand *et al.*, 1996).

Genotyping for GSTM1 was successful in 100/100 (100%) male controls, 52/54 (96%) controls with no endometriosis at hysterectomy, 75/101 (74%) sporadic cases and 57/57 (100%) familial cases. Genotyping for GSTT1 was successful in 100/100 (100%) male controls, 50/54 (93%) controls with no endometriosis at hysterectomy, 59/101 (58%) sporadic cases and 57/57 (100%) familial cases. As mentioned earlier, the genotyping had a low success rate in the sporadic cases, as DNA was extracted from formalin-fixed tissue.

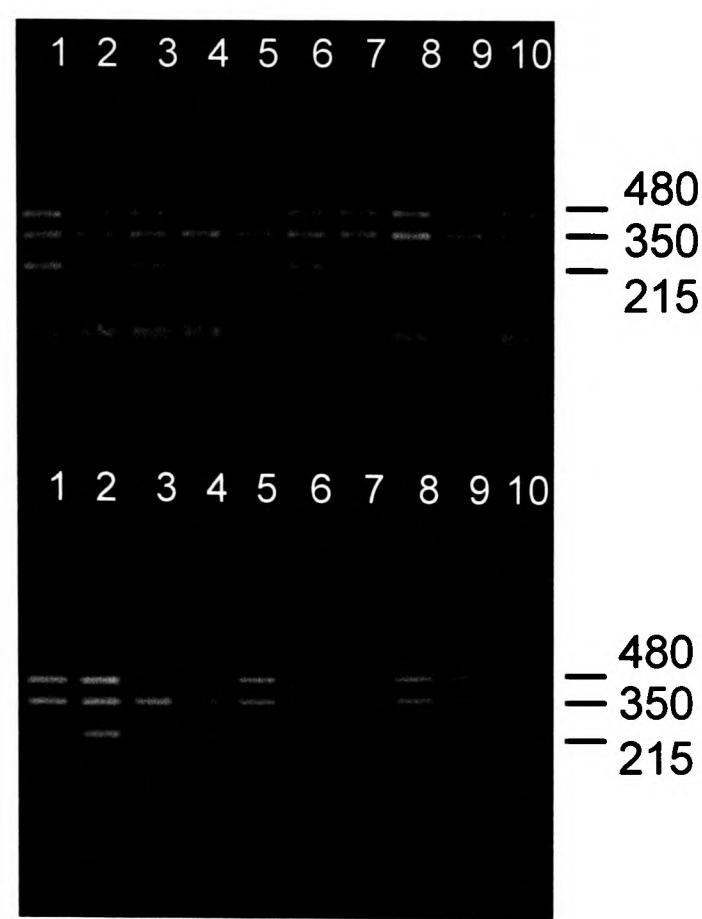


Figure 5.10. Agarose gel electrophoresis of the products of multiplex PCR stained with ethidium bromide and visualised under UV light. The 480bp band shows presence of the GSTT1 gene and absence indicates the individual is homozygous null (0/0) for this gene. Similarly, the 215bp band shows the presence of the GSTM1 gene. The 350bp band is an albumin gene fragment which gives a positive PCR control. The lower lane 10 shows a negative PCR control.

Linkage analysis

Three markers were selected from the region of chromosome 1 to which GSTM1 has been mapped (Zhong *et al.*, 1992) as shown in Figure 5.11. The markers were selected from the Généthon microsatellite maps (Dib *et al.*, 1996) and chosen for high heterozygosity to maximise the information content (Table 5.12).

Marker	Het	Label	Tm (°C)	Forward/reverse primers	Size range
D1S2844	81%	HEX	50.2	tctgaccttgcatg/aagaagtcactgagaacctggg	155-185
D1S2635	87%	NED	49.9	tagcagatccccgcg/tgaatcctaccctaagtagaat	142-159
D1S2762	81%	NED	45.0	ccttaattgtggtgttggt/aaaaatctggaaggcataaa	232-250

Table 5.12. Details of the three microsatellite markers from the 1p13 region chosen for linkage studies of GSTM1. Het = heterozygosity determined by Généthon using 28 unrelated individuals from the CEPH database. Tm = mean primer pair melting temperature.

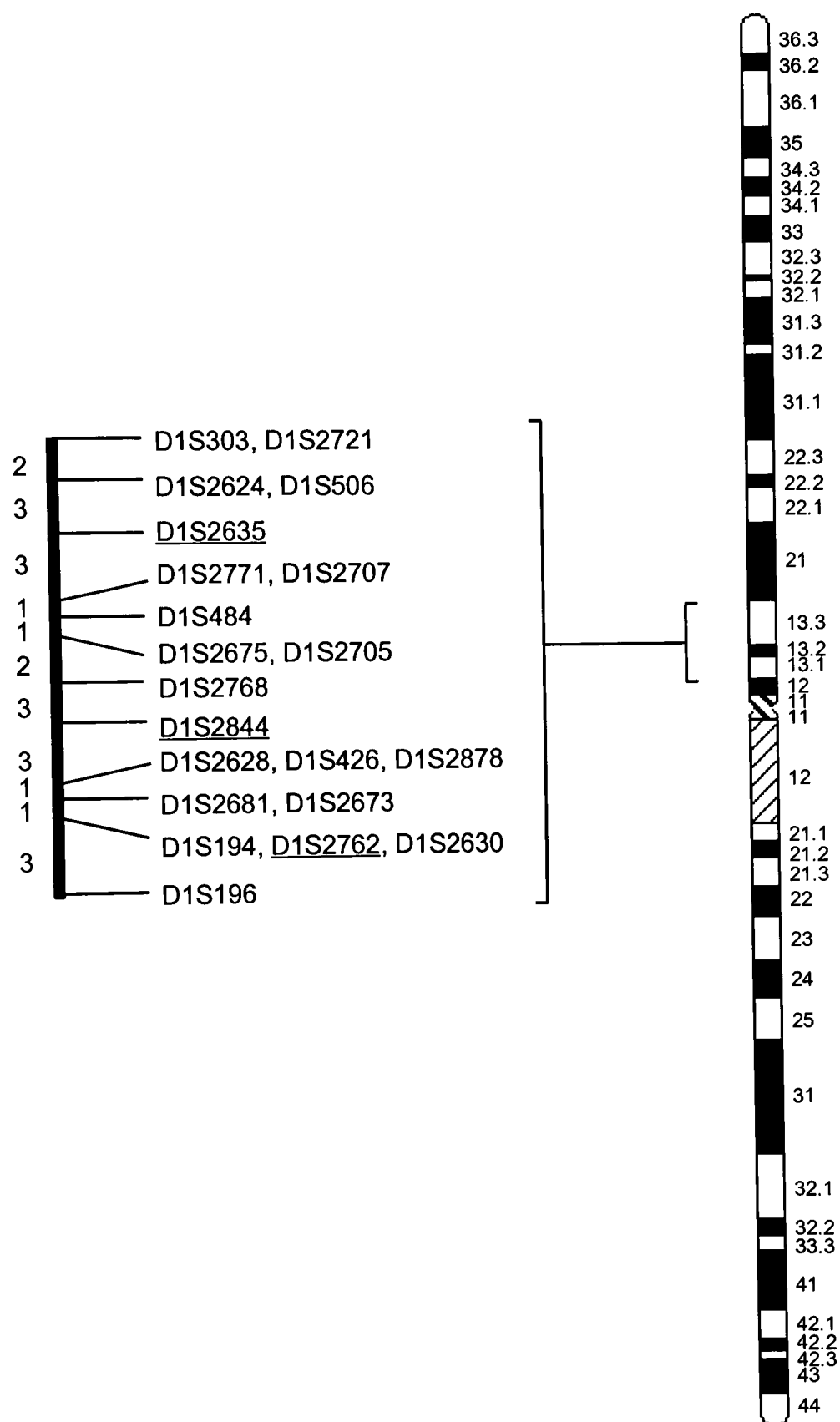


Figure 5.11. Markers were selected from the 1p13 region of chromosome 1 as shown. The underlined marker names denote those which were used in the linkage analysis. Map distances are in centimorgans (cM).

The genotyping method for linkage analysis is described in detail in Chapter Six. Briefly, primers were synthesised by PE Applied Biosystems and the forward primer fluorescence labelled as shown in Table 5.11. A separate PCR reaction mixture was prepared for each primer pair, containing 0.33 μM of each primer, 1x PCR buffer, 250 μM each dNTP, 0.04 units/ μL AmpliTaq GoldTM Taq polymerase and 2.5 μM MgCl_2 and 0.1 μg target DNA, to give a final PCR volume of 7.5 μL . All materials were purchased from PE Applied Biosciences. The reactions were prepared in 96-well reaction plates (PE Applied Biosciences) with individual sealing lids. PCR amplification was performed without mineral oil, using an ABI 9700 thermocycler (PE Applied Biosystems) with heated lid. The PCR profiles used are shown in Table 5.13.

DNA from the same patient was included in every run to control for gel to gel variation. The three PCR products from each patient were pooled by combining 4 μL of the products from D1S2844, 3 μL from D1S2635 and 3 μL from D1S2762 with water to give a total volume of 22 μL . The remainder of the genotyping and analysis procedure is described in Chapter Six, the only difference being that a 48-well square tooth comb was used for gel electrophoresis on an ABI 377 (PE Applied Biosystems).

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	12 min	
Denaturation	94 °C	15 sec	
Annealing	55/50 °C	15 sec	10
Extension	72 °C	30 sec	
Denaturation	89 °C	15 sec	
Annealing	55/50 °C	15 sec	20
Extension	72 °C	30 sec	
Final elongation	72 °C	10 min	

Table 5.13. PCR profile used for genotyping with an annealing temperature of 55 °C for markers D1S2844 and D1S2635, and an annealing temperature of 50 °C for D1S2762.

Statistical analysis of the genotype data was conducted, as fully described in Chapter Six, using SPLINK version 1.05 (Holmans, 1993), GENEHUNTER version 1.3

(Kruglyak *et al.*, 1996) and SimIBD version 1.2 (Cottingham *et al.*, 1993, Davis *et al.*, 1996, Ott, 1989, Weeks *et al.*, 1990).

Familial DNA samples for linkage analysis

Families for linkage analysis were collected as described in Chapter Four, and DNA was extracted from EDTA anti-coagulated blood samples. In total, 192 individuals from 43 nuclear families were genotyped for these three markers, containing in total 52 sister-pairs with Stage B endometriosis.

Statistical comparisons of proportions were made using a two-sided Fisher's exact test at the 95% level, unless otherwise stated.

Results

Association studies

The results of the association studies are presented in Tables 5.14 and 5.16. No significant differences were found between the four case groups compared to the two control groups for either the GSTM1 or GSTT1 null mutations. The *P* values are presented in Tables 5.15 and 5.17 respectively.

The proportion of individuals with null mutations did not differ significantly from population control groups in other studies. For example, amongst Caucasian female controls recruited in the United Kingdom, the prevalence of the GSTT1 null genotype was 61/325 (19%) (Sarhanis *et al.*, 1996). The prevalence of the GSTM1 null genotype among Caucasian controls was 94/225 (42%) in a UK based study (Zhong *et al.*, 1993), and 232/446 (52%) among controls consisting of friends or spouses of lung cancer patients in a US based study (García-Closas *et al.*, 1997).

Subjects	<i>n</i>	GSTM1 null (%)	95% CI
Controls:			
Male controls	100	45 (45%)	0.35 - 0.55
Women with a normal pelvis at hysterectomy	52	27 (52%)	0.38 - 0.66
Cases:			
All cases [†]	132	59 (45%)	0.36 - 0.54
Stage B disease only	96	46 (45%)	0.38 - 0.58
Sporadic cases only	75	33 (44%)	0.33 - 0.56
Familial cases only	57	26 (46%)	0.32 - 0.59

Table 5.14. Prevalence of the GSTM1 null mutation in women with endometriosis and controls without the disease. [†]Sporadic + familial cases.

<i>GSTM1</i> (<i>P</i> values)		Controls
Cases	Male controls	Women with a normal pelvis at hysterectomy
All cases	1.00	0.41
Stage B disease only	0.77	0.73
Sporadic cases only	1.00	0.47
Familial cases only	1.00	0.57

Table 5.15. *P* values (two-sided Fisher’s exact test) for comparisons between case and control groups for the GSTM1 null mutation.

Subjects	<i>n</i>	GSTT1 null (%)	95% CI
Controls:			
Male controls	100	20 (20%)	0.13 - 0.29
Women with a normal pelvis at hysterectomy	50	14 (28%)	0.16 - 0.42
Cases:			
All cases [†]	116	29 (25%)	0.17 - 0.34
Stage B disease only	87	20 (23%)	0.15 - 0.33
Sporadic cases only	59	19 (32%)	0.21 - 0.46
Familial cases only	57	10 (18%)	0.09 - 0.30

Table 5.16. Prevalence of the GSTT1 null mutation in women with endometriosis and controls without the disease. [†]Sporadic + familial cases.

<i>GSTT1</i> (<i>P</i> values)		Controls
Cases	Male controls	Women with a normal pelvis at hysterectomy
All cases	0.42	0.70
Stage B disease only	0.72	0.54
Sporadic cases only	0.09	0.68
Familial cases only	0.83	0.25

Table 5.17. *P* values (two-sided Fisher’s exact test) for comparisons between case and control groups for the GSTT1 null mutation.

CYP1A1 MspI polymorphism/GSTM1 null genotype combination

An analysis of the proportion of patients who were null for GSTM1 and also carried the CYP1A1 MspI polymorphism was conducted. Among male controls who were null for GSTM1, 5/44 (11.4%) also carried at least one MspI variant CYP1A1 allele. Among women with a normal pelvis at hysterectomy, 4/26 (15.4%) had the same combination of genotypes. In comparison, 8/29 (27.6%) of sporadic cases and 9/24 (37.5%) of familial cases were both null for GSTM1 and carried at least one MspI variant CYP1A1 allele. The difference between the proportion of male controls and familial cases with the genotype combination was statistically significant ($P = 0.025$). However, comparisons made between the other groups of cases and controls were not significantly different (for male controls compared to sporadic cases, $P = 0.11$; hysterectomy controls compared to familial cases $P = 0.11$ and sporadic cases $P = 0.34$).

The proportion of individuals in each group who were both GSTM1 null and GSTT1 null was not significantly different. In total, 10/45 (22%) male controls were null for both genes, compared to 5/29 (17%) sporadic cases ($P = 0.77$) and 5/26 (19%) familial cases ($P = 1.00$). Similarly, 9/27 (33%) hysterectomy controls had the null genotype combination, a proportion not significantly different when compared to sporadic and familial cases ($P = 0.22$ and 0.35 respectively).

Comparisons were made of individuals both GSTT1 null and CYP1A1 MspI heterozygous or homozygous, however, due to the low frequency of both of these genotypes the sample sizes were too small to draw any conclusions.

Linkage studies

The proportion of patients successfully genotyped was 99% for D1S2635, 93% for D1S2762, and 91% for D1S2844. The accuracy of the genotyping data was checked by comparing the calculated heterozygosity of each marker to published values from Génethon (Dib *et al.*, 1996). The observed and expected values were not significantly different for any of the three markers. The observed heterozygosity was 85% for D1S2635 (published value 87%), 83% D1S2762 (published value 81%) and 82% for D1S2844 (published value 81%).

The results of the statistical analysis using SPLINK are presented in Table 5.18. No significant increases from expected IBD (identical-by-descent) sharing were observed for any of the three markers studied. Similarly, no significant deviations from expected values were observed using the programs SimIBD or Genehunter (Table 5.19). The results of multipoint analysis using Genehunter are shown in Figure 5.20.

A fuller description of the different methods of statistical genetic analysis employed here is given in the following chapter.

Marker	N [†]	Estimated IBD sharing			LOD	P value
		0 alleles	1 allele	2 alleles		
D1S2635	49	0.25	0.49	0.26	0.007	0.52
D1S2844	48	0.23	0.48	0.29	0.063	0.38
D1S2762	50	0.23	0.47	0.30	0.103	0.32

Table 5.18. Analysis of the data using SPLINK version 1.05 (Holmans, 1993). [†]The number of affected sister-pair comparisons, weighted according to the number of siblings (e.g. in affected sib trios, each sib pair weighted by 2/3, 1/2 etc.). The sample size is reduced because some families were uninformative at each locus. LOD (logarithm of the odds) score = LOD score equivalent generated by SPLINK.

Marker	Het	Number of alleles	APM	SimIBD	Genehunter
		observed	P value	P value	P value
D1S2635	85%	13	0.65	0.56	0.33
D1S2844	83%	11	0.17	0.11	0.14
D1S2762	82%	12	0.66	0.56	0.26

Table 5.19. Analysis using SimIBD version 1.2 (Cottingham *et al.*, 1993, Davis *et al.*, 1996, Ott, 1989, Weeks *et al.*, 1990), APM statistics were also calculated with this package (Weeks & Lange, 1988), and Genehunter version 1.3 (multipoint analysis) (Kruglyak *et al.*, 1996). Het = heterozygosity.

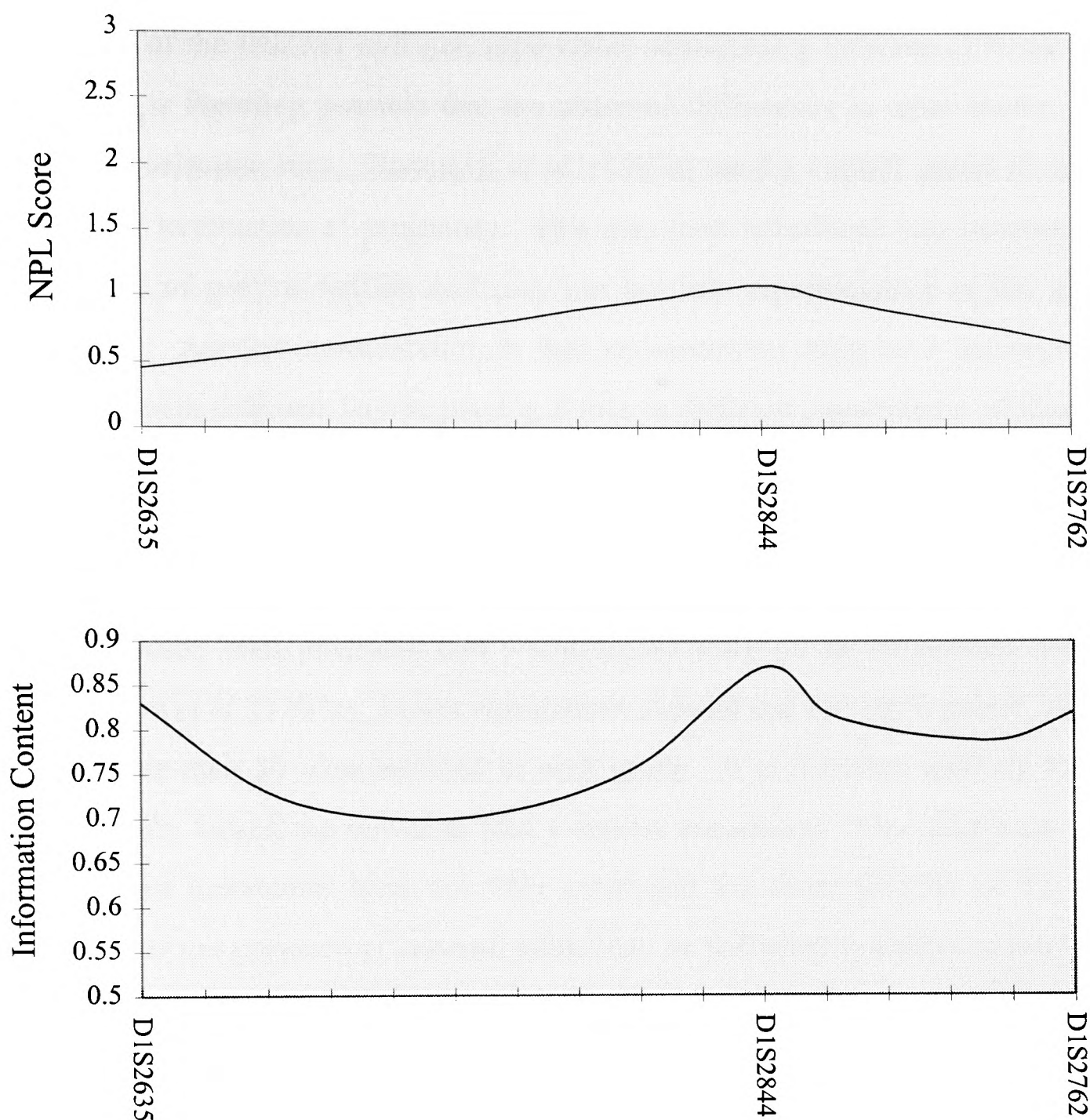


Figure 5.20. Graphical output from Genhunter analysis. The upper graph shows the microsatellite marker map versus non-parametric linkage (NPL) score generated by Genhunter. The lower graph shows the information content of each marker for comparison.

Discussion

In this study, no significant difference in the frequency of GSTM1 and GSTT1 null homozygotes between cases and controls was observed. This is in contrast to the two studies in the literature which have shown significant association between the GSTM1 null genotype and endometriosis (Baranov *et al.*, 1996, Baranova *et al.*, 1997a).

This difference can be explained in a number of different ways. Firstly, the prevalence of the GSTM1 null genotype varies considerably between different racial groups. It is therefore possible that the observed differences in other studies could arise from selection bias. Baranova *et al.* (1997a) used a control group of women undergoing termination of pregnancy. This may have introduced bias because these women are of proven fertility and may not be truly representative of the general population. Another consideration is that endometriosis may be a heterogeneous condition, with different factors playing a role in different populations. If causative environmental agents are important in disease development, then different agents may be involved in different regions.

The association study presented here was designed based on the frequencies observed by Baranova *et al.* (1997a). Power calculations showed that for >80% power, samples sizes greater than 50 were required in each group. It is therefore unlikely that the present study lacked the power to find a similar association. One drawback of the study is that individuals were not fully genotyped for either GSTM1 or T1. It is possible that the presence of one null allele may be sufficient to confer susceptibility for endometriosis. However, given the high frequency of GSTM1 null homozygotes any significant association should have been detected by genotyping in this way.

Similarly, there was no evidence of linkage to three markers in the 1p13 region. The power to detect linkage depends on the number of susceptibility loci for endometriosis, as discussed in the following Chapter, but if it is assumed that there are two susceptibility loci, each exerting equal effects, then there would be >80% power to detect linkage with the sample size of affected sib-pairs studied.

The combination of the GSTM1 null genotype and the CYP1A1 MspI polymorphism appeared to increase the risk of endometriosis, with 11% and 15% of controls having both genotypes compared to 28% and 38% of cases. This observed association may justify further investigation in a larger study, taking into account epidemiological factors such as caffeine intake, smoking and region of residence. Interestingly, the GSTM1 null genotype has been associated with high inducibility of CYP1A1 gene transcription by dioxin in *in vitro* studies (Vaury *et al.*, 1995). Vaury *et al.* demonstrated that the levels of CYP1A1 mRNA induced by dioxin were low when at

least one GSTM1 allele was present, whereas the GSTM1 null genotype resulted in the induction of high CYP1A1 mRNA levels by dioxin.

The region of chromosome 15 to which CYP1A1 has been mapped will therefore be of particular interest in the genome wide search for endometriosis susceptibility loci. The possibility of conducting association studies of other genes in the aryl hydrocarbon gene battery, including CYP1A2, NADPH:quinone oxidoreductase, aldehyde dehydrogenase and UDP-glucuronosyltransferase in relation to endometriosis should also be investigated.

5.6 CONCLUSION

The investigation of four candidate genes for endometriosis has been presented here. No association between endometriosis and the GALT N314D polymorphism was observed, in contrast to a previous study conducted in the USA. However, a possible association between this polymorphism and infertility in endometriosis patients was observed which warrants further investigation. No associations were observed between endometriosis and the GSTM1 null genotype, the GSTT1 null genotype and the CYP1A1 MspI polymorphism. The lack of an association between GSTM1 and endometriosis contrasts with studies conducted in French and Slavic populations. The linkage studies using three highly polymorphic markers from the 1p13 region also failed to provide evidence for the involvement of this region in endometriosis. However, based upon small sample sizes, an association was observed between endometriosis and the combination of the GSTM1 null genotype and the CYP1A1 MspI polymorphism. Another study is required to investigate this observation further.

The preliminary findings of the genome-wide search being conducted in affected sister-pairs with endometriosis are presented in the following chapter; chromosome one was the first to be analysed and the results are presented in this thesis.

Chapter Six

Genome-wide search for endometriosis susceptibility loci: Chromosome One

6.1 INTRODUCTION

Genetic analysis of complex traits

When the fundamental biochemical basis of a complex trait is unknown, as in endometriosis, then linkage analysis provides a useful starting point by defining the approximate chromosomal location of disease genes. Once the approximate location is determined, it can be refined by using additional markers and identifying those that show infrequent or no recombination with the disease locus. The next step is to use techniques such as chromosome jumping, chromosome walking and assembly of yeast artificial chromosome (YAC) and cosmid contigs etc. to produce a library of sequential DNA clones from the region identified. Finally, the disease gene may be isolated by saturation genetic and physical mapping.

A genome wide search for susceptibility loci for endometriosis was being conducted in parallel with the association and linkage studies presented in Chapter Five. In this chapter the results of screening chromosome one with 29 microsatellite markers, in over 100 affected relative-pairs, are presented. Chromosome one was the first chromosome investigated for two reasons, firstly because of the presence of the GSTM1 locus on the short arm of this chromosome (Zhong *et al.*, 1992). GSTM1 was one of the few candidate genes for which there is published evidence of a strong association with endometriosis (Baranova *et al.*, 1997, Baranov *et al.*, 1996). Secondly, chromosome one contains a number of candidate tumour suppressor genes which potentially could be involved in the aetiology of endometriosis (Pejovic *et al.*, 1996, Mai *et al.*, 1998, Bieche *et al.*, 1995, Mori *et al.*, 1998, Mertens *et al.*, 1997).

Linkage analysis

Linkage analysis relies on the fact that, because of recombination or crossing-over during meiosis, two loci on the same chromosome will cosegregate at a rate which is related to the distance between them. This rate is the probability of a recombination event occurring between two loci and is known as the recombination fraction, θ . The recombination fraction for two loci may range from $\theta = 0$, for closely linked loci, to $\theta = \frac{1}{2}$ for loci on different chromosomes or a long distance apart on the same chromosome. The recombination fraction is therefore a measure of the map distance between two loci, and a recombination fraction of $\frac{1}{100}$ corresponds to a map distance of approximately 1 centimorgan (cM). Over small distances the relationship between θ and the map distance, ω , is proportional; however, the relationship becomes non-linear over larger distances, partly because of multiple crossing-over events occurring between the two loci. Interference also affects the relationship because one cross-over event reduces the probability of another in the vicinity. A map function, such as the Kosambi function (Figure 6.1), must therefore be used to translate θ into a map distance. The Kosambi function is given as:

$$\omega = \frac{1}{4} \log \left[\frac{(1 + 2\theta)}{(1 - 2\theta)} \right]$$

Two loci are said to be in linkage if $\theta < \frac{1}{2}$. Linkage analysis tests whether the estimated recombination fraction is significantly less than expected using a likelihood ratio test. The formula for this ratio, expressed as a logarithm (base 10) is:

$$Z(\theta) = \log_{10} \frac{L(\theta)}{L(0.5)}$$

This ratio, $Z(\theta)$, is known as the lod (logarithm of the odds) score. Values of $Z(\theta) > 3.0$, corresponding to odds of 1:1000 in favour of linkage, are taken as significant evidence of linkage. A lod score of 3 corresponds to approximately 95% confidence that linkage exists, because the random probability that any two loci are linked is high at about 1 in 50. In a genome-wide screen, values of $Z(\theta)$ lower than 3 may still justify further investigation. A lod score of less than -2 provides evidence against linkage.

In practice, the initial step of linkage analysis presents serious practical difficulties for a complex multifactorial disease such as endometriosis. For parametric methods such

as this to be used effectively, the mode of inheritance, allele frequencies, penetrance and disease status must be known. Large numbers of families with multiple affected members must be collected for such an analysis. To avoid some of these problems, non-parametric, or model-free, methods such as affected sibling-pair analysis have been developed.

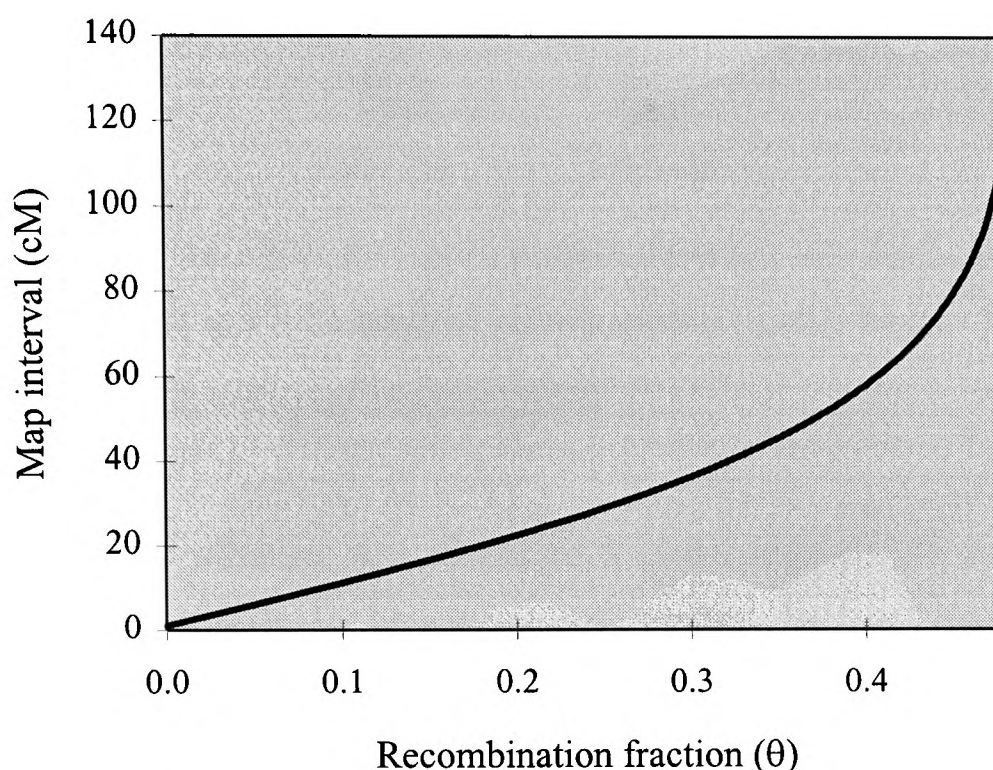


Figure 6.1. The Kosambi function illustrating the non-linear relationship between genetic map distance (ω) and recombination fraction (θ) over larger distances.

Affected sib-pair analysis involves determining whether affected siblings inherit identical copies of a chromosomal region from their parents more often than would be expected by chance. In this method, blood is collected and DNA isolated from pairs of affected siblings and their parents. At selected dinucleotide marker loci, the individuals are genotyped to determine whether the siblings share alleles identical-by-descent (IBD) (Figure 6.2).

According to Mendelian inheritance, the probability that the siblings will share both, one or neither marker alleles is $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{1}{4}$ respectively. However, if siblings are affected by the same disease they are likely to have inherited the same disease loci, and the chromosomal regions surrounding these loci will be more similar than expected, because recombination is less likely to occur between adjacent loci. Thus, for a given marker in the proximity of a disease locus, the frequency of alleles shared between affected sibs will be, on average, greater than the expected value. Across a

large number of affected sister pairs any statistically significant deviations from this expected value can be detected.

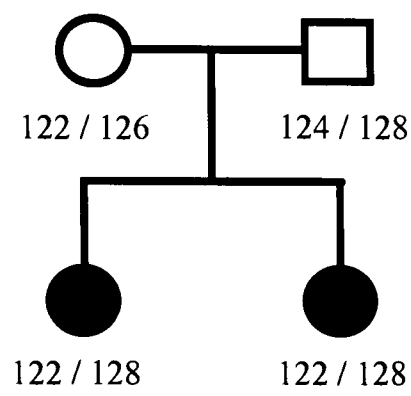


Figure 6.2. An affected sister pair and parents showing the fragment sizes in base pairs generated by PCR at a hypothetical microsatellite marker locus. The sisters have inherited both alleles identical by descent (IBD).

In order to determine whether the alleles of a given marker are shared IBD it is necessary to know the genotypes of both parents. If either parent is homozygous for a given marker, then the siblings would appear always to inherit that allele identically. It is, therefore, important for the dinucleotide markers to be highly heterozygous: the lower the heterozygosity, the higher the number of families which will be uninformative at that marker. If the parents are not genotyped, but the siblings have alleles which appear the same (but may not be, for the reasons above) they are termed identical by state (IBS). IBS methodology relies on an estimation of the allele frequency in the general population. If affected siblings are IBS for alleles that have a low frequency, then the probability that a parent is homozygous, or both parents carry the allele is small, and the chance that they are IBD is high. Additional information can therefore be gained in this way.

Power calculations

As presented in Chapter One, a precise λ_s is not known for endometriosis but can be estimated as approximately 10 for sisters of women with stage B endometriosis. The power to detect linkage using affected sib-pair analysis is directly related to the magnitude of λ_s . The power also depends on the number of susceptibility loci that exist for endometriosis (Figure 6.3). If one locus were responsible for all of the increased risk then the power to detect linkage would be very high, even for a small number of families. However, it is possible that endometriosis results from a number of different genes and hence the increased risk caused by each of these loci would be

diminished, as shown in Figure 6.3. For example, if there were three susceptibility loci, each exerting an equivalent effect, the risk caused by each would be approximately 2.2.

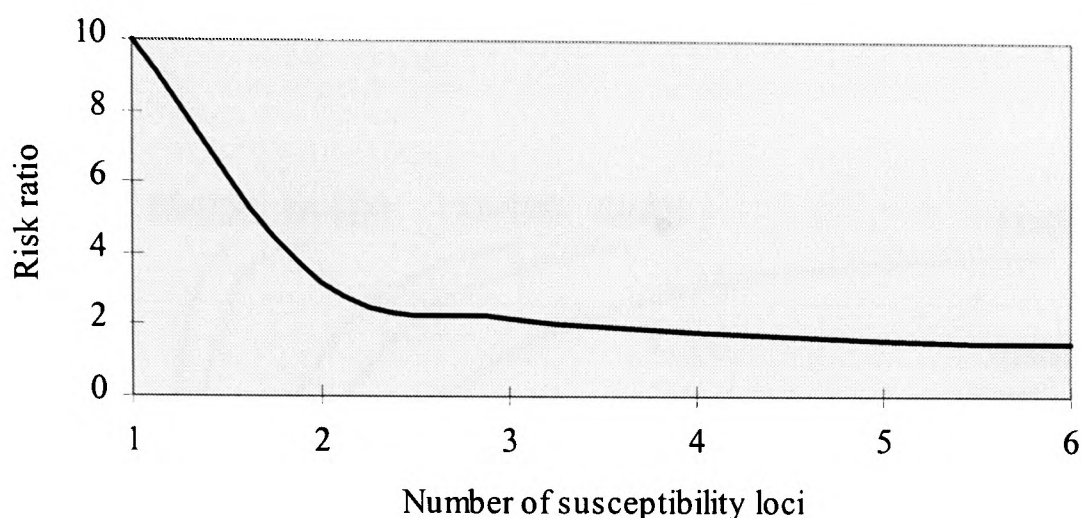


Figure 6.3. Graph illustrating the risk ratio at each locus for differing numbers of hypothetical susceptibility loci of equal effect given a λ_s of 10 .

The power to detect linkage as a function of λ_s , for varying numbers of affected sister pairs, with evidence for linkage taken as a LOD score of 3 or more, is shown in Figure 6.4 (Risch, 1990). The power to detect linkage, however, is dependent on a number of factors, other than λ_s and sample size, including the degree of recombination and the polymorphic information content of each marker. So, in reality, the graph in Figure 6.4 represents the minimum required sample size, because recombination is assumed to be zero and the marker is assumed to be 100% polymorphic.

Markers for linkage analysis

Maps of microsatellite markers covering the human genome with a 2-5cM resolution are readily available (e.g. Génethon, Dib *et al.*, 1996). Dinucleotide repeats, such as stretches of $(CA)_n$ repeats which make up 0.5% of the human genome and $(CT)_n/(AG)_n$ repeats, which account for 0.2% of the genome, are commonly used as genetic markers. Markers such as these occurring across the genome have been identified and the most polymorphic selected for use in chromosome linkage maps. The polymorphism information content (PIC) of a marker can be calculated, for a

marker with n alleles, as follows:
$$PIC = 1 - \sum_{i=1}^n p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^n 2 p_i^2 p_j^2$$

where p_i is the frequency of the i^{th} allele. The PIC of a marker can range from 0 (completely uninformative) to 1 (fully informative). For linkage analysis, markers with the highest possible PIC are selected to give the greatest power of detecting linkage.

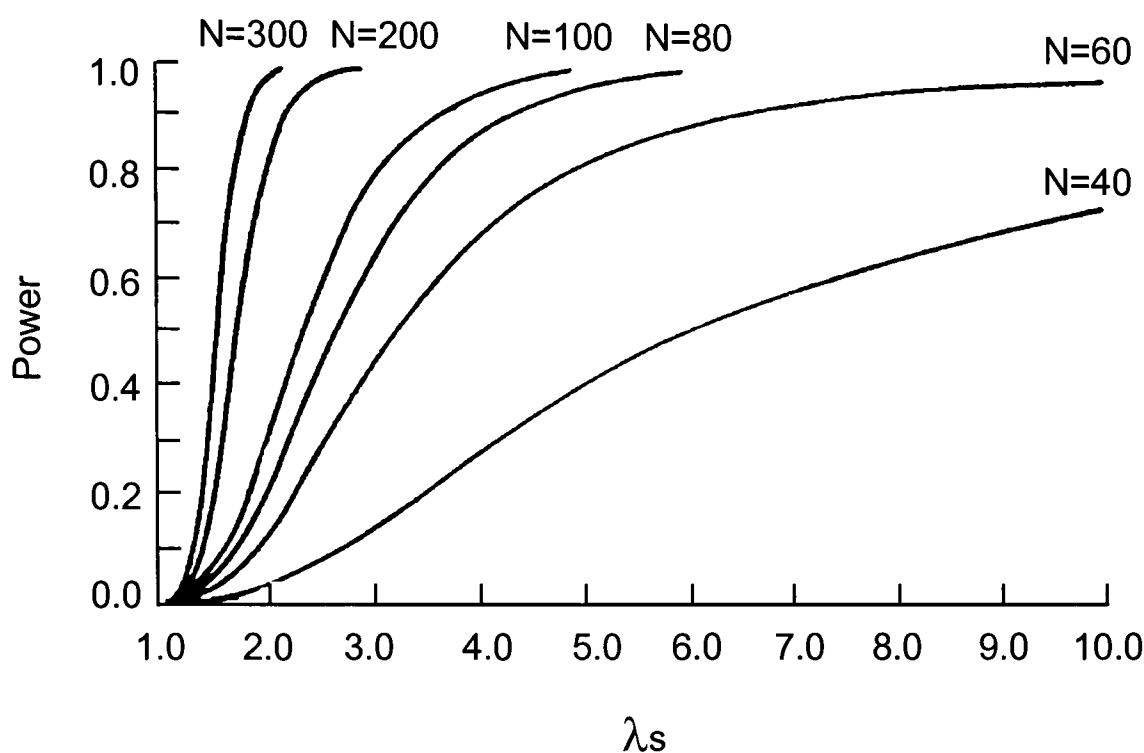


Figure 6.4. The power to detect linkage for different values of λ_s and varying numbers of sister-pairs (N). These power curves assume that the marker is fully informative and that there is no recombination ($\theta = 0$). Adapted from (Risch, 1990).

6.2 METHODS

Subjects

Patients and their families were identified as described previously in Chapter Four, Section 4.2. In total, 85 families containing 344 members and 128 affected sibling pairs were genotyped in this initial screen. Sister pairs both had stage B disease ($n = 77$), one had stage B and the other stage A ($n = 29$) or both had stage A disease ($n = 3$). Stage of disease could not be determined from the operative records in one or both affected siblings in 19 pairs. Other affected relative-pairs within the families included mother-daughter ($n = 17$), aunt-niece ($n = 10$), grandmother-granddaughter ($n = 1$) and first-cousin pairs ($n = 8$). The families included were from a number of different countries, as shown in Table 6.5. Families were collected either by the Oxford group

(n = 57) or by collaborating centres: Utah (n = 12), Norway (n = 3), Germany (n = 3), Russia (n = 3), Belgium (n = 2) or Australia (n = 4).

Country	Number of families
United Kingdom	36
United States of America	34
Norway	3
Germany	3
Russia	3
Belgium	2
Australia	4

Table 6.5. Origin of the first 85 families genotyped.

Laboratory methods

EDTA anti-coagulated blood samples (9ml) were collected from family members and stored frozen at -80 °C until use. The samples were thawed and genomic DNA was extracted either by the standard phenol-chloroform method (see Chapter Five) or by using Puregene™ kits (Flowgen) according to the manufacturer’s instructions. The DNA yield and level of protein contamination were determined by recording the UV absorption of the diluted sample at wavelengths of 260 nm and 280 nm. The following formula was used to calculate the nucleic acid concentration in the sample:

$$\text{Concentration } (\mu\text{g}/\mu\text{L}) = \text{Absorbance}_{260 \text{ nm}} \times \text{Dilution Factor} \times 0.05$$

The ratio of the absorbance at 260 nm over 280 nm was used to determine the amount of protein contamination, with ratios >1.75 showing acceptably low levels of protein. The DNA samples were diluted to a concentration of 50 ng/μL and arranged in a 8x12 format in 96-well microtitre plates.

Genotyping

Primers to microsatellite markers on chromosome one (Figure 6.6) were purchased from the ABI Prism linkage mapping set (Perkin Elmer (PE) Applied Biosystems). The primers were provided in two separate panels and the forward primer in each pair was labelled with one of the fluorescent markers FAM, HEX or NED. The forward

Panel 1

Locus [Dye Label]	Heterozygosity	Allele size range
D1S2797 [FAM]	0.74	102-138
D1S249 [FAM]	0.87	163-193
D1S2800 [FAM]	0.77	210-224
D1S234 [FAM]	0.81	268-288
D1S450 [FAM]	0.81	316-340
D1S255 [HEX]	0.75	91-111
D1S2667 [HEX]	0.82	128-156
D1S2785 [HEX]	0.76	173-187
D1S484 [HEX]	0.64	276-290
D1S196 [HEX]	0.74	322-338
D1S213 [NED]	0.86	107-133
D1S2878 [NED]	0.84	152-178
D1S206 [NED]	0.82	208-226
D1S2836 [NED]	0.79	246-260
D1S2726 [NED]	0.75	284-296
D1S2842 [NED]	0.76	338-358

Panel 2

Locus [Dye Label]	Heterozygosity	Allele size range
D1S199 [FAM]	0.83	99-125
D1S207 [FAM]	0.84	149-179
D1S2868 [FAM]	0.76	210-224
D1S413 [FAM]	0.76	254-270
D1S238 [FAM]	0.86	296-330
D1S252 [HEX]	0.81	90-115
D1S230 [HEX]	0.78	154-166
D1S468 [HEX]	0.76	193-213
D1S2841 [HEX]	0.78	234-254
D1S2697 [HEX]	0.70	291-305
D1S498 [NED]	0.82	190-212
D1S218 [NED]	0.83	271-295
D1S425 [NED]	0.81	336-360

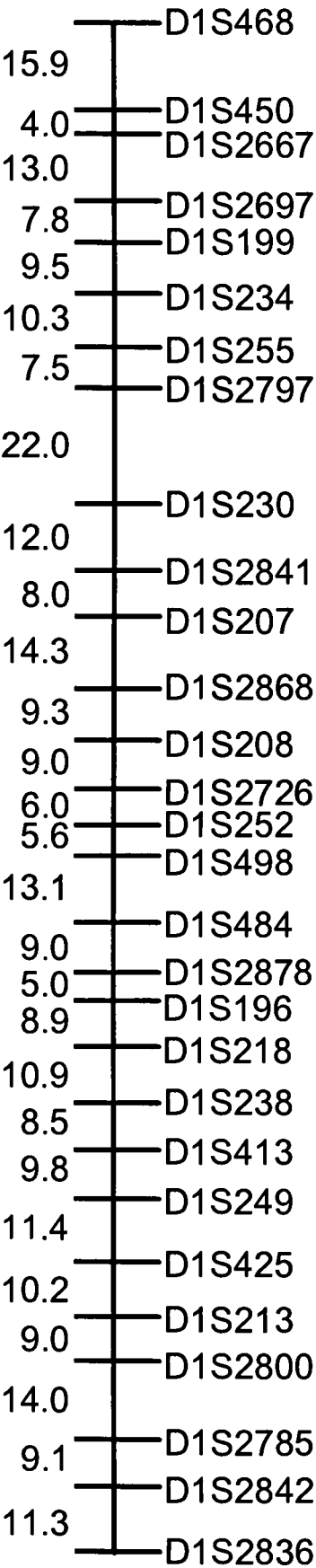


Figure 6.6. Microsatellite marker map showing the 29 markers used for chromosome one genotyping, with map distances shown in centimorgans (cM).

and reverse primers were combined in a 1:1 ratio to yield a 5 μ M solution. A separate PCR reaction mixture was prepared for each primer pair, with components as shown in Table 6.7, to give a final PCR volume of 15 μ L. All materials were purchased from PE Applied Biosciences.

PCR component	Volume	Final concentration
Primer Mix (5 μ M)	1.00	0.33 μ M
DNA (50 ng/ μ L)	1.20	60 ng
10x PCR Buffer	1.50	1x
dNTP mix (2.5 mM)	1.50	250 μ M each
AmpliTaq Gold™ (5 units/ μ L)	0.12	0.04 units/ μ L
MgCl ₂ (25 mM)	1.50	2.5 mM
H ₂ O	8.18	--
Total	15.00	--

Table 6.7. Components of PCR mixture and their concentrations.

The reaction mixture was prepared in 96-well microtitre plates (Thermowell™, Costar) corresponding to the layout of familial DNA samples. The mixtures were overlayed with mineral oil (Sigma) and PCR amplification was performed (MJ research thermocycler) as shown in Table 6.8.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	12 mins	
Denaturation	94 °C	15 s	10
Annealing	55 °C	15 s	
Extension	72 °C	30 s	
Denaturation	89 °C	15 s	20
Annealing	55 °C	15 s	
Extension	72 °C	30 s	
Final elongation	72 °C	10 mins	

Table 6.8. PCR profile used for genotyping.

A control DNA sample from CEPH individual 1347-02 was included on each plate to monitor PCR amplification efficiency and to control for any gel-to-gel variation. Following PCR amplification the reactions were pooled together by taking 1 μ L from each mixture. To avoid errors caused by pipetting small volumes, a pool was first made of the products in each of the three fluorescent label groups, and these were then

pooled to create a pool-of-pools. For each individual's samples a volume of 1.5 μL of the pooled products was mixed with 3.5 μL gel loading cocktail (containing 2.5 μL deionised formamide, 0.5 μL blue dextran 50 mg/mL, 50 mM EDTA and 0.5 μL GeneScan-500 [ROX] size standards). The samples were denatured by heating at 95 °C for 5 mins and then placed onto ice for immediate gel loading. Samples (4 μL) were loaded onto an ABI 377 automated sequencer for electrophoresis (PE Applied Biosystems) containing a 6%, 0.2 mm thick, 36 cm long polyacrylamide gel, with a 48-lane sharks-tooth comb fitted, which had been pre-run with TBE buffer for 30 mins. The samples were run for 2.5 hours using a standard gel matrix for the fluorescent dyes used. Data were saved into Genescan™ software (PE Applied Biosystems). After electrophoresis the product sizes generated by each primer pair were determined using Genescan™ and Genotyper™ software (PE Applied Biosystems). The allele size was determined by comparison to standards using the local southern method, which determines fragment size by using the reciprocal of the relationship between fragment length and mobility. A macro was recorded to semi-automate the analysis in Genotyper™. The macro automated the following analytical steps. Firstly, all category peaks were labelled with the size in base-pairs. The labels were then removed from peaks in the size range 0.00 to 75.00 bp; then from peaks whose height was less than 32% of the highest peak in a category's range; then from peaks preceded by a higher, labeled peak within 0.00 to 1.60 bp, and finally labels were removed from peaks followed by a higher, labeled peak within 0.00 to 3.00 bp. The output table was cleared and a new table was set up with one category and one lane per row, containing lane number, category, one label per peak for two peaks per category. If the category had no labeled peaks then an 'x' was placed in the table and the text 'overflow' was placed in a third column of the table if there were more than two labels per category. After running the macro, the set of peaks generated by each primer pair were manually checked for errors, and to ensure amplification was sufficient. Finally, the results were exported to Microsoft Excel and merged with patient codes, relationships and disease status for subsequent output to a variety of UNIX based linkage analysis programs.

Data analysis

The data were transformed by binning or down-coding alleles into groups according to size in base pairs. The program DOWNFREQ (Terwilliger & Ott, 1994, Ott, 1992) automates this process and estimates allele frequencies by looking at the alleles in founders only. The genotyping data were checked by comparing the calculated heterozygosity of each marker to published values from Généthron (Dib *et al.*, 1996). Mendelian inheritance was checked using the program PedCheck (O'Connell & Weeks, 1997), and families with consistent mis-inheritance were removed from the analysis. Nonparametric, statistical analysis of the genotype data was conducted using SPLINK (sib-pair linkage) version 1.05 (Holmans, 1993) and SimIBD (simulation-based identity-by-descent) version 1.2 (Davis *et al.*, 1996, Ott, 1989, Weeks *et al.*, 1990, Cottingham *et al.*, 1993) for single-point analysis and GENEHUNTER version 1.3 (Kruglyak *et al.*, 1996) for multipoint analysis.

Single point analyses

Single point statistics were generated for each of the 29 microsatellite markers studied using two UNIX based programs: SPLINK and SimIBD. SPLINK was developed to increase the power of analysis when either a marker is not fully informative or the parents are unavailable. One way of overcoming the loss of power in such situations is to use identity-by-state methods, such as the affected-pedigree-member method (Weeks & Lange, 1998) and another, as in the program SPLINK, is to use the method of maximum likelihood to estimate the probability that affected siblings share 0, 1 or 2 marker haplotypes. The null hypothesis for this test is $z_0 = 0.25$, $z_1 = 0.5$ and $z_2 = 0.25$ and the likelihood ratio chi-squared test is carried out against the “possible triangle” alternatives of $z_1 < 0.5$, $z_0 < 0.5 * z_1$.

The SimAPM statistic (generated by SimIBD) uses the simulation-based affected-pedigree-member method (Weeks, 1988) and is based on IBS allele sharing. The SimIBD statistic is based on IBD sharing. Both the SimIBD and SimAPM statistics are determined by first calculating an observed statistic, based upon the pedigree marker data. Many replicates are then simulated using new marker data, derived from the genotypes of unaffected individuals and these simulations are conducted independently of disease status. A statistic is calculated based on the simulated data for each replicate, and a null distribution is produced for each marker, independent of

disease status. A P value can then be calculated for each marker based on where the observed statistic falls within the simulated null distribution (Davis *et al.*, 1996).

SimIBD was used with the recommended family weighting function $\frac{1}{\sqrt{p}}$ (where p is the population frequency of a given allele) and 100 simulations were conducted to ensure accuracy of the P value to two decimal places.

Multipoint analysis

Multipoint analysis was conducted using the program GENEHUNTER (Kruglyak *et al.*, 1996). The non-parametric linkage (NPL) function used was the 'all' function which examines all individuals simultaneously, assigning a higher score when more affected individuals share the same allele IBD. The 'all' statistic has been shown to be more powerful than the 'pairs' statistic, which only looks at the degree of sharing between pairs of affected individuals. The multipoint NPL_{all} scores were determined for the set of 29 markers studied, and can be thought of like a LOD score, with values over three indicating evidence for linkage at that position. The corresponding P values and marker information content values were also determined using this package.

Comparison of linkage methods

The methods used in this analysis have been compared by Davis & Weeks (1997). They compared the power to detect linkage in families with two affected siblings and with either both, one or neither parents genotyped under eight different disease models. Each analysis involved 20 replicates of 100 families and the power to detect linkage was based on the proportion of replicates in which $P < 0.05$. These data are illustrated in Figure 6.9 A. False-positive rates were also determined using 1000 replicates of 100 families and a marker unlinked to disease. The false-positive rate was calculated based on the proportion of replicates which showed evidence of linkage. These data are illustrated in Figure 6.9 B.

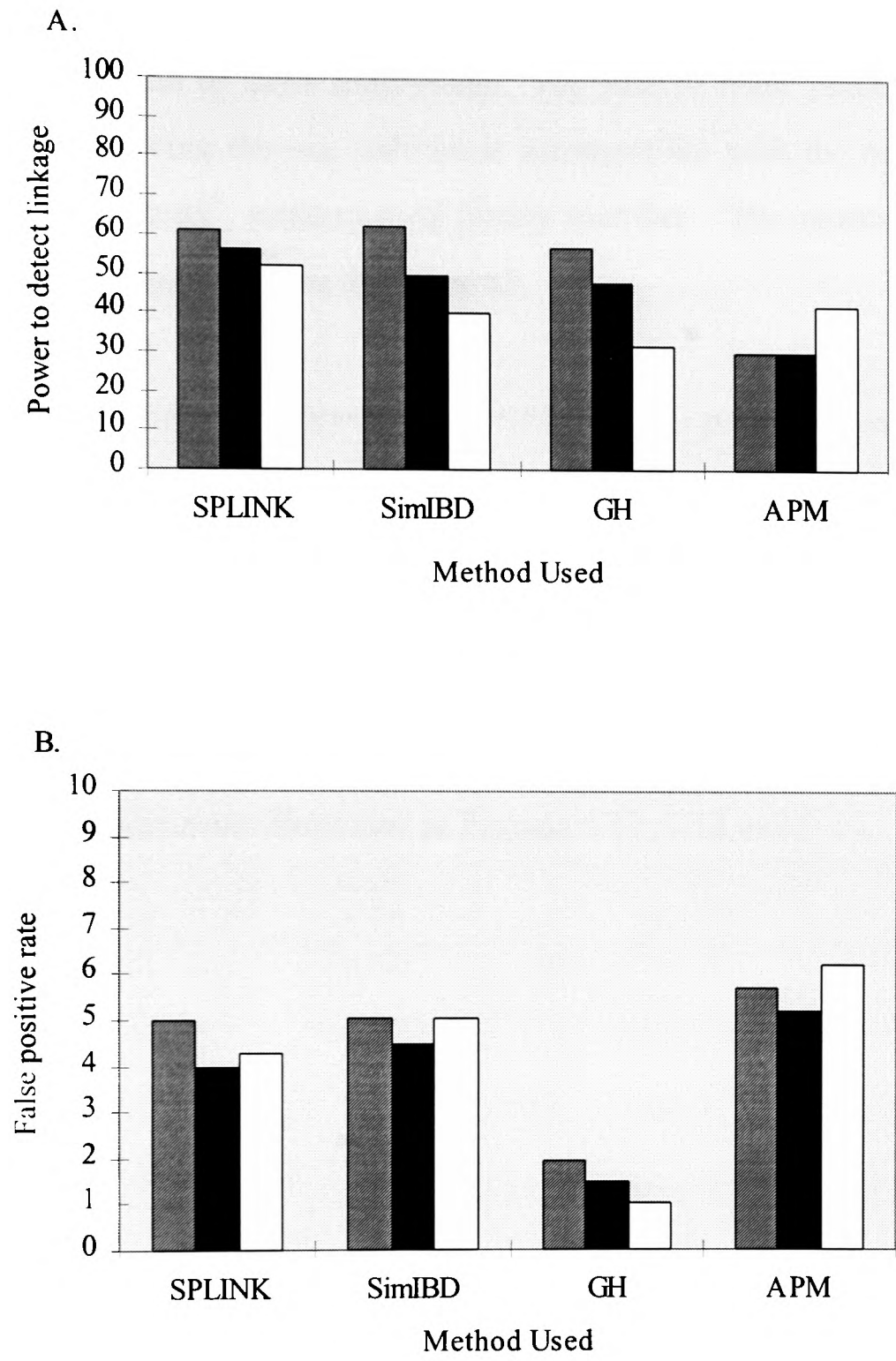


Figure 6.9. Graphs comparing the four different methods of single-point linkage analysis used (GH = GENEHUNTER single-point, APM = affected pedigree member method). Graph A shows the power to detect linkage in families with two affected sisters and both parents genotyped (▤), one parent genotyped (■) or neither parent genotyped (□). Similarly, Graph B shows the false positive rate in families with two affected sisters and both parents genotyped (▤), one parent genotyped (■) or neither parent genotyped (□). Adapted from Davis & Weeks, 1997.

6.3 RESULTS

Using PedCheck it was found that 6/85 (7%) families demonstrated consistent mis-inheritance in one or more individuals. For four of these families, the problem was solved by removing the one individual incompatible with the rest of the family, and inserting a ‘dummy’, ungenotyped family member. The number of families finally included in the analysis was therefore 83.

The results of linkage analysis using SPLINK are presented in Table 6.10. The P values generated ranged from 0.16 to 0.59, and no statistically significant evidence of linkage was found for any of the 29 markers tested. The results of linkage analysis using GENEHUNTER multipoint, GENEHUNTER singlepoint, SimIBD and SimAPM are presented in Table 6.11. None of the 29 markers studied gave rise to values of $P < 0.05$ in more than one test. The results of the GENEHUNTER multipoint analysis are illustrated in Figures 6.12, 6.13 and 6.14.

Table 6.10. Data analysis of 29 microsatellite markers mapping to chromosome one using SPLINK. Het. = heterozygosity.

Marker	Het.	IBD sharing			P value	LOD
		0	1	2		
D1S468	0.74	0.25	0.50	0.25	0.58	0
D1S450	0.80	0.24	0.48	0.28	0.43	0.04
D1S2667	0.83	0.22	0.49	0.29	0.31	0.11
D1S2697	0.69	0.22	0.50	0.28	0.35	0.08
D1S199	0.84	0.25	0.50	0.25	0.59	0
D1S234	0.82	0.25	0.50	0.25	0.59	0
D1S255	0.70	0.25	0.50	0.25	0.59	0
D1S2797	0.78	0.25	0.50	0.25	0.58	0
D1S230	0.76	0.23	0.50	0.28	0.39	0.05
D1S2841	0.79	0.21	0.50	0.29	0.24	0.18
D1S207	0.88	0.25	0.50	0.25	0.59	0
D1S2868	0.72	0.19	0.50	0.31	0.16	0.31
D1S206	0.74	0.25	0.50	0.25	0.58	0
D1S2726	0.73	0.21	0.50	0.29	0.25	0.17
D1S252	0.80	0.24	0.48	0.28	0.43	0.03
D1S498	0.79	0.24	0.49	0.27	0.47	0.02
D1S484	0.72	0.23	0.50	0.27	0.43	0.03
D1S2878	0.83	0.25	0.50	0.25	0.59	0
D1S196	0.76	0.25	0.50	0.25	0.58	0
D1S218	0.79	0.23	0.48	0.29	0.29	0.12
D1S238	0.86	0.25	0.50	0.25	0.59	0
D1S413	0.71	0.25	0.50	0.25	0.58	0
D1S249	0.86	0.20	0.50	0.30	0.24	0.18
D1S425	0.79	0.23	0.50	0.27	0.39	0.05
D1S213	0.85	0.25	0.50	0.25	0.58	0
D1S2800	0.76	0.24	0.50	0.26	0.47	0.02
D1S2785	0.83	0.25	0.50	0.25	0.59	0
D1S2842	0.80	0.21	0.50	0.29	0.28	0.14
D1S2836	0.73	0.25	0.50	0.25	0.58	0

Table 6.11. Data analysis of 29 microsatellite markers mapping to chromosome one using GENEHUNTER (multipoint and single point analyses), SimIBD and APM. Values of $P < 0.05$ are underlined.

Marker	Genehunter Multipoint <i>P</i> value	Genehunter Single Point <i>P</i> value	SimIBD <i>P</i> value	APM <i>P</i> value
D1S468	0.87	0.44	0.73	0.87
D1S450	0.66	0.31	0.50	0.62
D1S2667	0.53	0.32	0.20	0.20
D1S2697	0.43	0.62	0.29	0.22
D1S199	0.45	0.75	0.56	0.21
D1S234	0.72	0.36	0.79	0.85
D1S255	0.82	0.22	0.78	0.51
D1S2797	0.52	0.84	0.65	0.83
D1S230	0.09	0.54	0.16	<u>0.03</u>
D1S2841	0.14	0.56	0.54	<u>0.03</u>
D1S207	0.35	0.48	0.79	0.66
D1S2868	0.23	0.88	0.25	0.27
D1S206	0.18	0.42	0.56	0.30
D1S2726	0.20	0.33	0.07	0.04
D1S252	0.20	0.31	0.85	0.85
D1S498	0.27	0.75	0.48	0.72
D1S484	0.36	0.70	0.48	0.65
D1S2878	0.49	0.43	0.58	0.57
D1S196	0.50	0.84	0.20	0.08
D1S218	0.39	0.22	0.79	0.59
D1S238	0.47	0.40	0.38	0.34
D1S413	0.51	0.21	0.57	0.52
D1S249	0.60	0.32	0.23	0.40
D1S425	0.49	0.53	0.56	0.60
D1S213	0.76	0.35	0.79	0.59
D1S2800	0.44	0.86	0.61	0.31
D1S2785	0.53	0.81	0.70	0.57
D1S2842	0.46	0.49	0.46	0.38
D1S2836	0.67	0.36	0.99	0.98

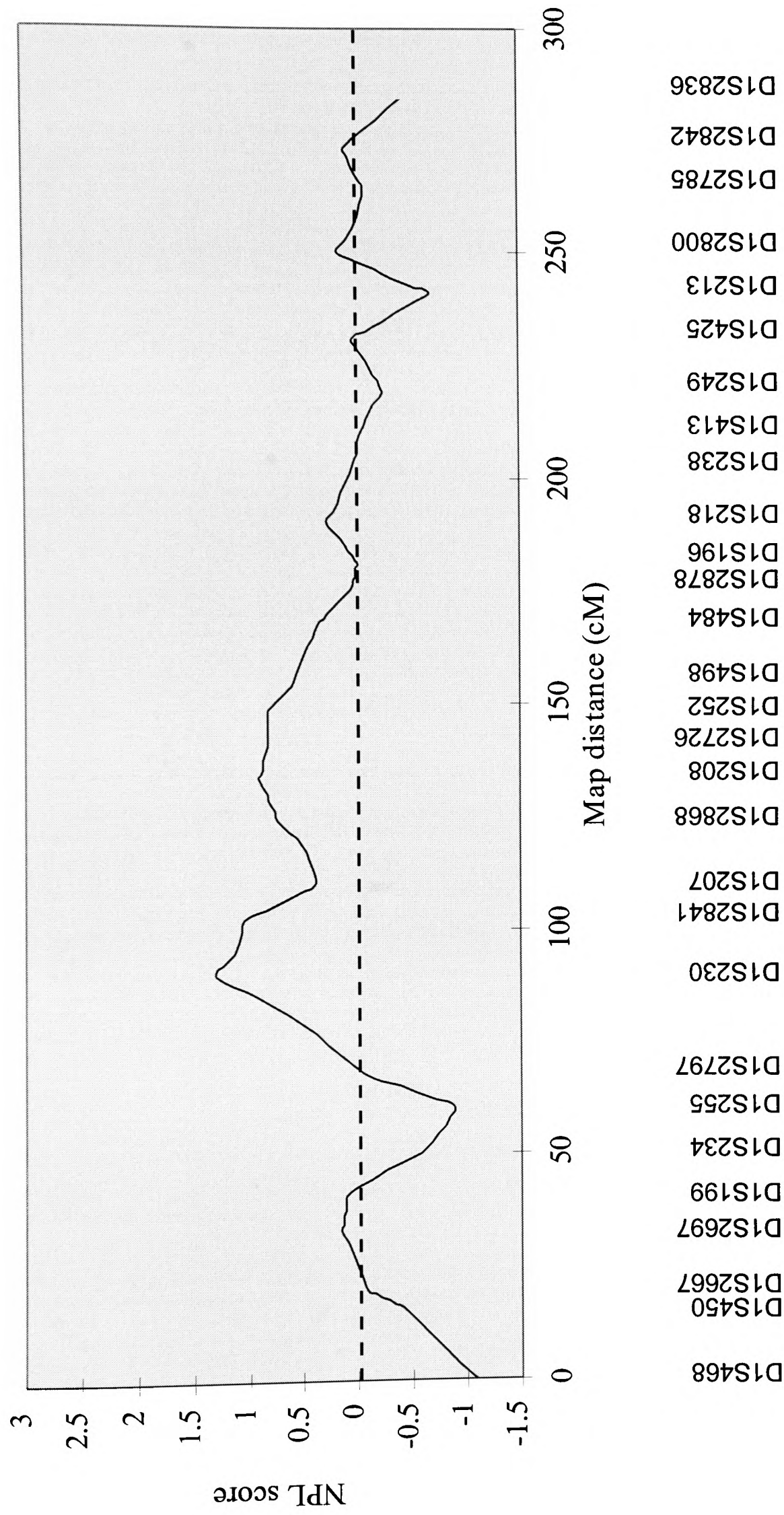


Figure 6.12. Graphical representation of the output from GENEHUNTER, showing the GENEHUNTER NPL score relative to map distance for markers selected from chromosome one.

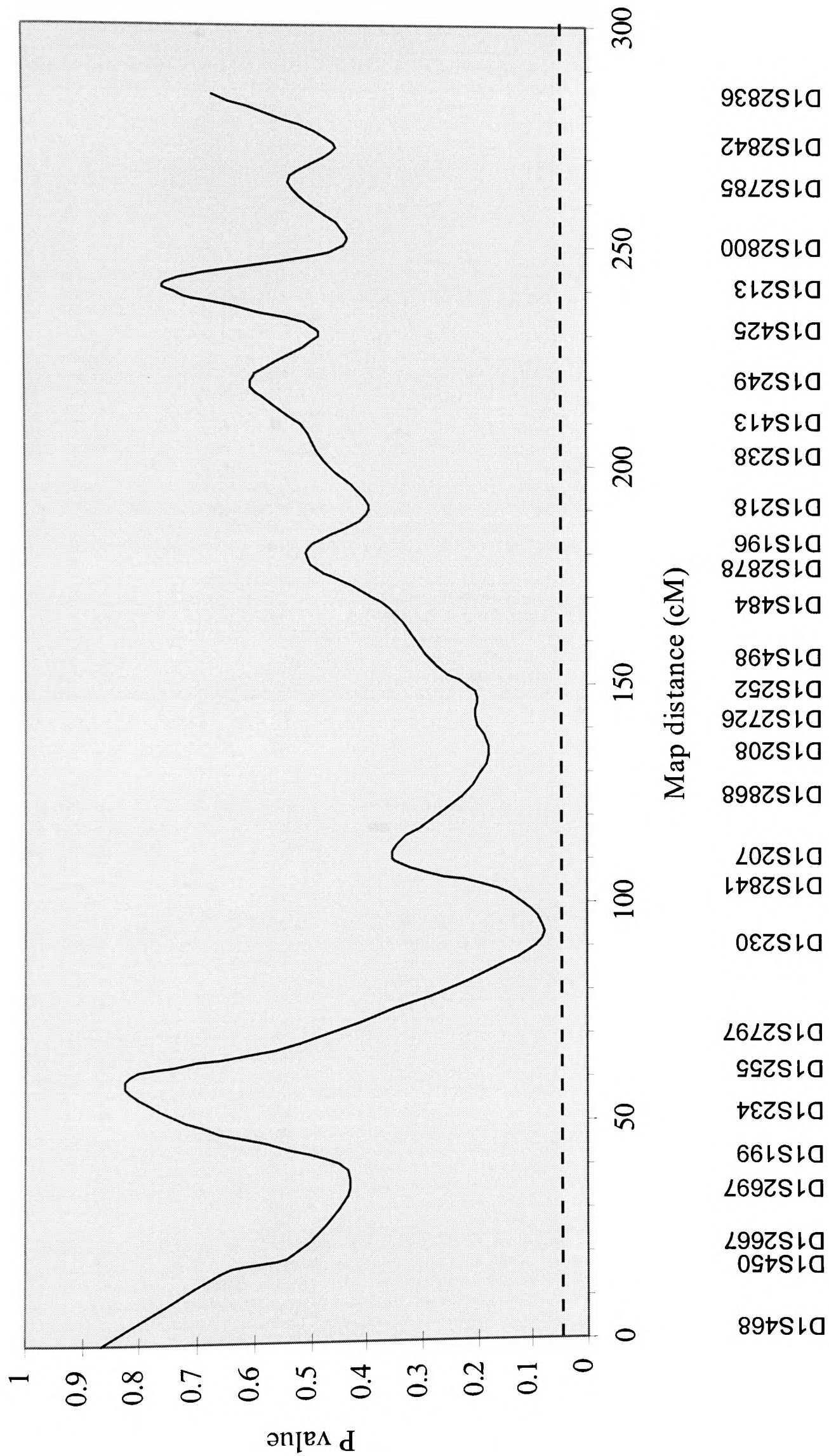


Figure 6.13. The GENEHUNTER multipoint *P* values for 29 microsatellite markers selected from chromosome one.

6.4 DISCUSSION

In this study, sib-pair analysis was conducted with 29 microsatellite markers chosen from across chromosome one. A number of different methods were used to analyse the data generated in this study. The methods vary in their power to detect linkage and in their false positive rate. For example, SPLINK has relatively high power to detect linkage, but also a high false positive rate (Figure 6.9). In comparison, single-point GENEHUNTER analysis has less power to detect linkage, but a far lower false positive rate (Davis *et al.*, 1997).

There was no consistent evidence of linkage between any of the markers studied and endometriosis. Markers D1S230 and D1S2841 both gave rise to P values of 0.03 when the data were analysed using APM. However, this method has been shown to have a high false-positive rate and is no longer a favoured method for affected sibling-pair analysis. The P values for these markers were not significant using any of the other methods, although multipoint GENEHUNTER analysis gave a value approaching significance ($P = 0.09$) for D1S230 and this was the only area of chromosome one yielding an NPL score >1 (Figure 6.13). Nevertheless, D1S230 has no markers nearby, with a 22cM space on one side and a 12cM space on the other (Figure 6.6) and this may give rise to false positive results in GENEHUNTER. Ideally more markers should be included in the chromosome one set to cover this region more fully. Interestingly, marker D1S230 is in region 1p32.1-33 of chromosome one, an area which was reported to be frequently deleted in malignant mixed mesodermal tumours of the uterus (Gil-Benso *et al.*, 1997).

As only a small number of families and sister-pairs were included in this study, which may have resulted in a lack of power to detect linkage, it is not possible to rule out completely the involvement of loci on chromosome one in the development of endometriosis. However, given the results of this study it seems unlikely that there are any loci on chromosome one which play a very significant role in the aetiology of endometriosis. This study has also provided further evidence against the association between endometriosis and GSTM1, as the marker D1S484 is from the 1p13 region.

Chapter Seven

Summary

Endometriosis causes pain and infertility in millions of women world-wide, yet surprisingly little is known about the aetiology or epidemiology of this disease. There is now mounting evidence that endometriosis is a complex trait, with genetic and environmental factors interacting to produce the phenotype. For a disease such as endometriosis, genetic dissection using genome-wide linkage analysis is a particularly attractive option as there is the potential for a major breakthrough in the understanding of this condition. In this thesis, data on both the genetic and environmental basis of endometriosis have been presented using studies in an animal model, case-control studies, segregation analysis, association studies and sib-pair analysis.

Risk factors for the development of spontaneous endometriosis in the rhesus monkeys at the WRPRC were studied in Chapter Two. Although it was known that a high prevalence of endometriosis occurred in captive colonies of *Macaca mulatta*, neither the exact prevalence nor the reason for the preponderance of the disease in captivity was known.

The frequency of endometriosis was 20% in animals that died aged four years or older in the 15 year period studied. Animals with endometriosis were significantly older than animals without the disease. No rhesus monkey less than 10 years old had endometriosis at autopsy, so a more accurate representation of the prevalence is 29% in animals that died over this age. It was concluded that age was a major risk factor for endometriosis in this colony, a finding supported by existing data indicating age as an important risk factor in women.

This study was the first analysis of risk factors for endometriosis using a large group of affected rhesus macaques and matched controls without the disease. Because the procedures and treatments were more often given in combination than independently,

logistic regression analysis was used to estimate the independent effect of each procedure on the development of endometriosis. Hysterotomy was associated with a significantly increased risk of endometriosis with an adjusted odds ratio of 5.8 ($P = 0.006$). The relationship between ovarian steroids and the development of an endometriotic lesion is well established in both human and non-human primates. In this study, having more than three oestradiol implants was the strongest risk factor for endometriosis in the rhesus macaque, with an adjusted odds ratio of 9.7 ($P < 0.001$). The number of offspring was not a significant factor in the development of disease. Consistent with studies in women, a significant difference in the length of time between last pregnancy and the finding of endometriosis was also observed, with a median of 7 years for cases and 3 years for controls ($P = 0.003$).

These findings confirm observations already made in women, suggesting that the rhesus macaque provides an excellent model for the disease, and give some insight into the aetiology of the disease.

Until now, no studies had been conducted to investigate the familial tendency of endometriosis in the non-human primate and this work was presented in Chapter Three. The detailed records held at the WRPRC provided the ideal opportunity for such an investigation to be conducted because 41% of affected animals were related to at least one other animal with the disease. MRI was conducted on a group of the living descendants of these animals to increase the sample size and subsequently segregation analysis was conducted. In the 113 animals scanned, the prevalence of endometriosis ranged from 7% (implants >1 cm) to 19% (all endometriosis).

The results of the segregation analysis were inconclusive. Although the raw data and family tree diagrams suggested that familial clustering does occur, the disease status was defined for only a small proportion of animals, resulting in small sample sizes. The predominance of half-sibling pairs confounded the problems and it was therefore difficult to draw conclusions from these data. Over time, as more animals are autopsied or diagnosed by other means, there may be sufficient data to confirm or disprove a genetic basis to a portion of the endometriosis in the colony.

The feasibility studies on developing a marker set in this species, using oligonucleotide primers for human microsatellite markers, on the rhesus macaque genome identified 92 new potential microsatellite markers in this species.

The identification of a large number of families with endometriosis for the OXEGENE study enabled the characteristics of a cohort of women with surgically confirmed disease to be examined and these data were presented in Chapter Four. A familial tendency was confirmed and a number of extended pedigrees were identified. The mean age at diagnosis was shown to be significantly higher for women with Stage B disease, but the mean age at symptom onset was not different for Stage B compared to Stage A disease. Significantly more women with Stage A disease were diagnosed in the 20-24 age group compared to Stage B, and for both disease stages a significant majority of women reported that their symptoms began between the ages of 25-29.

MRI studies of the first-degree relatives of women with Stage B disease estimated that the relative risk in first degree relatives (λ_R) may be as high as 14 (95% CI 4.8 - 30.3) and the relative risk in the sisters of affected women (λ_S) may be as high as 15 (95% CI 5.1 - 31.9). This work illustrated the use of MRI as a noninvasive method for screening women who may be at increased risk of developing endometriosis.

An analysis of the delay in diagnosis experienced by women revealed that the mean time between symptom onset and diagnosis was 9.4 ± 8.6 years (range 0 - 44) years. The mean delay in diagnosis was 8.0 ± 7.9 (range 0 - 33) years for women from the UK and 11.7 ± 9.1 (range 0 - 44) years for women from the USA, a difference that was statistically significant ($P = 0.0014$). The prevalence of endometriosis in the twin-pairs collected for this study showed that there was 87% concordance amongst the 16 MZ pairs and 56% of MZ twin pairs were concordant for stage B endometriosis.

Candidate gene association studies were presented in Chapter Five. No association was observed between the GALT N314D polymorphism and endometriosis, nor was there any evidence that mothers of women with disease were more likely to carry the polymorphism than the general population. The finding that 11/35 (31%) women with endometriosis and infertility were heterozygous for the N314D polymorphism in

this study, in combination with observations in previous studies, suggests that the relationship between GALT N314D, infertility and endometriosis warrants further investigation. No significant differences were observed between the frequency of the CYP1A1 MspI polymorphism in endometriosis patients compared to controls. Likewise, no significant difference in the frequency of GSTM1 and GSTT1 null homozygotes between cases and controls was observed. This is in contrast to two previous studies in the literature which have shown significant association between the GSTM1 null genotype and endometriosis. Linkage studies using three highly polymorphic microsatellite markers from the region to which GSTM1 has been mapped (1p13) showed no evidence of linkage to this region in 50 affected sib-pairs. The combination of the GSTM1 null genotype and the CYP1A1 MspI polymorphism appeared to increase the risk of endometriosis and needs further investigation.

Finally, in Chapter Six, sib-pair analysis was conducted with 29 microsatellite markers spanning chromosome one. There was no evidence of linkage between any of the markers studied and endometriosis. As only a relatively small number of families and sister-pairs were included in this study, which may have resulted in a lack of power to detect linkage, it is not possible to completely rule out the involvement of gene loci on chromosome one in the development of endometriosis.

Chapter Eight

References

- Ami, Y., Suzaki, Y., & Goto, N. (1993) Endometriosis in cynomolgus monkeys retired from breeding. *J.Vet.Med.Sci.* **55**, 7-11.
- Arand, M., Mühlbauer, R., Hengstler, J., Jäger, E., Fuchs, J., Winkler, L., & Oesch, F. (1996) A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphisms. *Analyt. Biochem.* **236**, 184-186.
- Arnold, L. L., Ascher, S. M., & Simon, J. A. (1994) Familial adenomyosis: a case report. *Fertil. Steril.* **61**, 1165-1167.
- Arnold, L. L., Meck, J. M., & Simon, J. A. (1995) Adenomyosis: Evidence for a genetic cause. *Am. J. Med. Genet.* **55**, 505-506.
- Arumugam, K., & Templeton, A. A. (1992) Endometriosis and race. *Aust. N.Z. J. Obstet. Gynaecol.* **32**, 164-165.
- Baranov, V. S., Ivaschenko, T., Bakay, B., *et. al.* (1996) Proportion of the GSTM1 0/0 genotype in some Slavic populations and its correlation with cystic fibrosis and some multifactorial diseases. *Hum. Genet.* **97**, 516-520.
- Baranova, H., Bothorishvilli, R., Canis, M., Albuisson, E., Perriot, S., Glowaczower, E., Bruhat, M., Baranov, V., & Malet, P. (1997a) Glutathione S-transferase M1 gene polymorphism and susceptibility to endometriosis in a French population. *Mol. Hum. Reprod.* **3**, 775-780.
- Baranova, H., Perriot, J., Albuisson, E., Ivaschenko, T., Baranov, V. S., Hemery, B., Mouraire, P., Riol, N., & Malet, P. (1997b) Peculiarities of the GSTM1 0/0 genotype in French heavy smokers with various types of chronic bronchitis. *Hum. Genet.* **99**, 822-826.
- Baranova, H., Canis, M., Ivaschenko, T., Albuisson, E., Bothorishvilli, R., Baranov, V., Malet, P., & Bruhat, M.A. (1999) Possible involvement of arylamine N-acetyltransferase 2, glutathione S-transferases M1 and T1 genes in the development of endometriosis. *Mol. Hum. Reprod.* **5**, 636-641.
- Barbieri, R. L., Callery, M., & Perez, S. E. (1992) Directionality of menstrual flow: cervical os diameter as a determinant of retrograde menstruation. *Fertil. Steril.* **57**, 727-730.

- Bertens, A. P. M. G., Helmond, F. A., & Hein, P. R. (1982) Endometriosis in rhesus monkeys. *Lab. Anim.* **16**, 281-284.
- Bieche, I., Champeme, M. H., & Lidereau, R. (1995) Loss and gain of distinct regions of chromosome 1q in primary breast cancer. *Clin. Cancer Res.* **1**, 123-127.
- Binhazim, A. A., Tarara, R. P., & Suleman, M. A. (1989) Spontaneous external endometriosis in a de Brazza's monkey. *J. Comp. Path.* **101**, 471-474.
- Bischoff, F. Z., Rossi, H. L., Nguyen, D. D., Marquez-Do, D. A., Bahler, A. A., Shin, J.-C., Simpson, J. L., & Elias, S. (1997) Loss of heterozygosity for chromosome 17 (p53) in endometriosis by molecular genetic and two-color FISH analysis. *J. Soc. Gynecol. Invest.* **4**, 205A.
- Board, P., Coggan, M., Johnston, P., Ross, V., Suzuki, T., & Webb, G. (1990) Genetic heterogeneity of the human glutathione transferases: a complex of gene families. *Pharmacol. Ther.* **48**, 357-369.
- Bocker, J., Tadmor, O. P., Gal, M., & Diamant, Y. Z. (1994) The prevalence of adenomyosis and endometriosis in an ultra-religious Jewish population. *Asia-Oceania J. Obstet. Gynaecol.* **20**, 125-129.
- Bois, F. Y., & Eskenazi, B. (1994) Possible risk of endometriosis for Seveso, Italy residents: An assessment of exposure to dioxin. *Environ. Health. Pers.* **102**, 476-477.
- Bonen, A., Belcastro, A. N., Ling, W. Y. & Simpson, A. A. (1981) Profiles of selected hormones during menstrual cycles of teenage athletes. *J. Appl. Physiol.* **50**, 545-551.
- Bricarelli, D. F., Magnani, M., Arslanian, A., Camera, G., Coviello, D. A., Di-Pietro, P., & Dallapiccola, B. (1981) Expression of GALT in two unrelated 9p- patients. Evidence for assignment of the GALT locus to the 9p21 band. *Hum. Genet.* **59**, 112-114.
- Brinton, L. A., Gridley, G., Persson, I., Baron, J., & Bergvist, A. (1997) Cancer risk after a hospital discharge diagnosis of endometriosis. *Am. J. Obstet. Gynecol.* **176**, 572-579.
- Candiani, G. B., Danesino, V., Gastaldi, A., Parazzini, F., & Ferraroni, M. (1991) Reproductive and menstrual factors and risk of peritoneal and ovarian endometriosis. *Fertil. Steril.* **56**, 230-234.
- Cary, C. J., Peter, G. K., & Schiffer, S. P. (1982) A case report and review of endometriosis in nonhuman primates. *Lab. Anim. Sci.* **32**, 426.
- Chan, K. W. A., Felson, D. T., Yood, R. A., & Walker, A. M. (1994) The lag time between onset of symptoms and diagnosis of rheumatoid arthritis. *Arth. Rheum.* **37**, 814-820.
- Chatterjee, S. K. (1980) Scar endometriosis: a clinicopathologic study of 17 cases. *Obstet. Gynecol.* **56**, 81-84.
- Chen, Y.-T., Mattison, D. R., & Feigenbaum, L. (1981) Reduction in oocyte number following prenatal exposure to a diet high in galactose. *Science* **214**, 1145-1147.

- Chin, S. C., 58-64. (1994) Endometriosis and pyometra in a Taiwan rhesus (*Macaca cyclopis*). *J. Chin. Soc. Vet. Sci.* **20**, 58-64.
- Cornillie, F. J., D'Hooghe, T. M., Bambra, C. S., Lauweryns, J. M., Isahakia, M., & Koninckx, P. R. (1992) Morphological characteristics of spontaneous endometriosis in the baboon (*Papio anubis* and *Papio cynocephalus*). *Gynecol. Obstet. Invest.* **34**, 225-228.
- Cottingham, J. R. W., Idury, R. M., & Schaffer, A. A. (1993) Faster sequential genetic linkage computations. *Am. J. Hum. Genet.* **53**, 252-263.
- Coxhead, D., & Thomas, E. J. (1993) Familial inheritance of endometriosis in a British population. A case control study. *J. Obstet. Gynaecol.* **13**, 42-44.
- Cramer, D. W., Goldstein, D. P., Fraer, C., & Reichardt, J. K. V. (1996a) Vaginal agenesis (Mayer-Rokitansky-Hauser syndrome) associated with the N314D mutation of galactose-1-phosphate uridyl transferase (GALT). *Mol. Hum. Reprod.* **2**, 145-148.
- Cramer, D. W., Harlow, B. L., Barbieri, R. L., & Ng, W. G. (1989) Galactose-1-phosphate uridyl transferase activity associated with age at menopause and reproductive history. *Fertil. Steril.* **51**, 609-615.
- Cramer, D. W., Hornstein, M. D., Ng, W. G., & Barbieri, R. L. (1996b) Endometriosis associated with the N314D mutation of galactose-1-phosphate uridyl transferase (GALT). *Mol. Hum. Reprod.* **2**, 149-152.
- Cramer, D. W., Ravnkar, V. A., Craighill, M., Ng, W. G., Goldstein, D. P., & Reilly, R. (1987) Müllerian aplasia associated with maternal deficiency of galactose-1-phosphate uridyl transferase. *Fertil. Steril.* **47**, 930-934.
- Cramer, D. W., Wilson, E., Stillman, R. J., Berger, M. J., Belisle, S., Schiff, I., Albrecht, B., Gibson, M., Stadel, B. V., & Schoenbaum, S. C. (1986) The relation of endometriosis to menstrual characteristics, smoking, and exercise. *J.A.M.A.* **255**, 1904-1908.
- Cummings, A., Metcalf, J. L., & Birnbaum, L. (1996) Promotion of endometriosis by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats and mice: time-dose dependence and species comparison. *Toxicol. Appl. Pharmacol.* **138**, 131-139.
- Davies, J.L., Kawaguchi, Y., Bennett, S.T., Copeman, J.B., Cordell, H.J., Pritchard, L.E., Reed, P.W., Gough, S.C., Jenkins, S.C., Palmer, S.M., *et al.* (1994) A genome-side search for human type 1 diabetes susceptibility genes. *Nature* **371**, 130-136.
- Davis, S., Schroeder, M., Goldin, L. R., & Weeks, D. E. (1996) Nonparametric simulation-based statistics for detecting linkage in general pedigrees. *Am. J. Hum. Genet.* **58**, 867-880.

- Davis, S., & Weeks, D. E. (1997) Comparison of nonparametric statistics for detection of linkage in nuclear families: Single-marker evaluation. *Am. J. Hum. Genet.* **61**, 1431-1444.
- DeVito, M. J., Thomas, T., Martin, E., Umbreit, T. H., & Gallo, M. A. (1992) Antioestrogenic action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Tissue-specific regulation of estrogen receptor in CD1 mice. *Toxicol. Appl. Pharmacol.* **113**, 284-292.
- D'Hooghe, T. M. (1994) *The baboon: a model for the study of human endometriosis?*, Catholic University of Leuven, Leuven, Belgium. *PhD Thesis*.
- D'Hooghe, T. M., Bambra, C. S., Cornillie, F. J., Isahakia, M., & Koninckx, P. R. (1991) Prevalence and laparoscopic appearance of spontaneous endometriosis in the baboon (*Papio anubis*, *Papio cynocephalus*). *Biol. Reprod.* **45**, 411-416.
- D'Hooghe, T. M., Bambra, C. S., DeJonge, I., Lauweryns, J. M., & Koninckx, P. R. (1996a) The prevalence of spontaneous endometriosis in the baboon (*Papio anubis*, *Papio cynocephalus*) increases with the duration of captivity. *Acta. Obstet. Gynecol. Scand.* **75**, 98-101.
- D'Hooghe, T. M., Bambra, C. S., Isahakia, M., & Koninckx, P. R. (1992) Evolution of spontaneous endometriosis in the baboon (*Papio anubis*, *Papio cynocephalus*) over a 12-month period. *Fertil. Steril.* **58**, 409-412.
- D'Hooghe, T. M., Bambra, C. S., Raeymaekers, B. M., & Koninckx, P. R. (1996b) Increased prevalence and recurrence of retrograde menstruation in baboons with spontaneous endometriosis. *Hum. Reprod.* **11**, 2022-2025.
- D'Hooghe, T. M., De Jonge, I., Bambra, C. S., Hill, J. A., Raeymaekers, B. M., & Koninckx, P. R. (1995) The effects of immunosuppression on development and progression of endometriosis in baboons (*Papio anubis*). *Fertil. Steril.* **64**, 172-178.
- D'Hooghe, T. M., Dunselman, G. A., Bambra, C. S., Evers, H. L., Suleman, M. A., & Koninckx, P. R. (1994) Development of a model of retrograde menstruation in baboons (*Papio anubis*). *Fertil. Steril.* **62**, 635-638.
- D'Hooghe, T. M., & Hill, J. A. (1995) in *Immunology of human reproduction* pp 133-162, BIOS Scientific.
- Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J., & Weissenbach, J. (1996) A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* **380**, 152-154.
- Dierschke, D. J., & Clark, J. R. (1976) Laparoscopy in *Macaca mulatta*: specialized equipment employed and initial observations. *J. Med. Primatol.* **5**, 100-110.

- Dierschke, D. J., Reinhold, J. H., & Wolf, R. C. (1985) Induced follicular atresia in rhesus monkeys: strength-duration relationships of the estrogen stimulus. *Endocrinology* **117**, 1397-1403.
- DiGiacomo, R. F. (1977) Gynecologic pathology in the rhesus monkey (*Macaca mulatta*) II. Findings in laboratory and free-ranging monkeys. *Vet. Pathol.* **14**, 539-546.
- Dizerega, G. S., Barber, D. L., & Hodgen, G. D. (1980) Endometriosis: role of ovarian steroids in initiation, maintenance, and suppression. *Fertil. Steril.* **33**, 649-653.
- Dmowski, W. P., Gebel, H. M., & Braun, D. P. (1994) The role of cell-mediated immunity in pathogenesis of endometriosis. *Acta. Obstet. Gynecol. Scand.* **S159**, 7-14.
- Dodge, S. T., Pumphrey, R. S., & Miyazawa, K. (1986) Peritoneal endometriosis in women requesting reversal of sterilization. *Fertil. Steril.* **45**, 774-777.
- Dore, M., & Lagace, A. (1985) Spontaneous external endometriosis in a gorilla (*Gorilla gorilla*). *Can. Vet. J.* **26**, 347-349.
- Durinzi, K. L., & DeLeon, F. D. (1993) Endometriosis in the adolescent and teenage female. *Adolesc. Pediatr. Gynecol.* **6**, 3-7.
- Ebers, G. C., & Dessa Sadovnick, A. (1994) Association studies in multiple sclerosis. *J. Neuroimmunol.* **53**, 117-122.
- Eisen, S., Neuman, R., Goldberg, J., Rice, J., & True, W. (1989) Determining zygosity in the Vietnam era twin registry: an approach using questionnaires. *Clin. Genet.* **35**, 423-432.
- Eisenfeld, A. J., Gardner, W. U., & Van Wagenan, G. (1971) Radioactive estradiol accumulation in endometriosis of the rhesus monkey. *Am. J. Obstet. Gynecol.* **109**, 124-130.
- Emmert, C., Romann, D., & Riedel, H. H. (1998) Endometriosis diagnosed by laparoscopy in adolescent girls. *Arch. Gynecol. Obstet.* **261**, 89-93.
- Ergun, A., Pabuccu, R., Atay, V., Kucuk, T., Duru, N. K., & Gungor, S. (1997) Three sisters with septate uteri: another reference to bidirectional theory. *Hum. Reprod.* **12**, 140-142.
- Erzen, M., & Kovacic, J. (1998) Relationship between endometriosis and ovarian cancer. *Eur. J. Gynaecol. Oncol.* **19**, 553-555.
- Eskenazi, B., & Warner, M. L. (1997) Epidemiology of endometriosis. *Obstet. Gynecol. Clin. Nth. Amer.* **24**, 235-258.
- Eugster, H.-P., Probst, M., Würgler, F. E., & Sengstag, C. (1993) Caffeine, estradiol and progesterone interact with human CYP1A1 and CYP1A2: Evidence from cDNA-directed expression in *Saccharomyces cerevisiae*. *Drug Metab. Dispos.* **21**, 43-49.
- Fanton, J. W., & Golden, J. G. (1991) Radiation-induced endometriosis in *Macaca mulatta*. *Radiat. Res.* **126**, 141-146.

- Fanton, J. W., & Hubbard, G. B. (1983) Spontaneous endometriosis in a cynomolgous monkey (*Macaca fascicularis*). *Lab. Anim. Sci.* **33**, 597-599.
- Fanton, J. W., Hubbard, G. B., & Wood, D. H. (1986) Endometriosis: Clinical and pathologic findings in 70 rhesus monkeys. *Am. J. Vet. Res.* **47**, 1537-1541.
- Fedele, L., Bianchi, S., DiNola, G., Franchi, D., & Candiani, G. B. (1992) Endometriosis and nonobstructive Müllerian anomalies. *Obstet. Gynecol.* **79**, 515-517.
- Folse, D. S., & Stout, L. C. (1978) Endometriosis in a baboon (*Papio doguera*). *Lab. Anim. Sci.* **28**, 217-219.
- Frey, P. A. (1996) The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J.* **10**, 461-470.
- Fujii, S. (1991) Secondary Müllerian system and endometriosis. *Am. J. Obstet. Gynecol.* **165**, 219-225.
- García-Closas, M., Kelsey, K. T., Wiencke, J. K., Xu, X., Wain, J. C., & Christiani, D. C. (1997) A case-control study of cytochrome P450 1A1, glutathione S-transferase M1, cigarette smoking and lung cancer susceptibility (Massachusetts, United States). *Cancer Causes & Control* **8**, 544-553.
- Gil-Benso, R., Lopez-Gines, C., Carda-Batalla, C., Monteagudo-Crespo, C., Torres, J. V., & Llombart-Bosch, A. (1997) Cytogenetic findings in malignant mixed mesodermal tumors of the uterus. *Cancer Genet. Cytogenet.* **99**, 121-125.
- Gray, I. C., & Jeffreys, A. J. (1991) Evolutionary transience of hypervariable minisatellites in man and the primates. *Proc. R. Soc. Lond. B* **243**, 241-253.
- Grosskinsky, C. M., & Halme, J. (1993) Endometriosis: the host response. *Bailliere's Clinical Obstetrics and Gynaecology* **7**, 704-713.
- Guengerich, F. P. (1988) Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.* **48**, 2946-2954.
- Hadfield, R., Mardon, H., Barlow, D., & Kennedy, S. (1996) Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. *Hum. Reprod.* **11**, 878-880.
- Hadfield, R. M., Mardon, H. J., Barlow, D. H., & Kennedy, S. H. (1997a) Endometriosis in monozygotic twins. *Fertil. Steril.* **68**, 941-942.
- Hadfield, R. M., Yudkin, P. L., Coe, C. L., Scheffler, J., Uno, H., Barlow, D. H., Kemnitz, J. W., & Kennedy, S. H. (1997b) Risk factors for endometriosis in the rhesus monkey (*Macaca mulatta*): a case-control study. *Hum. Rep. Update* **3**, 109-115.
- Hanson, J., & Khong, T. Y. (1996) An audit of hysterectomies in young women at the Queen Victoria Hospital, 1984-1994. *Aust. N.Z. J. Obstet. Gynaecol.* **36**, 441-443.

- Heaps, J. M., Nieverg, R. K., & Berek, J. S. (1990) Malignant neoplasms arising in endometriosis. *Obstet. Gynecol.* **75**, 1023-1028.
- Hendrickx, A. G., & Dukelow, W. R. (1995) in *Nonhuman primates in biomedical research: Biology and management* pp 147-191, Academic Press.
- Hendrickx, A. G., Prahallada, S., & Binkerd, P. E. (1988) Long-term evaluation of the diethylstilbestrol (DES) syndrome in adult female rhesus monkeys (*Macaca mulatta*). *Reprod. Toxicol.* **1**, 253-261.
- Hill, J.A. (1997) Immunology and endometriosis. Fact, artifact, or epiphenomenon? *Obstet. Gynecol. Clin. North. Am.* **24**, 291-306.
- Holmans, P. (1993) Asymptotic properties of affected-sib-pair linkage analysis. *Am. J. Hum. Genet.* **52**, 362-374.
- Hornstein, M. D., Thomas, P. P., Sober, A. J., Wyshak, G., Albright, N. L., & Frisch, R. (1997) Association between endometriosis, dysplastic naevi and history of melanoma in women of reproductive age. *Hum. Reprod.* **12**, 143-145.
- Houston, D. E., Noller, K. L., Melton, L. J., & Selwyn, B. J. (1988) The epidemiology of pelvic endometriosis. *Clin. Obstet. Gynecol.* **31**, 787-799.
- Hutz, R. J., Dierschke, D. J., & Wolf, R. C. (1985) Seasonal effects on ovarian folliculogenesis in rhesus monkeys. *Biol. Reprod.* **33**, 653-659.
- Hutz, R. J., Dierschke, D. J., & Wolf, R. C. (1986) Markers of atresia in ovarian follicular components from rhesus monkeys treated with estradiol-17 β . *Biol. Reprod.* **34**, 65-70.
- Inoue, M., & Takenaka, O. (1993) Japanese macaque microsatellite PCR primers for paternity testing. *Primates* **34**, 37-45.
- Jiang, X., Hitchcock, A., Bryan, E. J., Watson, R. H., Englefield, P., Thomas, E. J., & Campbell, I. G. (1996) Microsatellite analysis of endometriosis reveals loss of heterozygosity at candidate ovarian tumor suppressor gene loci. *Cancer Res.* **56**, 3534-3539.
- Jiang, X., Morland, S. J., Hitchcock, A., Thomas, R. J., & Campbell, I. G. (1998) Allelotyping of endometriosis with adjacent ovarian carcinoma reveals evidence of common lineage. *Cancer Res.* **58**, 1707-1712.
- Jimbo, H., Hitomi, Y., Yoshikawa, H., Yano, T., Momoeda, M., Sakamoto, A., Tsutsumi, O., Taketani, Y., & Esumi, H. (1997a) Evidence for monoclonal expansion of epithelial cells in ovarian endometrial cysts. *Am. J. Pathol.* **150**, 1173-1178.
- Jimbo, H., Yoshikawa, H., Onda, T., Yasugi, T., Sakamoto, A., & Taketani, Y. (1997b) Prevalence of ovarian endometriosis in epithelial ovarian cancer. *Int. J. Gynecol. Obstet.* **59**, 245-250.

- Jurka, J. (1995) Simple repetitive DNA sequences from primates: Compilation and analysis. *J. Mol. Evol.* **40**, 120-126.
- Kale, S., Shuster, M., & Shangold, J. (1971) Endometrioma in a cesarean scar: Case report and review of the literature. *Am. J. Obstet. Gynecol.* **111**, 596-597.
- Kawajiri, K., Nakachi, K., Imai, K., Yoshii, A., Shinoda, N., & Watanabe, J. (1990) Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P4501A1 gene. *FEBS* **263**, 131-133.
- Kennedy, S., Hadfield, R., Barlow, D., Weeks, D. E., Laird, E., & Golding, S. (1997) Use of MRI in genetic studies of endometriosis. *Am. J. Med. Genet.* **71**, 371-372.
- Kennedy, S., Hadfield, R., Westbrook, C., Weeks, D. E., Barlow, D., & Golding, S. (1998) Magnetic resonance imaging to assess familial risk in relatives of women with endometriosis. *Lancet* **352**, 1440-1441.
- Kennedy, S., Mardon, H., & Barlow, D. (1995) Familial endometriosis. *J. Assist. Reprod. Genet.* **12**, 32-34.
- Kennedy, S. H. (1991) What is important to the patient with endometriosis? *Br. J. Clin. Pract. Symp. Suppl.* **72**, 8-10.
- Kennedy, S. H., Brodribb, J., Godfrey, A. M., & Barlow, D. H. (1988) Pre-operative treatment of an abdominal wall endometrioma with nafarelin acetate. Case report. *Br. J. Obstet. Gynaecol.* **95**, 521-523.
- Kharat, I., & Saatcioglu, F. (1996) Antiestrogenic effects of 2,3,7,8-tetrachlordibenzo-*p*-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. *J.B.C.* **271**, 10533-10537.
- Kirshon, B., & Poindexter, A. N. (1988) Contraception: A risk factor for endometriosis. *Obstet. Gynecol.* **71**, 829-831.
- Kline, R. C., Wharton, J. T., Atkinson, E. N., Burke, T. W., Gershenson, D. M., & Edwards, C. L. (1990) Endometrioid carcinoma of the ovary: retrospective review of 145 cases. *Gynecol. Oncol.* **39**, 337-346.
- Koninckx, P. R., Braet, P., Kennedy, S. H., & Barlow, D. H. (1994) Dioxin pollution and endometriosis in Belgium. *Hum. Reprod.* **9**, 1001-1002.
- Koninckx, P. R. (1994) Is mild endometriosis a condition occurring intermittently in all women? *Hum. Reprod.* **9**, 2202-2205.
- Koumantaki, Y., Giziaki, E., Linos, A., *et al.* (1997) Family history as a risk factor for rheumatoid arthritis: A case-control study. *J. Rheumatol.* **24**, 1522-1526.
- Krajinovic, M., Labuda, D., Richer, C., Karimi, S., & Sinnett, D. (1999) Susceptibility to childhood acute lymphoblastic leukemia: Influence of CYP1A1, CYP2D6, GSTM1 and GSTT1 genetic polymorphisms. *Blood* **93**, 1496-1501.

- Krawczak, M., Berard, J. D., Bercovitch, F. B., Schmidtke, J., & Nurnberg, P. (1993) Power and limits of DNA-profiling in primate populations: paternity assessment in rhesus macaques from Cayo Santiago. *Primates* **34**, 395-402.
- Kresch, A. J., Seifer, D. B., Sachs, L. B., & Barrese, I. (1984) Laparoscopy in 100 women with chronic pelvic pain. *Obstet. Gynecol.* **64**, 672-674.
- Kruglyak, L., Daly, M. J., Reeve-Daly, M. P., & Lander, E. S. (1996) Parametric and nonparametric linkage analysis: A unified multipoint approach. *Am. J. Hum. Genet.* **58**, 1347-1363.
- Lamb, K., Hoffmann, R. G., & Nichols, T. R. (1986) Family trait analysis: A case-control study of 43 women with endometriosis and their best friends. *Am. J. Obstet. Gynecol.* **154**, 596-601.
- Lander, E. S., & Schork, N. J. (1994) Genetic dissection of complex traits. *Science* **265**, 2037-2048.
- Leslie, N. D., Immerman, E. B., Flach, J. E., Florez, M., Fridovich-Keil, J. L., & Elsas, L. J. (1992) The human galactose-1-phosphate uridyltransferase gene. *Genomics* **14**, 474-480.
- Lin, H.-C., Kirby, L. T., Ng, W. G., & Reichardt, J. K. V. (1994) On the molecular nature of the Duarte variant of galactose-1-phosphate uridyl transferase (GALT). *Hum. Genet.* **93**, 167-169.
- Liu, D. T. Y., & Hitchcock, A. (1986) Endometriosis: its association with retrograde menstruation, dysmenorrhoea and tubal pathology. *Br. J. Obstet. Gynaecol.* **93**, 859-862.
- Lorenzen, T., Pociot, F., Hougaard, P., & Nerup, J. (1994) Long-term risk of IDDM in first-degree relatives of patients with IDDM. *Diabetologia* **37**, 321-327.
- Lucas, D., Ménez, C., Floch, F., Gourlaouen, Y., Sparfer, O., & al., e. (1996) Cytochromes P4502E1 and P4501A1 genotypes and susceptibility to cirrhosis or upper aerodigestive tract cancer in alcoholic caucasians. *Alcohol. Clin. Exp. Res.* **20**, 1033-1037.
- Lykken, D. T., McGue, M., & Tellegen, A. (1987) Recruitment bias in twin research: The rule of two-thirds reconsidered. *Behav. Genet.* **17**, 343-362.
- Lykken, D. T., Tellegen, A., & DeRubeis, R. (1978) Volunteer bias in twin research: The rule of two-thirds. *Soc. Biol.* **25**, 1-9.
- MacKenzie, W. F., & Casey, H. W. (1975) Animal Model: Endometriosis in Rhesus Monkeys. *Am. J. Pathol.* **80**, 341-344.
- Magnus, P., Berg, K., & Nance, W. E. (1983) Predicting zygoty in Norwegian twin pairs born 1915-1960. *Clin. Genet.* **24**, 103-112.

- Mahmood, T. A., & Templeton, A. (1991) Prevalence and genesis of endometriosis. *Hum. Reprod.* **6**, 544-549.
- Mai, M., Huang, H., Reed, C., Qian, C., Smith, J. S., Alderete, B., Jenkins, R., Smith, D. I., & Liu, W. (1998) Genomic organization and mutation analysis of p73 in oligodendrogliomas with chromosome 1 p-arm deletions. *Genomics* **51**, 359-363.
- Maier, D., & Koch, E. (1990) Müllerian dysgenesis associated with a chromosomal anomaly and mental retardation: A case report. *J. Reprod. Med.* **35**, 301-302.
- Martin, R. H., & Higginbottom, J. (1973) Hysterotomy and endometriosis. *Lancet* **July 14**, 106.
- Matorras, R., Rodríguez, F., Ignacio Pijoan, K., Ramón, O., Gutierrez de Terán, G., & Rodríguez-Escudero, F. (1995) Epidemiology of endometriosis in infertile women. *Fertil. Steril.* **63**, 34-38.
- Maxwell, C., Kilpatrick, D. C., Haining, R., & Smith, S. K. (1989) No HLA-DR specificity is associated with endometriosis. *Tissue Antigens* **34**, 145-147.
- Mayani, A., Barel, S., Soback, S., & Almagor, M. (1997) Dioxin concentrations in women with endometriosis. *Hum. Reprod.* **12**, 373-375.
- Mayo-Smith, W. W., & Lee, M. J. (1995) MR imaging of the female pelvis. *Clin. Radiol.* **50**, 667-376.
- McCann, T. O., & Myers, R. E. (1970) Endometriosis in rhesus monkeys. *Am. J. Obstet. Gynecol.* **106**, 516-523.
- McMeekin, D. S., Burger, R. A., Manetta, A., DiSaia, P., & Berman, M. L. (1995) Endometrioid adenocarcinoma of the ovary and its relationship to endometriosis. *Gynecol. Oncol.* **59**, 81-86.
- Mertens, F., Johansson, B., Höglund, M., & Mitelman, F. (1997) Chromosomal imbalance maps of malignant solid tumors: A cytogenetic survey of 3185 neoplasms. *Cancer Res.* **57**, 2765-2780.
- Moen, M. H. (1987) Endometriosis in women at interval sterilization. *Acta. Obstet. Gynecol. Scand.* **66**, 451-454.
- Moen, M. H. (1991) Is a long period without childbirth a risk factor for developing endometriosis? *Hum. Reprod.* **6**, 1404-1407.
- Moen, M. H. (1994) Endometriosis in monozygotic twins. *Acta. Obstet. Gynecol. Scand.* **73**, 59-62.
- Moen, M. H. (1995) Is mild endometriosis a disease? Why do women develop endometriosis and why is it diagnosed? *Hum. Reprod.* **10**, 8-11.
- Moen, M. H., & Magnus, P. (1993) The familial risk of endometriosis. *Acta. Obstet. Gynecol. Scand.* **72**, 560-564.

- Moen, M. H., & Muus, K. M. (1991) Endometriosis in pregnant and non-pregnant women at tubal sterilization. *Hum. Reprod.* **6**, 699-702.
- Moen, M. H., & Schei, B. (1997) Epidemiology of endometriosis in a Norwegian country. *Acta Obstet. Gynecol. Scand.* **76**, 559-562.
- Mol, B.W., Bayram, N., Lijmer, J.G., Wiegerinck, M.A., Bongers, M.Y., van der Veen, F., & Bossuyt, P.M. (1998) The performance of CA-125 measurement in the detection of endometrisois: a meta-analysis. *Fertil. Steril.* **70**, 1101-1108.
- Moody, K. D., Papero, M. A., & Morgenstern, S. E. (1989) Use of leuprolide to treat spontaneous endometriosis in a rhesus macaque. *Lab. Anim. Sci.* **39**, 475.
- Mori, N., Morosetti, R., Spira, S., Lee, S., Ben-Yehuda, D., Schiller, G., Landolfi, R., Mizoguchi, H., & Koeffler, H. P. (1998) Chromosome band 1p36 contains a putative tumor suppressor gene important in the evolution of chronic myelocytic leukemia. *Blood* **92**, 3405-3409.
- Morin, P. A., Mahboubi, P., Wedel, S., & Rogers, J. (1998) Rapid screening and comparison of human microsatellite markers in baboons: Allele size is conserved, but allele number is not. *Genomics* **53**, 12-20.
- Morland, S. J., Jiang, X., Hitchcock, A., Thomas, E. J., & Campbell, I. G. (1998) Mutation of galactose-1-phosphate uridyl transferase and its association with ovarian cancer and endometriosis. *Int. J. Cancer* **77**, 825-827.
- Murata, M., Shiraishi, T., Fukutome, K., Watanabe, M., Nagao, M., Kubota, Y., Ito, H., Kawamura, J., & Yatani, R. (1998) Cytochrome P4501A1 and glutathione S-transferase M1 genotypes as risk factors for prostate cancer. *Jap. J. Clin. Oncol.* **28**, 657-660.
- Muyldermans, M., Cornillie, F. J., & Koninckx, P. R. (1995) CA125 and endometriosis. *Hum. Reprod. Update* **1**, 173-187.
- Nebert, D. W., & Gonzalez, F. J. (1987) P450 genes: structure, evolution and regulation. *Ann. Rev. Biochem.* **56**, 945-993.
- Neubert, R., Jacob-Müller, U., Helge, H., Stahlmann, R., & Neubert, D. (1991) Polyhalogenated debenzo-*p*-dioxins and dibenzofurans and the immune system 2. *In vitro* effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on lymphocytes of venous blood from man and a non-human primate (*Callithrix jacchus*). *Arch. Toxicol.* **65**, 213-219.
- Novak, E. (1926) The significance of uterine mucosa in the fallopian tubes with a discussion of the origin of aberrant endometrium. *Am. J. Obstet. Gynecol.* **12**, 484.
- O'Connell, J. R., & Weeks, D. E. (1997) PedCheck: a program for identifying marker typing incompatibilities in linkage analysis. *Am. J. Hum. Genet.*, A288.

- Olive, D.L., & Henderson, D.Y. (1987) Endometriosis and mullerian anomalies. *Obstet. Gynecol.* **63**, 412-415.
- Orholm, M., Munkholm, P., Langholz, E., Nielsen, O. H., Sorensen, I. A., & Binder, V. (1991) Familial occurrence of inflammatory bowel disease. *N. Engl. J. Med.* **324**, 84-88.
- Ott, J. (1989) Computer-simulation methods in human linkage analysis. *Proc. Natl. Acad. Sci. USA* **86**, 4175-4178.
- Ott, J. (1992) Strategies for characterizing highly polymorphic markers in human gene mapping. *Am. J. Hum. Genet.* **51**, 283-290.
- Pejovic, T., Alm, P., Iosif, S. C., Mitelman, F., & Heim, S. (1996) Cytogenetic findings in four malignant mixed mesodermal tumors of the ovary. *Cancer Genet. Cytogenet.* **88**, 53-56.
- Perelman, R. H., Engle, M. J., Palta, M., Kemnitz, J. W., & Farrell, P. M. (1986) Fetal lung development in male and female non-human primates. *Pediat. Res.* **20**, 987-991.
- Petersen, D. D., McKinney, C. E., Ikeya, K., Smith, H. H., Bale, A. E., McBride, O. W., & Nebert, D. W. (1991) Human CYP1A1 gene: Cosegregation of the enzyme inducibility phenotype and an RFLP. *Am. J. Hum. Genet.* **48**, 720-725.
- Provinciali, M., DiStefano, G., Muzzioli, M., Garzetti, G. G., Ciavattini, A., & Fabris, N. (1995) Relationship between 17- β estradiol and prolactin in the regulation of natural killer cell activity during progression of endometriosis. *J. Endocrinol. Invest.* **18**, 645-652.
- Reichardt, J. K. V., Belmont, J. W., Levy, H. L., & Woo, S. L. C. (1992) Characterization of two missense mutations in human galactose-1-phosphate uridyl transferase: different molecular mechanisms for galactosemia. *Genomics* **12**, 596-600.
- Reichardt, J. K. V., & Woo, S. L. C. (1991) Molecular basis of galactosemia: Mutations and polymorphisms in the gene encoding human galactose-1-phosphate uridylyltransferase. *Proc. Natl. Acad. Sci. USA* **88**, 2633-2637.
- Rier, S. E., Martin, D. C., Bowman, R. E., Dmowski, W. P., & Becker, J. L. (1993) Endometriosis in rhesus monkeys (*Macaca mulatta*) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Fundam. Appl. Toxicol.* **21**, 433-441.
- Rippy, M. K., Lee, D. R., Pearson, S. L., Bernal, J. C., & Kueht, T. J. (1996) Identification of rhesus macaques with spontaneous endometriosis. *J. Med. Primatol.* **25**, 346-355.
- Risch, N. (1990) Linkage strategies for genetically complex traits. II. The power of affected relative pairs. *Am. J. Hum. Genet.* **46**, 229-241.
- Rojas, M., Alexandrov, K., Cascorbi, I., Brockmoller, J., Likhachev, A., Pazharisski, K., Bouvier, G., Auburtin, G., Mayer, L., KoppSchneider, A., Roots, I., & Bartsch, H. (1998) High benzo[a]pyrene diol-epoxide DNA adduct levels in lung and blood cells

- from individuals with combined CYP1A1 MspI/MspI-GSTM1 *0/*0 genotypes. *Pharmacogenetics* **8**, 109-118.
- Safe, S., Astroff, B., Harris, M., Zacharewski, T., Dickerson, R., Romkes, M., & Biegel, L. (1991) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as anti-oestrogens: Characterization and mechanism of action. *Pharmacol. Toxicol.* **69**, 400-409.
- Safe, S. H. (1995) Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. *Pharmac. Ther.* **67**, 247-281.
- Sainz de la Cuesta, R., Eichhorn, J. H., Rice, L. W., Fuller, A. F., Nikrui, N., & Goff, B. A. (1996) Histologic transformation of benign endometriosis to early epithelial ovarian cancer. *Gynecol. Oncol.* **60**, 238-244.
- Sampson, J.A. (1927) Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am. J. Obstet. Gynecol.* **14**, 422.
- Sangi-Haghpeykar, H., & Poindexter, A. N. (1995) Epidemiology of endometriosis among parous women. *Obstet. Gynecol.* **85**, 983-992.
- Sarhanis, P., Redman, C., Perrett, C., Brannigan, K., Clayton, R. N., & al., e. (1996) Epithelial ovarian cancer: influence of polymorphism at the glutathione *S*-transferase GSTM1 loci on p53 expression. *Br. J. Cancer* **74**, 1757-1761.
- Schaerdel, A. D. (1986) Pelvic endometriosis associated with infarctions of the colon in a rhesus monkey. *Lab. Anim. Sci.* **36**, 533-536.
- Schenken, R. S., Williams, R. F., & Hodgen, G. D. (1987) Effect of pregnancy on surgically induced endometriosis in cynomolgus monkeys. *Am. J. Obstet. Gynecol.* **157**, 1392-1396.
- Schisselbauer, J. C., Hogan, W. M., Buetow, K. H., & Tew, K. D. (1992) Heterogeneity of glutathione *S*-transferase enzyme and gene expression in ovarian cancer. *Pharmacogenetics* **2**, 63-72.
- Schmidt, R. E., & Hartfiel, D. A. (1978) Endometriosis in a gray-cheeked mangaby (*Cercocebus albigena*). *J. Zoo. Anim. Med.* **9**, 42-45.
- Schrodt, G. R., Alcorn, M. O., & Ibanez, J. (1980) Endometriosis of the male urinary system: A case report. *J. Urol.* **124**, 722-723.
- Shalev, M., Ciurea, D., & Deligdisch, L. (1992) Endometriosis and stromal tumor in a baboon (*Papio Hamadryas*). *Lab. Anim. Sci.* **42**, 204-208.
- Shin, J.-C., Ross, H. L., Elias, S., Nguyen, D. D., Mitchell-Leef, D., Simpson, J. L., & Bischoff, F. Z. (1997) Detection of chromosomal aneuploidy in endometriosis by multi-color fluorescence in situ hybridization (FISH). *Hum. Genet.* **100**, 401-406.

- Simpson, J. L., Elias, S., Malinak, I. R., & Buttram, V. C. (1980) Heritable aspects of endometriosis I. Genetic studies. *Am. J. Obstet. Gynecol.* **137**, 327-331.
- Simpson, J. L., Malinak, L. R., Elias, S., Carson, S. A., & Radvany, R. A. (1984) HLA associations in endometriosis. *Am. J. Obstet. Gynecol.* **148**, 395-397.
- Sivaraman, L., Leatham, M. P., Yee, J., Wilkens, L. R., Lau, A. F., & Le Marchand, L. (1994) CYP1A1 genetic polymorphisms and *in situ* colorectal cancer. *Cancer Res.* **54**, 3692-3695.
- Smith, D. G. (1982) Use of genetic markers in the colony management of nonhuman primates: A review. *Lab. Anim. Sci.* **32**, 540-546.
- Sparkes, R. S., Epstein, P. A., Kidd, K. K., Klisak, I., Sparkes, M. C., Crist, M., & Morton, L. A. (1980) Probable linkage between the human galactose-1-P uridyl transferase locus and 9qh. *Am. J. Hum. Genet.* **32**, 188-193.
- Spitz, M. R., Currier, R. D., Fueger, J. J., Babaian, R. J., & Newell, G. R. (1991) Familial patterns of prostate cancer: a case-control analysis. *J. Urol.* **146**, 1305-1307.
- Stone, W. H., Ely, J. J., Manis, G. S., & Van de Berg, J. L. (1993) Classical genetic markers and DNA markers: A commensal marriage. *Primates* **34**, 363-376.
- Strathy, J. H., Molgaard, C. A., Coulam, C. B., & Melton, L. J. (1982) Endometriosis and infertility: a laparoscopic study of endometriosis among fertile and infertile women. *Fertil. Steril.* **38**, 667-672.
- Takahashi, K., Kitao, M., Okada, S., Sugimura, K., & Ozaki, T. (1994) Diagnosis of pelvic endometriosis by magnetic resonance imaging using 'fat-saturation' technique. *Fertility and Sterility* **62**, 973-977.
- Takahashi, K., Okada, M., Okada, S., Kitao, M., Imaoka, I., & Sugimura, K. (1996) Studies on the detection of small endometrial implants by magnetic resonance imaging using a fat saturation technique. *Fertil. Steril.* **41**, 203-206.
- Tanimoto, K., Hayashi, S., Yoshiga, K., & Ichikawa, T. (1999) Polymorphisms of the CYP1A1 and GSTM1 gene involved in oral squamous cell carcinoma in association with a cigarette dose. *Oral Oncol.* **35**, 191-196.
- Te Linde, R. W., & Scott, R. B. (1950) Experimental endometriosis. *Am. J. Obstet. Gynecol.* **60**, 1147-1173.
- Terwilliger, J. D., & Ott, J. (1994) *Handbook of human genetic linkage*, John Hopkins University Press.
- The American Fertility Society. (1985) Revised American fertility society classification of endometriosis. *Fertil. Steril.* **43**, 351-352.
- Thomas, E. J. (1995) Endometriosis, 1995 - confusion or sense? *Int. J. Gynecol. Obstet.* **48**, 149-155.

- Togashi, K., Nishimura, K., Kimura, I., Tsuda, Y., Yamashita, K., Shibata, T., Nakano, Y., Konishi, J., Konishi, I., & Mori, T. (1991) Endometrial cysts: Diagnosis with MR Imaging. *Radiol.* **180**, 73-78.
- Toki, T., Fujii, S., & Silverberg, S. G. (1996) A clinicopathologic study on the association of endometriosis and carcinoma of the ovary using a scoring system. *Int. J. Gynecol. Cancer* **6**, 68-75.
- Treloar, S. A., O'Connor, D. T., O'Connor, V. M., & Martin, N. G. (1999) Genetic influences on endometriosis in an Australian twin sample. *Fertil. Steril.* **71**, 701-710.
- Trimbos, J. B., Trimbos-Kemper, G. C. M., Peters, A. A. W., van der Does, C. D., & van Hall, E. V. (1990) Findings in 200 consecutive asymptomatic women having laparoscopic sterilization. *Arch. Gynecol. Obstet.* **247**, 121-124.
- Ugur, M., Turan, C., Mungan, T., Kuscu, E., Senoz, S., Agis, H., & Gokmen, O. (1995) Endometriosis in association with Müllerian anomalies. *Gynecol. Obstet. Invest.* **40**, 261-264.
- Unic, I., Stalmeier, P. F., Peer, P. G., & van Daal, W. A. (1997) A review on family history of breast cancer: screening and counseling proposals for women with familial (non-hereditary) breast cancer. *Patient Educ. Couns.* **32**, 117-127.
- Vaury, C., Lainé, R., Noguiez, P., de Coppet, P., Jaulin, C., & al., e. (1995) Human glutathione S-transferase M1 null genotype is associated with a high inducibility of cytochrome P450 1A1 gene transcription. *Cancer Res.* **55**, 5520-5523.
- Vercellini, P., Parazzini, F., Bolis, G., Carinelli, S., Dindelli, M., Vendola, N., Luchini, L., & Crosignani, P. G. (1993) Endometriosis and ovarian cancer. *Am. J. Obstet. Gynecol.* **169**, 181-182.
- Vercellini, P., De-Giorgi, O., Aimi, G., Panazza, S., Uglietti, A., & Crosignani, P.G. (1997) Menstrual characteristics of women with and without endometriosis. *Obstet. Gynecol.* **90**, 264-268.
- Vercellini, P., Parazzini, F., Oldani, S., Panazza, S., Bramante, T., & Crosignani, P. G. (1995) Adenomyosis at hysterectomy - a study on frequency-distribution and patient characteristics. *Hum. Reprod.* **10**, 1160-1162.
- Verp, M. S., Simpson, J. L., Elias, S., Carson, S. A., Sarto, G. E., & Feingold, M. (1983) Heritable aspects of uterine anomalies. I. Three familial aggregates with Müllerian fusion anomalies. *Fertil. Steril.* **40**, 80-85.
- Vessey, M. P., Villard-Mackintosh, L., & Painter, R. (1993) Epidemiology of endometriosis in women attending family planning clinics. *B.M.J.* **306**, 182-184.
- Waterton, J. C., Breen, S. A., Dukes, M., Horrocks, M., & Wadsworth, P. F. (1993) A case of adenomyosis in a pigtailed monkey diagnosed by magnetic resonance imaging and treated with the novel pure antiestrogen, ICI 182,780. *Lab. Anim. Sci.* **43**, 247-251.

- Waterton, J. C., Miller, D., Morrell, J. S. W., Dukes, M., West, C. D., & Wadsworth, P. F. (1992) A case of endometriosis in the macaque diagnosed by nuclear magnetic resonance imaging. *Lab. Anim.* **26**, 59-64.
- Wedekind, J. E., Frey, P. A., & Rayment, I. (1995) Three-dimensional structure of galactose-1-phosphate uridylyltransferase from *Escherichia coli* at 1.8 Å resolution. *Biochemistry* **34**, 11049-11061.
- Weeks, D. E., & Lange, K. (1988) The affected-pedigree-member method of linkage analysis. *Am. J. Hum. Genet.* **42**, 315-326.
- Weeks, D. E., Ott, J., & Lathrop, G. M. (1990) SLINK: a general simulation program for linkage analysis. *Am. J. Hum. Genet.* **47**, A204.
- Wood, D. H. (1991) Long-term mortality and cancer risk in irradiated rhesus monkeys. *Radiat. Res.* **126**, 132-140.
- Wood, D. H., Yochmowitz, M. G., Salmon, Y. L., Eason, R. L., & Boster, R. A. (1983) Proton irradiation and endometriosis. *Aviat. Space Environ. Med.* **54**, 718-724.
- Woodworth, S. H., Sanfilippo, J. S., Singh, M., Cook, C. L., Yussman, M. A., & Lincoln, S. R. (1995) A prospective study on the association between red hair color and endometriosis in infertile patients. *Fertil. Steril.* **64**, 651-652.
- Xu, X., Kelsey, K. T., Wiencke, J. K., Wain, J. C., & Christiani, D. C. (1996) Cytochrome P450 CYP1A1 MspI polymorphism and lung cancer susceptibility. *Cancer Epidemiol. Biomark. Prev.* **5**, 687-692.
- Zhong, S., Roland Wolf, C., & Spurr, N. K. (1992) Chromosomal assignment and linkage analysis of the human glutathione S-transferase μ gene (GSTM1) using intron specific polymerase chain reaction. *Hum. Genet.* **90**, 435-439.
- Zhong, S., Wyllie, A. H., Barnes, D., Wolf, C. R., & Spurr, N. K. (1993) Relationship between the GSTM1 genetic polymorphism and susceptibility to bladder, breast and colon cancer. *Carcinogenesis* **14**, 1821-1824.