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The Impact of Lifestyle Factors on the Clinical Outcomes of *in vitro* Fertilisation-Embryo Transfer (IVF) Treatment

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Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

Signature: ..

Date: ..... 10th November 2003

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Abstract

Objectives: To determine the effect of female and male cigarette smoking, caffeine and alcohol consumption, stress and indicators of dietary status on the clinical outcomes of IVF treatment.

Design: Prospective cohort study.

Setting: PIVET Medical Centre, Perth, Western Australia.

Patients: Of 351 couples who commenced IVF treatment at PIVET Medical Centre between January 1997 and August 1998, 281 females and 247 males participated in this study, generating participation rates of 80.1% and 70.4%, respectively.

Main Outcome Measures: Multivariate methods of data analyses were used to control for patient and treatment variables in the examination of the effect of lifestyle factors on the following clinical outcomes: 1) number of oocytes retrieved by transvaginal oocyte aspiration (oocyte production), 2) fertilisation, measured as the number of oocytes fertilised weighted by the number of oocytes inseminated, 3) β-hCG pregnancy, 16 days post-embryo transfer, and 4) <12 week pregnancy loss following confirmation of β-hCG pregnancy. As a measure of ovarian reserve, serum basal FSH levels were also investigated as a dependent variable. Lifestyle factors included years of cigarette smoking (smoke years), tobacco, alcohol, caffeine and fruit and vegetable consumption, and stress from daily living and IVF treatment.

Results: Daily stress, tobacco consumption and smoke years were the female lifestyle factors shown to have a significant effect on IVF treatment. Oocyte production decreased with increasing levels of daily stress (P=0.039). However, female patients with high daily stress levels experienced higher than average rates of fertilisation *in vitro* (P=0.0059) and pregnancy (P=0.0207). Smoke years had an adverse effect on ovarian reserve (P=0.035), which in turn, compromised oocyte production. Female smoke years was negatively associated with rates of fertilisation (P<0.0001), and this effect was exacerbated by cigarette smoking at the time of treatment (P=0.0187).

Of the male lifestyle factors, caffeine, alcohol and fruit and vegetable consumption and IVF stress affected fertilisation *in vitro*. Fertilisation increased with

alcohol consumption (P<0.0001), and with fruit and vegetable consumption (P<0.0001). A significant interaction term between these two factors (P=0.0144) implied a threshold of benefit from the combined effect of the consumption of alcohol and fruit and vegetables. Caffeine consumption negated the beneficial effect of alcohol consumption, as shown by a significant interaction term between alcohol consumption and caffeine consumption (P=0.0007). Male stress from IVF treatment had an adverse effect on rates of fertilisation *in vitro* (P<0.0001).

Cigarette smoking by the male partner increased the likelihood of the female partner experiencing a <12 week pregnancy loss (P=0.0084).

Conclusions: In meeting with its principal objective, this study has demonstrated that specific lifestyle factors impact on the clinical outcomes of IVF treatment. It confirms the findings from former studies, namely the adverse effect of female smoking on ovarian reserve, and daily stress on ovulation. Moreover, this study has identified numerous new and unexpected relationships. Of note, the positive effect of male alcohol consumption on fertilisation *in vitro* and the elevated risk of early pregnancy loss associated with male smoking. This study has paved the way for future research into the identification of specific mechanisms of effect, including those suggested.

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List of Abbreviations

1-ET	one-embryo transfer
2-ET	two-embryo transfer
3-day ET	embryo-transfer on the third day post-insemination
3-ET	three embryo transfer
4-ET	four-embryo transfer
2PN	pronucleus
5-methyl-THF	5-methyltetrahydrofolate
8-OHdG	8-hydroxydeoxguanosine
adjusted R^2	adjusted coefficient of multiple determination
ANOVA	analysis of variance
β-hCG	beta-human chorionic gonadotrophin
BPDE-I-dG-DNA	benzo(a)pyrene-induced guanosine adduct
BMI	body mass index
CCCT	clomiphene citrate challenge test
CD	Cook's distance
CES	Cumulative Embryo Score
CI	confidence interval
cig	cigarette
CL	centred leverage
CRF	cortiocotrophin releasing factor
CUHREC	Curtin University Human Research Ethics Committee
Dept	Department
df	degrees of freedom
DFBETA	change in logistic coefficient
dks	drinks
DNA	deoxyribonucleic acid
E_2	oestradiol
eFSH	exogenous follicle stimulating hormone
ET	embryo transfer
FF-C	follicle fluid cotinine
FSH	follicle stimulating hormone
GPX	glutathione peroxidase

GnRH	gonadotrophin releasing hormone
GSD	geometric standard deviation
GSH	reduced glutathione
GSSG	oxidised glutathione
H·	hydrogen
hCG	human chorionic gonadotrophin
hMG	human menopausal gonadotrophin
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
ICSI	intra-cytoplasmic sperm injection
ID	identification
IQR	inter-quartile range
Ins(1,4,5)P_3	inositol 1,4,5-triphosphate
IVF	*in vitro* fertilisation-embryo transfer
LDL	low-density lipoprotein
LH	luteinizing hormone
ln	natural logarithm
Ins(1,4,5)P_3	1,4,5-triphosphate
LQ/D	Lifestyle Questionnaire/Diary
LR	likelihood ratio
mCES	modified Cumulative Embryo Score
MESA	micro-epididymal sperm aspiration
MI	myo-inositol
MLR	multiple linear regression
MW	Mann-Whitney non-parametric statistical test
n	sample size
no.	number
non-methyl-THF	non-methyltetrahydrofolate
$O_2^{-}\cdot$	superoxide
OR	odds ratio
OH·	hydroxyl
OHSS	ovarian hyperstimulation syndrome

OTC	'over the counter'
oxo^{8}dG	8-oxo-deoxyguanosine
P_4	progesterone
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphoinositides
PI	phosphatidylinositol
(PI)PI3K	phosphatidylinositol 3-kinase
POMS	Bi-polar Profile of Mood States
PS	phosphatidylserine
PtdIns	phosphatidylinositol
PUFA	polyunsaturated fatty acids
Q/QH_2	quinone/hydroquinone complex
NO	nitric oxide
NO_2	nitrogen dioxide
NSW	New South Wales
NTD	neural tube defect
r	Pearson's correlation coefficient
RBC	red blood cell
RC	relative chance
RNA	ribonucleic acid
ROS	reactive oxygen species
RYO	'roll your own'
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SOD	superoxide dismutase
SD	standard deviation
SDFBETAS	standardised change in regression coefficient
SDFFITS	standardised change in fitted value
SDR	studentised deleted residual
SE	standard error
SM	sphingomyelin

SPIDA	Statistical Package for Interactive Data Analysis
SPSS	Statistical Program for Social Sciences
STAI	State-Trait Anxiety Inventory
std	standard
TESA	testicular sperm aspiration
TBAR	thiobarbituric acid reactive substances
TVOA	transvaginal oocyte aspiration
vs	versus
VIF	variance inflation factor
WA	Western Australia
WARTC	Western Australian Reproductive Technology Council
WHO	World Health Organization
wk	week
χ^2	chi-square

Chapter 1
Introduction

In vitro fertilisation-embryo transfer (IVF) was first administered as a treatment for infertility in the late 1970's. Since its advent, and in parallel with significant advances in assisted reproductive technology, our understanding of human fertility and reproduction has grown immeasurably. IVF treatment provides a unique opportunity to investigate aspects of reproduction that otherwise would not be possible through natural procreation, in particular, ovarian function, fertilisation and embryogenesis. However, significant gaps remain in the literature on the effect of lifestyle factors on reproduction, fertility and the clinical outcomes of IVF.

Cigarette smoking is the lifestyle factor that has been the most studied in regard to the clinical outcomes of IVF treatment. Most studies examined only the effect of female smoking, with no regard to the smoking habit of the male partner. Only a few of these studies included caffeine and alcohol consumption in the investigation. Stress is the second most studied lifestyle factor, of which there exists only a handful of studies on the its effect on IVF clinical outcomes. The majority of these lifestyle studies had flawed methodology (Hughes & Brennan 1996; Klonoff-Cohen et al. 2001), and the findings to date lack consistency; thus, limiting their application to clinical practice.

For ethics reasons, the effect of tobacco consumption in humans can only be examined in observational, epidemiological studies. It is well established that smokers and non-smokers differ significantly in terms of demographic, socioeconomic and other lifestyle factors. Given this and the fact that such factors cannot be controlled for by the application of randomisation in an experimental design, it is paramount that such potentially confounding differences between smokers and non-smokers are controlled by the use of multivariate statistical methods of data analysis. It is also imperative that attention to detail is paid to the collection of data on lifestyle factors. All of these issues have been adhered to in this study.

1.1 Aim and Objectives

The overall aim of the present study is to gain a better understanding of the impact of lifestyle factors on the clinical outcomes following in IVF treatment.

The specific objectives are as follows:

- To determine the effect of female cigarette smoking, caffeine and alcohol consumption, stress and dietary indicators on the number of oocytes retrieved.

- To determine the effect of female and male cigarette smoking, caffeine and alcohol consumption, stress and indicators of dietary status on the rates of fertilisation *in vitro*.

- To determine the effect of female and male cigarette smoking, caffeine and alcohol consumption, stress and indicators of dietary status on β-hCG pregnancy outcome at 16 days post-embryo transfer (ET).

- To determine the effect of female and male cigarette smoking, caffeine and alcohol consumption, stress and indicators of dietary status on <12 week pregnancy loss.

1.2 Benefits of the Study

The findings from former studies that investigated the effect of lifestyle factors on clinical outcomes of IVF treatment have lacked consistency, and in some instances, the direction of effect is conflicting. In part, this appears to be due to poor methodology and simplistic statistical techniques. By comparison, this observational, epidemiological study has sound methodology and statistical techniques; potential bias and confounding is minimal. Information gained from this study will contribute toward an understanding of how the clinical outcomes of IVF treatment are affected by lifestyle factors.

In terms of clinical practice, information regarding the effect of specific lifestyle factors, which at present is lacking, is highly sought after by most patients

undertaking IVF treatment. The majority of patients are at least willing to modify their lifestyle habits to optimise the success of having a healthy baby after IVF treatment. This study has the potential to generate reliable information about the effect of both female and male lifestyle factors on the clinical outcomes of IVF. This information can be used by clinicians, nurses and embryologists in advising patients about lifestyle behaviours that optimise their likelihood of a successful outcome. Patients and clinicians welcome any improvement in the likelihood of a pregnancy, as treatment failure for a given IVF treatment cycle is still relatively high.

The clinical practice of IVF treatment provides a unique opportunity to investigate human reproductive process that otherwise would not be possible through natural conception. The findings of this study will contribute to the current and broader understanding of infertility and reproduction. As the focus of the present study is lifestyle, any significant findings could contribute to the wider knowledge base used in health promotion and prevention programmes. When targeting young adults, prevention programmes may be more effective if the focus is on fertility and reproductive issues. Unlike chronic diseases, such as cardiovascular disease and cancers, which are associated with (relative) old age, concerns of virility, fertility and reproduction are more relevant to younger adults.

1.3 Limitations of the Study

- Total caffeine intake was calculated from multiple sources including coffee, tea and kola beverages. Specific caffeine products were not included as separate independent variables in the data analysis.

- Alcohol consumption was self-reported as number of standard alcoholic beverages. There was no provision for the discrimination of specific types of alcoholic beverages, e.g. beer, wine or spirits.

- Exercise and (paid and unpaid) working hours were not included as lifestyle variables in the data analysis.

- Data were acquired on medications (other than prescribed for IVF treatment) and 'over-the-counter' medications, which included mineral and vitamin supplements. Only vitamin E supplementation by males was included in the data analysis and that being only in relation to fertilisation. This was because it was the antioxidant that was the focus of attention at the time of data analysis. It was beyond the study objectives to include specific vitamins and minerals supplements as independent variables in the data analysis.

- Due to the clinical nature of IVF treatment, couples 'dropped-out' of the study as they proceed through the treatment. Coupled with a pregnancy rate of 31.3%, the sample of couples examined in terms of pregnancy loss was smaller than desired.

- In order to be seen as engaging in 'socially desirable' behaviour, patients may have under-reported negative and over-reported positive lifestyle factors.

- The temporal relationship between lifestyle and pregnancy outcomes is questionable among the female patients. Specifically, whether lifestyle during the first week of treatment reflects that post-ET, and then following confirmation of a pregnancy.

- All the subjects in this study were patients who attend PIVET Medical Centre in Perth, Western Australia. Of note in this sample is the variability in ethnicity. The extent to which one can extrapolate the findings from this sample of IVF couples to that of other IVF populations is noteworthy of consideration.

1.4 Outline of the Thesis

Chapter 2 is a detailed review of the relevant literature to date. This chapter is sectioned by clinical outcomes, and the order reflects the sequential nature of IVF treatment: oocyte production, fertilisation *in vitro*, pregnancy and pregnancy loss. Of the lifestyle factors, cigarette smoking has received the most attention, and is reviewed in detailed in each section. Within each section, the findings of epidemiological studies are first presented. Thereafter, relevant non-epidemiological studies are reviewed.

Chapter 3 is an expanded account of the materials used and methods undertaken in this study. Because of its reliance on self-reported data, the manner in which the lifestyle data is quantified into weekly measures is detailed in depth. The techniques used to enhance patient recruitment and participation are also described, as is patient follow-up. Validation of self-reported smoking data using cotinine analyses in a sub-sample of the study sample is reported at the end of this chapter.

Chapter 4 reports the results of this study. Rates of patient participation are presented in the first of the seven sections. The second section provides a description of the study sample by patient, treatment and lifestyle variables that are not reported in later sections. Like that of Chapter 2, this chapter is sectioned by clinical outcomes: oocyte production, fertilisation *in vitro*, pregnancy and pregnancy loss. The findings in regard to the respective clinical outcome are presented in a uniform manner: 1) descriptive statistics, 2) univariate data analysis, 3) a detailed step-by-step account of the hierarchical multiple regression method of data analysis, and 4) interpretation of the final regression model. There is an additional section on basal follicle stimulating hormone (FSH) levels, which is an indirect measure of ovarian reserve.

In Chapter 5, the findings of this study are discussed in relation to that of earlier studies. Reasons for inconsistencies and conflicting findings between studies are suggested. For the most part, this chapter focuses on the biological plausibility of the observed findings. Finally, conclusions are drawn and implications of the study discussed.

Chapter 2

Review of the Literature

More than 3000 different compounds have been identified in the smoke of cigarettes (Bos & Henderson 1984). At least 40 of these are carcinogenic and mutagenic to humans (International Agency for Research on Cancer 1986 cited in Zenzes 2000). Therefore, it is not surprising that cigarette smoking is the lifestyle factor most studied in regard to the clinical outcomes of IVF treatment. The majority of studies to date have focused on the female patient, especially in terms of oocyte production and pregnancy. Numerous of studies have been published on male smoking and sperm quality, but little research has been conducted on the effect of smoking on rates of fertilisation *in vitro*. Only a few studies have simultaneously examined caffeine and alcohol consumption in their investigation of smoking. Stress is the second most studied lifestyle factor in regard to IVF treatment, yet there are fewer studies involving males than females. The effect of diet on IVF treatment has scarcely been studied. There remains a definite need for well designed, prospective studies in this context to 1) clarify findings from previous studies on cigarette smoking and stress, and 2) examine other lifestyle factors that have received little attention to date.

Given the dominance of studies on the effect of cigarette smoking, a comprehensive search of the Medline electronic database was undertaken. Studies were selected for review if they fulfilled the following criteria: 1) presented original data from a sample of patients whom had undertaken IVF treatment, 2) examined smoking as the principal factor (independent variable), 3) included oocyte production, fertilisation, pregnancy or pregnancy loss as a clinical outcome of interest (dependent variable), and 4) were published in English. Twenty-two studies were identified and are detailed in Table 2.1. Appendix A is a systematic critique of these 22 studies; presented in chronological order by year of publication, each study is reviewed in terms of the study design, sample source and demographics, measure of smoking exposure and clinical outcomes. The findings of these studies are discussed in the subsequent sections on oocyte production, fertilisation, pregnancy and pregnancy loss, accordingly.

Table 2.1: Studies on the Effect of Smoking on the Clinical Outcomes of IVF Treatment

authors	year	period -ical	country	study design	data	selection criteria	exclusion criteria	sample size	smoking status	clinical outcomes	co-variates
Trapp, Kemeter & Feichtinger	1986	HR	Austria	a	C	<40 yrs	-	114 females	76 non-smoke (67%) 38 smoke (33%)	mean no. oocytes mean no. fert oocytes mean fert rate β-hCG pregnancy	nil
Harrison, Breen & Hennessey	1990	ANZ JOG	Aust-ralia	a	C	<40 yrs	smokers with unstable smoking habit[1]	650 females	542 non-smoke (83%) 72 1-10 cig/day (11%) 31 11-20 cig/day (5%) 5 21-30 cig/day (1%)	mean no. oocytes % oocytes fertilised clinical pregnancy clinical preg loss	nil
Elenbogen et al.	1991	HR	Israel	b	C	<37 yrs; tubal infert; NOC; NSS	-	41 females	21 non-smoke (51%) 20 >15 cig/day (49%)	mean no. oocytes % oocytes fertilised pregnancy (nd)	nil
Pattinson, Taylor & Pattinson	1991	F&S	Canada	c	C	first TC	-	360 females	236 non-smoke (66%) 17 <10 cig/day (5%) 46 10-20 cig/day (13%) 62 >20 cig/day (17%)	mean no. oocytes mean fert rate pregnancy (nd) clinical preg loss	nil
Van Voorhis et al.	1992	F&S	USA	b	C	-	-	54 females	(2:1 ratio) 36 non-smoke 18 smoke	mean no. oocytes % oocytes fertilised clinical pregnancy	age; weight (matched design)
Rosevear et al.	1992	Lancet	UK	c	A	<40 yrs; NOC; RCM; NNS	-	45 females 161 oocytes	13 NCFF (29%) 32 COFF (71%) 116 NCFF (72%) 45 COFF (28%)	prop fert oocytes mdn fert rate	nil

Table 2.1: Studies on the Effect of Smoking on the Clinical Outcomes of IVF Treatment (cont.)

authors	year	period -ical	country	study design	data	selection criteria	exclusion criteria	sample size	smoking status	clinical outcomes	co-variates
Rowlands, McDermott & Hull	1992	Lancet	UK	b	C	<40 yrs; NOC; NNS	-	71 couples	30 non-smoke (42%) 15 female only (21%) 13 male only (18%) 13 both smoke (18%)	median no. oocytes % oocytes fertilised clinical pregnancy	nil
Hughes et al.	1994	F&S	Canada	a	B	-	-	315 females	119 never smoke (38%) 101 ex-smoke (32%) 61 <15 cig/day (19%) 35 >15 cig/day (11%)	mean no. oocytes mean fert rate clinical pregnancy clinical preg loss	female age; infertility aetiology; infertility duration;
								462 female cycles	182 never smoke cycles 165 ex-smoke cycles 115 smoke cycles		female caffeine; male smoking
Sharara et al.	1994	F&S	USA	a	C	35-39 yrs; tubal infert; no DOR	ex-smokers; ovarian surgery	102 females	73 non-smoke (72%) 29 smoke (28%)	mean no. oocytes mean fert rate pregnancy (nd)	nil
Maximovich & Beyler	1995	JARG	USA	c	C	-	-	253 females	210 non-smoke (83%) 43 smoke (17%)	β-hCG pregnancy ongoing pregnancy β-hCG preg loss	nil
Zenzes, Wang & Casper	1995	HR	Canada	a	C	-	-	156 females	102 non-smoke (65%) 21 passive smoke (13%) 19 <15 cig/day (12%) 14 >15 cig/day (9%)	mean no. oocytes % oocytes fertilised	nil
								286 oocytes	197 non-smoke (69%) 23 passive smoke (8%) 33 light smoke (12%) 33 heavy smoke (12%)		

Table 2.1: Studies on the Effect of Smoking on the Clinical Outcomes of IVF Treatment (cont.)

authors	year	period -ical	country	study design	data	selection criteria	exclusion criteria	sample size	smoking status	clinical outcomes	covariates
Gustafson, Nylund & Carlström	1996	AOG Scand	Sweden	b	C	NOC; NSS	-	100 females	50 non-smoke 50 smoke >10 cig/day	median no. oocytes median fert rate clinical pregnancy	nil
Sterzik et al.	1996	F&S	Germany	a	A	first TC; tubal infert; NOC	-	197 females	68 non-smoke (34%) 26 passive smoke (13%) 103 smoke (52%)	mean no. oocytes % oocytes fertilised clinical pregnancy	nil
Van Voorhis et al.	1996	O&G	USA	d	C	first TC	-	499 females	351 non-smoke (70%) 111 ex-smoke (22%) 37 smoke (7%)	mean no. oocytes mean fert rate clinical pregnancy ongoing pregnancy	age
Zenzes, Reed & Casper	1997	HR	Canada	a	C	one TC per couple	-	234 females	130 non-smoke (56%) 30 passive smoke (13%) 74 smoke (32%)	total no. oocytes % oocytes fertilised individual % oocytes fertilised	age
Feichtinger et al.	1997	JARG	Austria	c	C	first TC	-	541 females	399 non-smoke (74%) 142 smoke (26%)	clinical pregnancy	nil
Joesbury et al.	1998	HR	Aust-ralia	c	C	-	donor cycles	465 female cycles	391 non-smoke (84%) 74 smoke (16%)	mean no. oocytes mean fert rate β-hCG pregnancy	
								422 male cycles	321 non-smoke (80%) 101 smoke (20%)	12 week pregnancy β-hCG preg loss	female age; male age; infert aetiology; ICSI
								416 couple cycles	287 non-smoke (69%) 30 female only (7%) 59 male only (14%) 39 both smoke (9%)		

Table 2.1: Studies on the Effect of Smoking on the Clinical Outcomes of IVF Treatment (cont.)

authors	year	period -ical	country	study design	data	selection criteria	exclusion criteria	sample size	smoking status	clinical outcomes	covariates
El-Nemr et al.	1998	HR	England	c	C	non-ICSI	ICSI cycles	173 female cycles	108 non-smoke (62%) 65 smoke (38%)	mean no. oocytes % failed fertilisation clinical pregnancy	age (by stratification)
Weigert et al.	1999	JARG	Austria	c	C	-	-	834 females	634 non-smoke (76%) 200 smoke (24%)	mean no. oocytes mean no. fert oocytes pregnancy (nd)	nil
Klonoff-Cohen et al.	2001	HR	USA	a	C	aged 20+ years; first cycle during study time frame	pre-existing illness[2]; donor or surrogate cycles	221 females 166 males couples	111 never smoked (50%) 104 ever smoked (47%) 6 unknown (3%) 70 never smoked (42%) 70 ever smoked (42%) 26 unknown (15%) ? never smoked (22%) ? ever smoked (62%) ? unknown (16%)	no. oocytes clinical pregnancy	female age; education; IVF or GIFT; no. of previous attempts; parity; female alcohol, marijuana & recreation drugs
Crha et al.	2001	CEJPH	Czech Repub	a	A	-	-	159 females	90 non-smokers (57%) 29 ex-smokers (18%) 16 occasional smokers; 0-1 cig/day (10%) 24 smokers; 2+ cig/day (15%)	% oocytes fr follicles mean no. oocytes mean fert rate % failed fertilisation % ≥60% oocytes fert. pregnancy (nd)	
Paszkowski, Clarke & Hornstein	2002	HR	USA	a	A	<40 years; tubal or male infert	-	108 females	72 non-smokers (66%) 21 passive smokers (19%) 15 active smokers (14%)	median no. oocytes	-

AOB Scand = Acta Obstetricia et Gynecologica Scandinavica

HR = Human Reproduction

F&S = Fertility & Sterility

ANZJOG = Australian & New Zealand Journal of Obstetrics & Gynaecology

JARG = Journal of Assisted Reproduction & Genetics

CEJPH = Central European Journal of Public Health

a = prospective cohort, with clearly defined patient groups at inception and data collection in a prospective fashion (Hughes & Brennan 1996).

b = prospective cohort by retrospective data review/analysis with smoking status ascertained before clinical outcomes and subject selection based on smoking status

c = prospective cohort by retrospective data review/analysis with smoking status ascertained before clinical outcomes

d = retrospective cohort with smoking status ascertained post-clinical outcomes

NOC = normal ovulatory cycle

RCM = receptive cervical mucus

TC = treatment cycle

NSS = normal spermatozoa sample

DOR = diminished ovarian reserve

cig/day = number of cigarettes per day

NCFF = non-detectable cotinine levels in follicular fluid

COFF = detectable cotinine levels in follicular fluid

fert = fertilised

infert = infertility

nd = not defined by author(s)

mdn = median

preg = pregnancy

prop = proportion

ever smoked = had smoked in their lifetime (past or present)

A = biochemical cotinine levels in bodily fluid samples

B = self-reported smoking status with biochemical validation

C = self-reported smoking status without biochemical validation

? = not reported by author(s)

[1] one month preceding treatment and throughout ovulation induction

[2] high blood pressure, heart disease, diabetes thyroid or renal disease

2.1 Oocyte Production

2.1.1 Cigarette Smoking

2.1.1.1 Epidemiological IVF Studies

Nineteen of the 22 studies in Table 2.1 examined female smoking and oocyte production. The findings of these 19 studies are detailed in Table 2.2. The majority compared smokers and non-smokers by the average number of oocytes retrieved: 17 of 19 studies. Excluding the study by Sharara et al. (1994), as the women in this study were selected based on evidence of a normal ovarian reserve, 13 studies reported the mean value and three the median. Only four of these 16 studies reported a significantly lower yield of oocytes from smokers (Crha et al. 2001; El-Nemr et al. 1998; Paszkowski, Clarke & Hornstein 2002; Van Voorhis et al. 1992). Of particular relevance, however, three studies found smokers to be significantly younger (Van Voorhis et al. 1996; Joesbury et al. 1998; Weigert et al. 1999). It is well established that oocyte production decreases with age. Detection of a smoking-related compromise in oocyte production could be negated if smokers are, on average, younger. In fact, in not one of the three studies in which smokers were significantly younger was a reduction in oocyte production shown.

Despite the significance of age, it was only taken into account by two studies that examined smoking and oocyte production (Van Voorhis et al. 1996; Zenzes, Reed & Casper 1997). Having found smokers to be significantly younger than past and never smokers (P=0.004), Van Voorhis et al. (1996) used multiple linear regression to control for age. A *significant* age-adjusted association between the number of oocytes retrieved and cigarette pack-years was reported (P-value not quoted). Specifically, 2.5 fewer mature oocytes were retrieved for every 10 cigarette-pack years. By contrast, Zenzes, Reed and Casper (1997) showed no age-adjusted correlation between log follicular fluid cotinine values and the number of retrieved oocytes (P<0.05). Cotinine is a metabolite of the nicotine alkaloid, and is considered cigarette smoke-specific (Benowitz et al. 1983).

Table 2.2: Average Number of Oocytes Retrieved by Female Smoking Status

author	age restriction	smoke categories (n)	age mean (SD)	*P*	no. of oocytes retrieved mean (SD)	*P*
Trapp, Kemeter & Feichtinger 1986	<40yrs	non-smoker (76) smoker (38)	34.2 (8.6) 33.8 (4.4)	-	4.3 (3.2) 3.6 (2.4)	>0.05
Harrison, Breen & Hennessey 1990	<40yrs	non-smoker (542) 1-10 cig/day (72) 11-20 cig/day (31) 21-30 cig/day (5)	? ? ? ?	-	5.1 (-) 5.4 (-) 4.4 (-) 3.8 (-)	>0.05
Elenbogen et al. 1991	<37yrs	non-smoker (21) smoker >15cig/day (20)	32.6 (1.6) 33.5 (1.8)	-	6.8 (1.4) 6.2 (1.5)	>0.05
Pattinson, Taylor & Pattinson 1991	-	non-smoker (236) smokers (124)	32.6 (2.9) 33.1 (3.8)	>0.05	5.4 (3.2) 5.1 (2.6)	>0.05
Van Voorhis et al. 1992	-	non-smokers (36) smokers (18)	32.3 (?) 32.6 (?)	>0.05	16.4 (7.8) 13.4 (4.7)	**<0.05**
Sharara et al. 1994	35-39yrs[1]	non-smoker (73) smoker (29)	37.2 (1.3) 37.8 (1.1)	>0.05	8.6 (4.7) 8.4 (4.2)	>0.05
Hughes et al. 1994	-	never smoke (119) ex-smoke (101) smoke <15cig/day (61) smoke >15cig/day (35)	34.3 (2.9) 33.5 (3.3) 34.3 (4.2) 33.5 (3.3)	>0.05	5.8 (3.7) 7.0 (4.8) 6.6 (3.8)[2]	>0.05
Zenzes, Wang & Casper 1995	-	non-smoker (102) passive smoker (21) light smoker (19) heavy smoker (14)	? ? ? ?	>0.05	10.0 (6.8) 9.5 (3.5) 8.0 (3.7) 7.3 (3.8)	>0.05
Sterzik et al. 1996	<40yrs	non-smoker (68) passive smoke (26) smoker (103)	32.5 (4.1) 32.7 (4.4) 32.4 (4.3)	>0.05	5.4 (3.3) 5.4 (2.9) 5.5 (2.7)	>0.05
Van Voorhis et al. 1996	-	non-smoker (351) ex-smoker (111) smoker (37)	32.9 (4.2) 33.8 (3.6) 31.5 (3.6)	**<0.05**	15.4 (9.0) 15.1 (9.7) 14.0 (6.2)	>0.05

Table 2.2: Average Number of Oocytes Retrieved by Female Smoking Status (cont.)

author	age restriction	smoke categories (n)	age mean (SD)	*P*	no. of oocytes retrieved mean (SD)	*P*
Joesbury et al. 1998	-	non-smoker (419) smoker (76)	34.6 (4.7) 33.1 (4.7)	**<0.05**	12.2 (6.8) 13.1 (7.7)	>0.05
El-Nemr et al. 1998	-	non-smoker (108) smoker (65)	33.2 (4.5) 32.3 (4.5)	>0.05	11.1 (6.3) 6.2 (3.4)	**<0.0001**
Weigert et al. 1999	-	non-smoker (634) smoker (200)	34.2 (5.3) 33.3 (4.9)	**0.0195**	7.0 (4.1) 6.4 (3.7)	>0.05
Crha et al. 2001	-	non-smokers (n=?) smokers (n=?)	? ?	>0.05	10.9 (5.6) 7.3 (4.8)	**<0.05**

author	age restriction	smoke categories (n)	age median (range)	*P*	no. of oocytes retrieved median (range)	*P*
Rowlands, McDermott & Hull 1992	<40yrs	both non-smokers (30) female smoke only (15) male smoke only (13) both smokers (13)	mean 31.8 (-) mean 30.6 (-) mean 33.3 (-) mean 31.3 (-)	-	8 (2-24) 14 (6-26) 6 (1-11) 10 (6-29)	>0.05
Gustafson, Nylund & Carlström 1996	-	non-smoker (50) smoker (50)	34 (25-37) 33 (25-37)	-	5 (2-28) 6 (2-15)	>0.05
Paszkowski. Clarke & Hornstein 2002	<40 yrs	non-smoker (72) passive smoker (21) active smoker (15)	36 (25-39) 35 (26-38) 37 (24-39)	>0.05	16 (7-38) 12 (2-52) 8 (2-27)	**0.010**

[1] normal ovarian reserve
[2] n=96 all smokers (smokers <15cig/day and >15cig/day)
? not reported by author(s)

In a recent study, Crha et al. (2001) used a novel measure of oocyte production: the percentage of ovarian follicles >15mm that elicit an oocyte. Mean percentages were presented by smoking category and stratified by age, ≤35 and >35 years. Among women >35 years, the mean percentage of follicles that failed to produce an oocyte was significantly higher than non-smokers and ex-smokers (specific values not quoted; $P<0.05$). By contrast, no differences were noted between smoking groups for women ≤35 years of age.

2.1.1.2 Ovarian Reserve

The fundamental factor in aging ovaries is depletion of primordial follicles (Lass 2001). Having accrued in number up to as many as seven million, oogonia cease cell division at around week 20 of fetal life (Fauser 2000). At this point, non-proliferating flat cells surround each oogonia and form the resting or primordial follicle (Canipari 2000). This pool of primordial follicles is termed the ovarian reserve. From birth to menopause, depletion of the ovarian reserve occurs at a logarithmic rate (Lass 2001), so as only 250,000 to 500,000 remain present at menarche. There is a notable increase in the depletion rate at approximately the age of 37 years, and few follicles exist at the onset of menopause (Fauser 2000; Lass 2001; Westhoff, Murphy & Heller 2000). However, there is considerable variability in the depletion rate of primordial follicles among women (Lass 2001). The ovarian reserve is measured by the following indirect methods: basal serum FSH concentrations, the gonadotrophin releasing hormone (GnRH) agonist stimulation test and the clomiphene citrate challenge test (CCCT)(Scott & Hofmann 1995).

The status of the ovarian reserve is a strong determinant of the number of oocytes retrieved in IVF treatment. In this regard is the noteworthy study undertaken by Sharara et al. (1994), which endeavoured to unravel the triad relationship between ovarian reserve, oocyte production and smoking. Smokers with normal ovarian reserve aged 35-39 years were compared to non-smokers also with normal ovarian reserve of the same age range. The mean number of oocytes retrieved from these two groups was not significantly different, 8.4 (standard deviation (SD) 4.2) versus (vs) 8.6 (SD 4.7; $P>0.05$). Sharara et al. (1994) concluded that cigarette smoking *per se* does not affect the number of oocytes retrieved; rather that the adverse effect of

smoking on oocyte production is only likely to become evident when the ovarian reserve shows age-related signs of depletion.

A number of studies excluded female patients of advanced reproductive age from their investigation of smoking and oocyte production (Elenbogen et al. 1991; Harrison, Breen & Hennessey 1990; Sterzik et al. 1996; Trapp, Kemeter & Feichtinger 1986). None of these four studies reported a significant difference in oocyte production between smokers and non-smokers. If, as suggested by Sharara et al. (1994), the adverse effect of smoking on oocyte production only becomes evident when the ovarian reserve shows signs of depletion, restricting women of advance reproductive age from inclusion in such studies may negate the detection of a smoking-related effect.

In a previous study by the Investigator, which involved a different sample of IVF couples (n=498) to that of the current study, the relationship between oocyte production, age and smoking status was investigated (see Joesbury et al. 1998 in Appendix B). As expected, oocyte production decreased with female age (Pearson's correlation coefficient (r) =-0.42; $P<0.01$); however, smokers showed an accelerated rate of oocyte depletion with age ($P<0.05$). On average, the number of oocytes retrieved from smokers decreased by 0.75 (95% (confidence interval (CI) 0.040 - 1.10) per one year increase in age as compared to 0.55 (95% CI 0.42 - 0.68) for non-smokers. Of note, the correlation between oocyte production and age was moderate, at best (r=-0.42). This confirms the role of factors other than age at play in regard to oocyte production. Based on the evidence to date, the effect of cigarette smoking on ovarian reserve is strongly implicated as one such factor.

The adverse effect of female smoking on ovarian reserve is believed to be irreversible upon cessation of the habit, and further evidence stems from laboratory-based studies. Recently, Westhoff, Murphy and Heller (2000) undertook a study to identify factors associated with ovarian follicle density in women undergoing hysterectomy who had morphologically normal ovaries. Age, parity, oral contraception, body size, cigarette smoking and alcohol consumption were examined in this regard. The mean number of follicles counted per slide of ovarian tissue sectioned from each patient

was used as the measure of follicle density. The expected decrease in follicle density with age was observed (P=0.001). Adjusting for age by stratifying women into two strata, 35-44 (sample size (n)=20) and 45-54 (n=65) years, smoking status was shown to be a significant factor in relation to follicle density. Moreover, both current and ex-smokers within each age strata had follicle counts that were less than half that of never smokers (P=0.04); a finding consistent with the suspected cigarette smoke-induced irreversible and accelerated depletion of ovarian follicles (Westhoff, Murphy & Heller 2000).

2.1.1.3 Ovarian Follicle Steroidogenesis

Chemical compounds specific to cigarette smoke have been detected in ovarian follicular fluid. This demonstrates that oocytes are exposed to at least some of the chemicals found in cigarette smoke, as well as the support cells of the ovarian follicle, which play a vital role in oocyte maturation and growth (Zenzes et al. 1996). A number of studies have detected cotinine in the follicular fluid of female smokers (Rosevear et al. 1992; Sterzik et al. 1996; Weiss & Eckert 1989; Zenzes, et al. 1996). Zenzes (2000) stated that exposure to the toxic compounds within cigarette smoke presents a potential threat to the viability of oocytes and the functionality of ovarian follicular cells.

Rhodanide and cadmium have also been detected in human follicular fluid. The former, a metabolite of cyanide, has been measured in the follicular fluid of both smokers and non-smokers. However, it was not unexpected for concentrations to be significantly higher in smokers (P<0.05), as cigarette smoke is the dominant source of cyanide levels found *in vivo* (Trapp, Kemeter & Feichtinger 1986). Zenzes et al. (1995) showed smokers to have significantly higher follicular fluid concentrations of the heavy metal, cadmium, than non-smokers (P=0.0012). Yet, no such difference between smokers and non-smokers was replicated in a similar study by Crha et al. (2001).

In a laboratory-based, experimental-design study, Barbieri, McShane and Ryan (1986) examined the effect of cigarette smoke on ovarian steroidogenesis, specifically its effect on aromatisation, the conversion of androgens to oestrogens.

Human granulosa cells cultured *in vitro* were exposed to aqueous extracts of cigarette smoke. In a dose-dependent manner, inhibition of aromatisation was observed. No concomitant reduction in progesterone production was evident, nor was there a decrease in the number of granulosa cells. Oestrogen production resumed when the aqueous extract of cigarette smoke was removed from the culture media. Barbieri, McShane and Ryan (1986) concluded that constituents of cigarette smoke inhibit granulosa cell aromatisation, and in turn, suppress oestrogen production.

In a further study by Barbieri and colleagues, oestrogen production by choriocarcinoma cells and term placental microsomes cultured *in vitro* was examined (Barbieri, Gochberg & Ryan 1986). Aqueous extracts of cigarette smoke suppressed the conversion of androstenediol to oestradiol in a dose-dependent manner, with no accompanying alteration in progesterone production or culture cell numbers. Normal rates of aromatisation were observed when the cigarette smoke extract was removed from the culture media. The addition of nicotine and other alkaloids (cotinine and anabasine; the latter, a minor metabolite of nicotine) were also shown to inhibit aromatisation. Again, aromatisation resumed upon withdrawal of these alkaloids. Barbieri, Gochberg and Ryan (1986) concluded that nicotine directly inhibits the aromatase enzyme system, and that other nicotinic alkaloids present in cigarette smoke may act in an additive fashion.

The effects of nicotine, cotinine, anabasine and cigarette smoke extract on progesterone and oestradiol production by *in vitro* cultures of human granulosa cells were examined in a more recent study (Gocze, Szabo & Freeman 1999). Cellular deoxyribonucleic acid (DNA) was also measured, expressed per μg of cell DNA. Progesterone increased marginally when exposed to nicotine (1.23-fold), but decreased by 34%, 61% and 54% on exposure to cotinine, all three alkaloids and smoke extract, respectively. Oestradiol production increased on exposure to cotinine and anabasine, but no change was evident when exposed to nicotine. Of significance, however, overall DNA content decreased in the cells exposed to nicotine, cotinine, anabasine, all three alkaloids and crude smoke extract by 8%, 36%, 27%, 66% and 65%, respectively. It was concluded that alkaloids in cigarette smoke inhibit

progesterone synthesis largely as a result of impaired growth or destruction of the granulosa cells (Gocze, Szabo & Freeman 1999).

The effect of cadmium on morphology and steroidogenesis of human granulosa cells cultured *in vitro* was investigated by Paksy et al. (1997). Progesterone production was shown to decrease with increasing concentrations of cadmium. However, the lowest cadmium concentration at which progesterone suppression became evident was 16μM, which is 3.5 times higher than that reported in follicular fluid of smokers. FSH offered marginal protection against the cadmium-induced suppression of progesterone production; an effect that only became evident at the highest level of cadmium exposure, 64μM.

Paksy et al. (1997) observed morphological changes to the cell-cell junctions and cytoplasmic extensions of granulosa cells following cadmium exposure. Gap junctions link adjacent granulosa cells and provide connections between the oocyte and the granulosa cells of the follicle. They are essential for oocyte growth through the transfer of metabolic precursors or small regulatory molecules (Canipari 2000). Gap junctions are also present between the oocyte and cumulus cells, and play a beneficial role in fertilisation (Canipari 2000).

Collectively, the findings from non-IVF, epidemiological studies support the conviction that cigarette smoking alters steroid production. It has been shown that in pre-menopausal women, luteal-phase urinary oestrogens levels are lower (MacMahon et al. 1982) and androstenedione levels higher in smokers than non-smokers (Michnovicz et al. 1986 cited in Vermeulen 1993). Lower oestrogen levels have also been reported in pregnant women who smoke as compared to non-smokers (Mochizuki et al. 1984 cited in Barbieri, Gochberg & Ryan 1986). Furthermore, female smokers and non-smokers differ in relation to oestrogen-associated diseases and reproductive events. For example, smokers tend to enter menopause at an earlier age (Jick, Porter & Morrison 1977; Midgette & Baron 1990). Whilst the risk of post-menopausal osteoporosis is higher among smokers (Daniell 1976 and Williams et al. 1982 cited in Barbieri, Gochberg & Ryan 1986), the risk of endometrial cancer (Lesko et al. 1985 cited in Barbieri, Gochberg & Ryan 1986) and endometrosis is

lower (Cramer et al. 1986). The hypothesis that cigarette smoking suppresses oestrogen production is supported by these differing rates of oestrogen-related conditions between female smokers and non-smokers.

2.1.1.4 Chromosomal Abnormalities

During gametogenesis, errors in chromosome distribution (non-disjunction) result in the production of gametes that carry extra or missing chromosomes. These numerical chromosomal abnormalities are referred to as aneuploidies (Zenzes, Wang & Casper 1992). Aneuploidy occurs at a frequency of about 0.3% among livebirths, 5% among stillbirths and 25% among spontaneous abortions (Hassold & Jacobs 1984 cited in Zenzes, Wang & Casper 1992). Zenzes et al. (1997) showed that the occurrence of diploid (46) instead of haploid chromosomal complements is elevated in the oocytes from female smokers ($P<0.001$). From observations of unfertilised oocytes, diploid oocytes are believed to result from failed extrusion of the first polar body chromosomes or oocytes arrested at syngamy after sperm entry (Edirisinghe, Murch & Yovich 1992). Smoking appears to interfere with the meiotic spindles by impairing their functional role of pulling the chromosomes from the equator to the poles (Zenzes 2000). Alkaloids are known to bind to tubulin, the protein comprising the tubules of meiotic spindles (Zenzes 2000). It is in this manner that Zenzes (2000) suggested that cigarette smoking may disrupt meiotic spindle function and cause chromosomal aneuploidy.

2.1.1.5 Oxidative Stress

Attention of late has focused on oxidative stress that results from the destructive activity of free radicals. Most molecules are non-radicals because they contain only paired electrons in their orbits and, therefore, are relatively stable. Free radicals are unstable molecules because they have one or more unpaired electrons and, as a consequence, react with other molecules. Free radicals react with non-radicals usually by theft of a hydrogen (H·) atom. This sets off a chain of reactions, whereby one radical begets another, that terminates only when two free radicals combine (Halliwell 1994; Halliwell, Gutteridge & Cross 1992). Non-radical derivatives of oxygen also have the potential to react (e.g., hydrogen peroxide (H_2O_2)). These metabolites and free radicals are collectively termed reactive oxygen species (ROS).

ROS are normal by-products of cellular metabolism, but are detrimental when present in excess (Bedaiwy et al. 2002). Superoxide ($O_2^{-}\cdot$), hydroxyl (OH·) and H_2O_2 have been implicated as the major contributors to oxidative stress in gametes and embryos (Guérin, El Mouatassim & Ménézo 2001). However, ROS vary in terms of their reactivity with other molecules. OH· is highly reactive because it has a short half-life of ≈0.3ns and, therefore, combines with any molecule at or very close to its site of formation. Consequently, OH· generated in close proximity to vital cellular components can cause serious, even lethal, damage (Lenzi et al. 1996). To be effective against highly reactive ROS such as OH·, defense mechanisms must act promptly and be present at the site of ROS generation (Guérin, El Mouatassim & Ménézo 2001). In contrast to OH·, $O_2^{-}\cdot$ has limited reactivity. Although H_2O_2, can readily permeate plasma membranes almost as freely as water and is cytotoxic, its oxidative capacity is relatively weak. Of significance, however, the highly reactive and lethal OH· is formed when H_2O_2 combines with $O_2^{-}\cdot$ or with transitional metals (e.g., manganese, copper, iron, cobalt and copper). Cellular damaged caused by ROS is that referred to as oxidative stress, and is often due to over-production or inadequate clearance of ROS.

Defenses systems protect against ROS and their oxidative damage by prevention of formation, interception and repair (Guérin, El Mouatassim & Ménézo 2001). The degree of cellular dysfunction and damage depends on the nature and extent of the ROS-induced oxidative stress (Halliwell, Gutteridge & Cross 1992). Deleterious effects of oxidative stress include DNA strand breaks, protein damage and elevated levels of intracellular concentrations of calcium. Within the ovarian follicle, both enzymatic and non-enzymatic defense systems exist. Glutathione appears to be the main non-enzymatic antioxidant, and is also the substrate of glutathione peroxidase (GPX), the main intracellular antioxidant enzyme (Guérin, El Mouatassim & Ménézo 2001). Other non-enzymatic antioxidants include dietary-derived vitamin A, vitamin C (ascorbic acid or L-ascorbate) and vitamin E (Guérin, El Mouatassim & Ménézo 2001).

Tobacco smoke is a major source of toxins, including free radicals (Frei et al. 1991). According to Halliwell (1994), toxins fall into four categories based on the manner in which they impose oxidative stress: the toxin 1) itself is a free radical, 2) when metabolised to a free radical, 3) generates oxygen-free radicals when metabolised, and 4) depletes antioxidant defenses. The relationship between smoking and antioxidants was investigated by Paszkowski and Clarke (1999). Vitamin C was measured in the follicular fluid of 65 women: 25 non-smokers (follicular fluid cotinine (FF-C) ≤20ng/ml), 22 passive smokers (FF-C 20-50ng/ml) and smokers (FF-C >50ng/ml). Median L-ascorbate levels (μM) were 107.9 (range 34.0-356.0), 96.2 (40.1-149.0) and 87.2 (33.4-148.2), respectively. Although levels tended to be lower among the smokers, follicular fluid levels of vitamin C did not differ significantly across the smoking groups (P=0.091)(Paszkowski & Clarke 1999).

The effect of smoking on antioxidative activity within the ovarian follicle was also investigated by Sabatini et al. (1999). Another important enzymatic antioxidant in the follicle is superoxide dismutase (SOD), which acts by converting $O2^{-}\cdot$ into O_2 and H_2O_2. SOD activity in 46 samples of follicular fluid from 13 smokers was compared with that in 68 samples from 15 non-smokers. Mean SOD activity did not differ significantly between smokers and non-smokers, 1.17 U/mg of protein (range 0.69-3.65) and 1.54 (0.60-4.92), respectively (P>0.05). A moderate correlation between SOD activity in follicular fluid and serum was shown, r=0.55 (P<0.0001), with levels in follicular fluid almost always higher than in serum. Considerable variation in SOD activity in ovarian follicles was evident, which Sabatini et al. (1999) states may reflect varying degrees of $O_2^{-}\cdot$ scavenging.

In a further study on antioxidative activity by Paszkowski, Clarke and Hornstein (2002), cotinine, total antioxidative capacity and thiobarbituric acid reactive substances (TBAR) were measured in samples of follicular fluid. The latter, TBAR, is a measure of lipid peroxidation. Patients were classified based on cotinine levels: non-smoker (FF-C <20ng/ml, n=72), passive smoker (FF-C 20-50ng/ml, n=21) and smoker (FF-C>50ng/ml, n=15). TBAR levels were shown to increase with cotinine levels (P<0.001). Of significance, smokers had a two-fold increase in the final products of peroxidation as compared to non-smokers. Paszkowski, Clarke and

Hornstein (2002) suggested that this is a result of a pro-oxidant to antioxidant imbalance. They concluded that oxidative stress may be yet another cause of the impaired follicular function observed among smokers.

2.1.2 Diet

Vitamin C is a potent, non-enzymatic antioxidant (Halliwell, Gutteridge & Cross 1992). Humans are one of the few species unable to synthesise L-gulonolactone oxidase, an enzyme required for vitamin C biosynthesis; therefore, reliant on intake of vitamin C from dietary sources (Paszkowski & Clarke 1999). Vitamin C is thought to increase the resistance of lipoproteins to metal ion-induced oxidation and protect DNA against oxidative damage (Guérin, El Mouatassim & Ménézo 2001). Recently, Paszkowski amd Clarke (1999) reported that vitamin C was 1.7 times higher in follicular fluid than blood serum, and that levels were strongly correlated. It was surmised that levels of vitamin C within the ovarian follicle, and in turn the resultant degree of protection from ROS, is dietary-related (Paszkowski & Clarke 1999).

For the first time, Chui et al. (2002) demonstrated the presence of myo-inositol (MI) in follicular fluid. MI is an isomer of the C_6 alcohol of the vitamin B complex group. It is a precursor of inositol 1,4,5-triphosphate [Ins(1,4,5)P_3]. Ins(1,4,5)P_3 is a second messenger of the phosphatidylinositol signal transduction system, which regulates many cellular processes by modulating the release of intracellular calcium (Chui et al. 2002). There is increasing evidence that Ins(1,4,5)P_3 is involved in oocyte maturation, fertilisation and early embryonic development (Chui et al. 2002). This is not surprising as biochemical functions of phosphoinositol in cell membranes include regulation of cellular response to external stimuli, as well as mediation of enzyme activity (Colodny & Hoffman 1998). Inositol monophosphate can be synthesis from glucose-6-phosphate via a receptor mediated salvage system (Colodny & Hoffman 1998). Whilst it is plausible that the sub-optimal levels of follicular MI may originate from dietary deficiencies of vitamin B, as a source of inositol, diet plays only a minor role (Colodny & Hoffman 1998).

2.1.3 Caffeine Consumption

Caffeine is extracted from the leaves, seeds or fruits of more than 60 plant species. Interestingly, caffeine and its related compounds (e.g., theophylline and theobromine) function in the leaves of these plants as natural insecticides (Caffeine Survey 1995). Caffeine is contained in many food products, including tea, coffee and kola drinks, cocoa, chocolate and guarana (Caffeine Survey 1995).

The quantification of caffeine consumption was undertaken in only two of the studies that investigated lifestyle factors and the clinical outcomes of IVF treatment (Hughes et al. 1994; Klonoff-Cohen, Bleha & Lam-Kruglick 2002). In an examination of the effect of female and male smoking, Hughes et al. (1994) included female caffeine consumption as a covariate in the multiple logistic regression analysis on pregnancy. The effect of female caffeine consumption on oocyte production was not investigated. Hughes et al. (1994) did, however, report that female smokers had significantly higher levels of caffeine intake than both never and ex-smokers ($P<0.001$).

In the recent study by Klonoff-Cohen, Bleha and Lam-Kruglick (2002), caffeine intake among the 221 participating couples was measured in relative detail. Data on the consumption of coffee, tea, cocoa and kola sodas beverages (caffeinated and decaffeinated) and chocolate products were collected by questionnaires that were completed by the female patients prior to, during and after treatment, and by the male patients on the initial visit and at sperm collection. Self-reported caffeine consumption was converted into average caffeine intake in milligrams per day (mg/day). In this study by Klonoff-Cohen, Bleha and Lam-Kruglick (2002), caffeine consumption was not shown to compromise oocyte production.

It is relevant to note that Klonoff-Cohen, Bleha and Lam-Kruglick (2002) observed differences in behavioural changes in caffeine consumption between female and male patients. With treatment, there was an increase in the proportion of female patients that abstained from caffeine products, and intake declined among the caffeine consumers. The majority of female patients (94%) reported consuming caffeine beverages during their lifetime (mean 106 mg/day), and 83% were regular caffeine

consumers at the time of the initial clinic visit (mean 72 mg/day). Yet, only 49% consumed caffeine one week prior to treatment (mean 66 mg/day) and 37% during treatment (40 mg/day). By contrast, behavioural modification in caffeine consumption among the male patients was minimal: 88% reported caffeine consumption during their lifetime (192 mg/day), 87% during the week of the initial clinic visit (180 mg/day), and 91% consumed caffeine one week prior to sperm collection (174 mg/day). From these values, it could be construed that 3% of males commenced caffeine consumption one week prior to semen collection for the first time in their lifetime. As no alternative explanation was offered by the authors, a relatively illogical inference is one in which the difference is attributed to IVF; however, it is more feasible that this is an artefact from a change in the sample composition due to 'drop-out' from earlier stages of treatment. Whilst female patients abstained or at least reduced their caffeine consumption, little change in such behaviour was evident among the males.

2.1.4 Alcohol Consumption

To date, no study has specifically examined the effect of female alcohol consumption on the clinical outcomes of IVF treatment, though a few studies included it as a covariate in regard to the effects of smoking on pregnancy (Hughes et al. 1994; Klonoff-Cohen, Bleha & Lam-Kruglick 2002). Hughes et al. (1994) demonstrated significantly higher alcohol consumption among female smokers as compared to non-smokers; mean number of alcoholic drinks per day for active, ex- and never smokers was 3.2 (SD 2.4), 2.0 (SD 1.9) and 1.5 (SD 1.6), respectively ($P<0.001$). However, neither of these two studies showed alcohol consumption to have a significant effect on oocyte production, fertilisation, pregnancy or first-trimester miscarriages.

2.1.5 Stress

The conceptualisation of *stress* has caused much confusion. It is viewed by some as both an event (a distressing circumstance external to that person) and a response (the disturbance of a person's normal state)(Newton, Sherrard & Glavac 1999). Others argued it is neither, but rather an experience of the 1) perceived meaning of an event, and 2) self-appraisal of one's coping resources (Newton, Sherrard & Glavac 1999). Facchinetti et al. (1997) stated that stress is a 'healthy' reaction (that is, it is healthy

to feel stressed in stressful situations), and the individual's coping style is the factor that best determines health status (Facchinetti et al. 1997). Similarly, Thiering et al. (1993) surmise that anxiety is a natural response to IVF treatment rather than a reflection of an 'anxious' personality *per se*. Regardless, it is agreed that chronic stress occurs when stress-related events and conditions persist over time (Newton, Sherrard & Glavac 1999).

It is well recognised that the experience of IVF treatment is stressful (Ardenti et al. 1999). Furthermore, IVF treatment is usually proceeded by a period of time during which couples have dealt psychologically with the gradual realisation and final diagnosis of infertility. Psychosocial consequences of infertility may include anxiety, depression, bad marital relationship, social isolation and feelings of guilt and inferiority (Visser et al. 1994). In addition, most couples have a history of infertility-related clinical investigations and pre-IVF treatment (Slade, Emery & Lieberman 1997). Considered the final treatment option for infertility, IVF is undertaken with the threat of impending sterility and the loss of future plans for having children (Verhaak et al. 2001). IVF treatment is stressful, and reasons include 1) compliance with the treatment regimen, 2) scheduling treatment around work and family commitments (or vice versa), 3) treatment side-effects, 4) threat of treatment failure, 5) unrealistic expectations, 6) awaiting results, 7) fear of pain from the oocyte retrieval procedure, and 8) related relationship issues, and 9) financial pressures (Ardenti et al. 1999; Facchinetti et al. 1997; Merari et al. 1992; Sanders & Bruce 1999). In effect, acute stress from IVF treatment is superimposed over pre-existing chronic stress associated with the diagnosis of infertility.

It is well established that the hormonal stress response impacts on the female reproductive system by way of the hypothalamic-pituitary-ovarian axis, though the specific mechanisms of effect are not clearly understood (Thiering et al. 1993). It is known, however, that there is an increase in the secretion of cortiocotrophin releasing factor (CRF), adrenocorticotrophin hormone and cortisol in response to stress (Negro-Vilar 1993). Acting on the neurons in the hypothalamus, these elevated levels of CRF suppress the release of GnRH, resulting in decreased secretion of the gonadotrophins, FSH and luteinizing hormone (LH)(Negro-Vilar 1993; Vermeulen,

1993). In evidence, women infused with CRF exhibited subsequent declines in plasma levels of LH and FSH (Barbarino et al. 1989). Depression and anxiety are associated with elevated levels of prolactin (Merari et al. 1992). It is thought that hyperprolactinaemia disrupts ovarian development and function, leading to anovulation or luteal phase inadequacy (Merari et al. 1992). Demyttenaere et al. (1991) demonstrated that plasma cortisol levels at the time of oocyte retrieval were significantly higher than baseline levels. And Harlow et al. (1996) showed stress-related hormones to be elevated throughout the duration of IVF treatment.

Hypogonadism or hypothalamic amenorrhea - spontaneous cessation of menstruation for a period ≥6 months - is characterised by dysfunction or absence of pulsatile gonadotrophin secretion, impaired maturation of ovarian follicles and anovulation (Gallinelli et al. 2000; Ledger & Baird 1995). Amenorrhea is associated with periods of chronic depression and stress (Thiering et al. 1993). Women most afflicted are those with stressful lives and occupations, underweight, single or a psychoactive drug history (Negro-Vilar 1993). With IVF treatment, female patients with stress-related hypothalamic amenorrhea respond well to exogenous GnRH in terms of oocyte production (Vermeulen 1993). This demonstrates functional integrity of the adenohypophysis and confirms the dysfunction is hypothalamic in origin (Vermeulen 1993). The onset of this so-called functional hypothalamic secondary amenorrhea has been linked to stressful life events (Gallinelli et al. 2000).

There is no consensus as to the most appropriate method or instrument for measuring stress in IVF patients (Harlow et al. 1996). Physiological measures of stress include cortisol, prolactin, epinephrine and salivary α-amylase. However, stress is more commonly measured using validated psychometric tests. Variability in the manner by which stress has been measured in IVF studies to date is demonstrated in Table 2.3.

Only a few studies have examined the relationship between stress and oocyte production (Boivin & Takefman 1995; Demyttenaere et al. 1998). The 40 female patients in the study by Boivin and Takefman (1995) prospectively recorded daily stress levels for one complete IVF treatment cycle. With a resultant correlation of r=-

0.30 (P>0.05), the relationship between stress and oocyte production is doubtful. Having first identified that 'expression of negative emotions' and 'palliative coping' were significant in relation to pregnancy, Demyttenaere et al. (1998) then investigated each further in regard to oocyte production. Patients were categorised into two groups based on low and high score for 'expression of negative emotions'. The groups did not differ in terms of age, oocyte production or the number of oocytes that fertilised (P>0.05). Patients were also categorised into low and high groups based on 'palliative coping'. Similarly, no difference in age, oocyte production or fertilised oocytes was evident between these two groups (P>0.05). To date, there is little to suggest that stress impacts on oocyte production. It has been suggested that exogenous administration of gonadotrophins during IVF treatment compensates for infertility caused by psychological factors (Harlow et al. 1996).

Table 2.3: Studies Investigating Stress and Clinical Outcomes of IVF

author	instrument	measure of stress phenomena	IVF clinical outcome
Merari et al., 1992	State and Trait Anxiety Inventory (STAI)	present and proneness to anxiety	β-hCG pregnancy
	Lubin's Depression Adjective Checklist	depressive mood	
	plasma biochemical markers	cortisol and prolactin (PRL)	
Thiering et al., 1993	STAI	present and proneness to anxiety	cumulative (8 week) pregnancy rate
	Centre for Epidemiological Studies Depression Scale	depression	
Boivin & Takefman, 1995	STAI	present and proneness to anxiety	number of oocytes
	Marital Adjustment Scale	marital compatibility and satisfaction	β-hCG pregnancy
	Social Desirability Scale	identifies individuals perceived social desirability	
	Miller Behavioral Scale	coping style	
	Infertility Questionnaire	adjustment to infertility	
	Childbearing Focus	desire to have a child and its focus of one's life	
	Daily Record Keeping Sheet	negative emotions and physical reactions	
Harlow et al., 1996	STAI	present and proneness to anxiety	pregnancy (not defined)
	plasma biochemical marker	cortisol and PRL	

Table 2.3: Studies Investigating Stress and Clinical Outcomes of IVF (cont.)

author	instrument	measure of stress phenomena	IVF clinical outcome
Slade et al., 1997	STAI	present and proneness to anxiety	pregnancy status following ≤ 3 cycles
	Beck Depression Inventory	severity of depression	
	Dyadic Adjustment Scale	quality of marital relationship	
	Golombok-Rust Inventory of Sexual Satisfaction	quality of sexual relationship	
	Bi-polar Profile of Mood States (POMS)	mood states	
	Rosenberg Self-Esteem Scale	self esteem	
Facchinetti et al., 1997	STAI	present and proneness to anxiety	β-hCG pregnancy
	Stroop Color Word Test + cardiovascular responses, SBP, DBP & HR	coping ability, cognitive dissonance and psychological tension	
Demyttenaere et al., 1998	Zung Depression Scale	depressive symptomatology	no. of oocytes
	Utrechtse Coping List	coping style	no. of fertilised oocytes
			clinical pregnancy at 7 weeks
Milad et al., 1998	STAI	present and proneness to anxiety	<20 week pregnancy loss
	Pregnancy Anxiety Scale	pregnancy-related anxiety	
	saliva biochemical markers	salivary amylase and cortisol	
	plasma biochemical markers	prolactin	

Table 2.3: Studies Investigating Stress and Clinical Outcomes of IVF (cont.)

author	instrument	measure of stress phenomena	IVF clinical outcome
Sanders & Bruce, 1999	STAI	present and proneness to anxiety	cycles to achieve clinical
	POMS	mood states	pregnancy at 8 weeks
Ardenti et al., 1999	STAI	present and proneness to anxiety	pregnancy (not defined)
	IPAT Anxiety Scale	anxiety	
	Experiental World Inventory	personality traits	
Csemiczky, Landgren & Collins 2000	Karolinska Scales of Personality	anxiety, aspects of extraversion and agression-hostility	pregnancy (not defined)
	serum biochemical marker	cortisol and prolactin	
Gallinelli et al., 2001	STAI	present and proneness to anxiety	β-hCG pregnancy
	Stroop Color Word Test + cardiovascular responses, SBP, DBP & HR	coping ability, cognitive dissonance and psychological tension	
Verhaak et al., 2001	STAI	present and proneness to anxiety	pregnancy (not defined)
	Beck Depression Index		
	POMS	mood states	
	Maudsley Marital Questionnaire	marital satisfaction	

2.2 Fertilisation

Zamboni (1992) stated that the term fertilisation is loosely used too often. Fertilisation proper is said to have occurred after the male and female chromatins have combined within the oocyte (Zamboni 1992). Yet viewed as a process of events, this is the finale that precedes sperm capacitation, hyperactivation, the acrosome reaction and sperm-oolemma fusion. After penetrating the oocyte, the spermatozoon's densely compacted chromatin must first decondense before being able to attain the final stage of fertilisation (Zamboni 1992). The result is zygotic genome formation (Peluso, Luciano & Nulsen 1992). In clinical practice, however, the appositional alignment of the male and female pronucleus within the oocyte (pronucleus (2PN)) at 18-24 hours post-insemination is used as confirmation of fertilisation *in vitro*.

Entry of the spermatozoon triggers the final stage of oocyte meiosis and nuclear maturation. A rise in intracellular calcium is fundamental to this process of oocyte activation, although it is not yet understood how spermatozoa trigger this increase in calcium (Wilding & Dale 1997). There are, however, two major categories of calcium release mechanisms: $Ins(1,4,5)P_3$-induced calcium release and calcium induced calcium release (Wilding & Dale 1997). It is hypothesised that the calcium increase that activates oocytes at fertilisation is a soluble component of sperm cytoplasm (Wilding & Dale 1997). Whatever the mechanism, only oocytes that have successfully undergone this spermatozoon-triggered maturation process are capable of being fertilised. This includes not only nuclear maturity, but also functionality of cytoplasmic mechanisms that develop during oocyte growth. Even though spermatozoa can bind and penetrate the zona pellucida of immature oocytes, normal fertilisation does not ensue (Liu & Baker 2000).

To understand factors affecting fertilisation, the contribution of both the oocyte and spermatozoon must be taken into account, such is the advantage of fertilisation *in vitro*; both male and female factors can be examined simultaneously. Oocytes vary in their capacity to be fertilised (Ben-Shlomo et al. 1992), with failure mainly attributed to immaturity or abnormalities (Liu & Baker 2000). However, oocyte factors are unlikely to account for complete failure of fertilisation, as stimulation regimens

rarely produce uniformly abnormal or immature oocytes (Liu & Baker 2000). When semen analysis is normal, the major cause of complete or low rates of fertilisation *in vitro* is a disordered zona pellucida-induced acrosome reaction of the spermatozoa and not a problem associated with the oocytes (Liu & Baker 2000). It is indicated that up to one-third of normozoospermic presenting for infertility treatment men are afflicted as such (Liu & Baker 2000).

2.2.1 Smoking

Eighteen of the 22 studies detailed in Table 2.1 examined the effect of smoking on fertilisation *in vitro* (Table 2.4). The majority ascertained smoking status by self-reports from patients, and the minority by cotinine levels of biological fluid samples. All studies examined fertilisation *in vitro* by female smoking status. In the only study to examine fertilisation rates by male smoking status, no effect was evident (Joesbury et al. 1998). Only Rowlands, McDermott and Hull (1992) simultaneously examined male and female smoking exposure, whereby fertilisation was reported by couple smoking status. A collective comparison of study findings is difficult because fertilisation was measured in different ways. Furthermore, the unit used for statistical analysis varied, and included the oocyte, female or male patient, and couple.

2.2.1.1 Female Smoking

The findings from studies that compared fertilisation *in vitro* by female smoking are compiled in Table 2.4. Including the study by Rowlands McDermott and Hull (1992), a significant effect was detected in ten of 24 sets of findings. However, statistical significance appeared to vary with the measure of fertilisation used. In only two of the nine study findings was there a statistically significant difference between female smokers and non-smokers when the average (mean or median) fertilisation rate was reported (Crha et al. 2001; Rosevear et al. 1992). Whereas, there was a difference of significance in five of the nine study findings when the percentage of oocyte fertilised (% oocytes fertilised) was presented (Elenbogen et al. 1991; Rosevear et al. 1992; Rowlands, McDermott & Hull 1992; Zenzes et al. 1995; Zenzes et al. 1997). This, in part, may reflect the fertilisation measures differing degrees of sensitivity to the effect, if any, of smoking.

Table 2.4: Studies on Smoking and Fertilisation *in vitro* among IVF Patients

unit	study	smoking status[1]	findings	*P*
mean fertilisation rate % (SD)				
female:	Trapp et al, Kemeter & Feichtinger 1986	non-smokers (n=38)	49.9% (38.2)	>0.05
		smokers (n=76)	45.1% (39.6)	
	Pattinson, Taylor & Pattinson 1991	non-smokers (n=236)	68.5% (-)	>0.05
		smokers (n=124)	65.9% (-)	
	Sharara et al., 1994[2]	non-smokers (n=73)	82% (14)[3]	>0.05
		smokers (n=29)	78% (11)	
	Hughes et al., 1994	never smoked (c=182)	64.3% (25.4)	>0.05
		ex-smokers (c=165)	58.9% (36.4)	
		active smokers (c=115)	65.7% (37.0)	
	Van Voorhis et al., 1996	non-smokers (n=351)	49.9% (25.4)	>0.05
		ex-smokers (n=111)	54.1% (24.0)	
		smokers (n=37)	54.1% (25.0)	
	Joesbury et al., 1998	non-smokers (c=419)	60% (20)[3]	>0.05
		smokers (c=76)	61% (20)	
	Crha et al., 2001	non-smokers (n=90)	68.2% (33.2)	**<0.01**
		ex-smokers (n=29)	65.8% (35.2)	
		smokers (n=40)	47.8% (40.3)	
median fertilisation rate % (range)				
female:	Rosevear et al., 1992	non-smokers (n=32)	75% (0-100)[3]	**<0.05**
		smokers (n=13)	57% (0-100)	
	Gustafson, Nylund & Carlström 1996	non-smokers (n=50)	68% (17-84%)[3]	>0.05
		>10 cig/day (n=50)	57% (11-89%)	

Table 2.4: Studies on Smoking and Fertilisation *in vitro* among IVF Patients (cont.)

unit	study		smoking status[1]	findings	*P*
<u>% oocytes fertilised</u>					
female:	Harrison et al., 1990		non-smokers (o=?)	66%[3]	>0.05
			1-10 cig/day (o=?)	65%	
			11-20 cig/day (o=?)	75%	
			21-30 cig/day (o=?)	79%	
	Elenbogen et al., 1991		non-smokers (o=?)	61.7%	**<0.05**
			>15 cig/day (o=?)	40.9%	
	Rosevear et al., 1992		non-smokers (o=116)	72%[3]	**<0.01**
			smokers (o=45)	44%	
	Van Voorhis et al., 1992		non-smokers (o=?)	76.6%	>0.05
			smokers (o=?)	78.6%	
	Zenzes, Reed & Casper 1995		non-smokers (o=1022)	37.0%	**<0.01**
			passive smokers (o=200)	47.0%	
			<15 cig/day (o=151)	46.4%	
			≥15 cig/day (o=102)	34.3%	
	Sterzik et al., 1996		non-smokers (o=?)	67.6%	>0.05
			passive smokers (o=?)	57.7%	
			active smokers (o=?)	67.9%	
	Zenzes et al. 1997	<35 yrs:	ff ln cotinine <-0.4 (o=172)	69.4%	>0.05
			-0.4 to 1.2 (o=304)	64.4%	
			>1.2 (o=271)	67.9%	
		≥35 yrs:	ff ln cotinine <-0.4 (o=226)	67.5%	**0.0016**
			-0.4 to 1.2 (o=174)	61.3%	
			>1.2 (o=121)	78.1%	

Table 2.4: Studies on Smoking and Fertilisation *in vitro* among IVF Patients (cont.)

unit	study	smoking status[1]	findings	*P*
mean no. of fertilised oocytes (SD)				
female:	Trapp, Kemeter & Feichtinger 1986	non-smokers (n=76)	2.2 (2.4)	>0.05
		smokers (n=38)	1.8 (1.8)	
	Van Voorhis et al., 1992	non-smokers (n=36)	10.4 (4.8)	**<0.05**
		smokers (n=18)	8.2 (5.1)	
	Weigert et al., 1999	non-smokers (n=634)	3.9 (2.6)	>0.05
		smokers (n=200)	4.3 (2.8)	
failed fertilisation rate %				
female:	El-Nemr et al., 1998	non-smokers (c=108)	8.3%	>0.05
		smokers (c=65)	18.5%	
	Crha et al. 2001	non-smokers (n=90)	1.3%	**<0.01**
		ex-smokers (n=29)	2.0%	
		smokers (n=40)	8.8%	
≥60% fertilisation rate (%)				
female:	Crha et al. 2001	non-smokers (n=90)	60.8%	**<0.01**
		ex-smokers (n=29)	68.0%	
		smokers (n=40)	38.2%	
% oocytes fertilised				
couple:	Rowlands, McDermott & Hull 1992	neither smoked (o=317)	69.1%	**<0.05**
		female only smoke (o=204)	60.3%	
		male only smoke (o=86)	58.1%	
		both smoked (o=157)	59.8%	
mean fertilisation rate % (SD)				
male:	Joesbury et al., 1998	non-smokers (c=321)	61% (20)[3]	>0.05
		smokers (c=101)	61% (20)	

[1] n=no. of cases; c=no. of treatment cycles; o=no. of oocytes
[2] aged 35-39 years with normal ovarian reserve
[3] not presented to 1 decimal place as only whole numbers were reported by author

The ten studies that showed a statistically significant difference in fertilisation by female smoking status lacked consistency in regard to the direction of effect (Table 2.4). Although eight study findings suggested a detrimental effect of female smoking, the findings of two notable studies by Zenzes and colleagues did not. Specifically, Zenzes et al. (1997) showed female heavy smokers aged ≥35 years had significantly higher rates of fertilisation than light smokers and non-smokers, 78.1%, 61.3% and 67.5% (P=0.0016), respectively. In the other Zenzes et al. (1995) study, passive smokers (47.0%) and smokers of <15 cig/day (46.4%) had significantly higher oocyte fertilisation rates than non-smokers (37.0%), although heavy smokers had the lowest (34.3%; P<0.01). From the collective sum of these study findings, it is surmised that the effect of female smoking on fertilisation *in vitro* is clearly far from understood.

2.2.1.2 Male Smoking

2.2.1.2.1 Sperm Quality

Numerous studies have been published on the effect of smoking on semen parameters, involving both fertile and infertile men. Over the past two decades, these and related studies have been reviewed in detail (Little & Vainio 1994; Mattison 1982; Stillman, Roseberg & Sachs 1986; Vine 1996; Vine et al. 1994; Zenzes 1995). Based on a meta-analysis of 27 of such studies, Vine (1996) concluded that smoking is associated with a reduction of 13% in sperm count, 10% in motility and 3% in morphology. In a recent study that involved a large sample of 1104 men undertaking clinical investigation for infertility, no significant differences in sperm concentration, morphology or motility were found between smoker, ex- and non-smokers (Trummer et al. 2002). However, standard semen analysis - sperm count, motility and morphology - is not a measure of fertilisation potential.

2.2.1.2.2 Leukocytes

Within the male reproductive tract, polymorphic leukocytes play an important role in immunosurveillance and removal of defective sperm by phagocytosis (Alvarez et al. 2002). Through normal metabolic processes, leukocytes release ROS within the immediate vicinity of spermatozoa (Alvarez et al. 2002). Leukocytes are prolific producers of $O_2^{-}\cdot$ due to respiratory burst oxidases (Kim & Parthasarathy 1998).

Recently studies show that male smoking is associated with leukocytospermia (>1 × 10^6/mL). Trummer et al. (2002) reported that the prevalence of leukocytospermia was significantly higher among smokers than non-smokers (P<0.001). Saleh et al. (2002a) compared smokers and non-smokers, both in a group of men with and without varicocele. In both groups, smokers had significantly higher levels of seminal leukocytes (P=0.003). This implies that the reason for the elevated production of leukocytes in male smokers is not genital tract abnormality.

The exact mechanism for the increased concentrations of leukocytes in the semen of infertile smokers is not known (Saleh et al. 2002a). Three potential explanations are proposed by Saleh et al (2002a): 1) Metabolites inhaled in the cigarette smoke induce an inflammatory response in the male genital tract. In effect, leukocytes are recruited and activated, and in turn generate high levels of ROS that overwhelm ROS defense mechanisms. 2) The toxic metabolites in cigarette smoke may adversely affect spermatogenesis that results in the production of defective spermatozoa. For example, retention of the residual cytoplasm following spermiation is associated with an excessive production of ROS (Aitken 1997). In which case, leukocytes infiltrate the male reproductive tract to eliminate defective spermatozoa by phagocytosis. 3) ROS originate from the cigarette smoke *per se*. Whatever, the reason for the increased leukocytes in semen of male smokers, their spermatozoa are exposed to higher levels of ROS than their non-smoking counterparts. In evidence, it was demonstrated that leukocytospermic men had significantly elevated levels of spermatozoa with DNA damage (Alvarez et al. 2002), and smokers had significantly higher levels of seminal plasma ROS (Saleh et al. 2002a).

2.2.1.2.3 Reactive Oxygen Species

Physiologically normal levels of ROS appear to play an important role in sperm function (Lenzi et al. 1996; Pasqualotto et al. 2000). Superoxide anion ($O_2^{-}\cdot$) is involved in hyperactivated motility and capacitation, and hydrogen peroxide (H_2O_2) may be involved in the latter (Lenzi et al. 1996; Kim & Parthasarathy 1998). These positive effects of ROS are strictly related to equilibrium between ROS and scavenging systems (Lenzi et al. 1996). In excess, however, ROS cause oxidative stress. It is now believed that oxidative stress from excessive ROS underlies many

aspects of male factor infertility (O'Connell, McClure & Lewis 2002), in particular, cases classified as idiopathic (Sherins 1995 cited in Ollero et al. 2001). In turn, male factor infertility is a significant problem accounting for approximately 50% of couples attending infertility clinics (Morris et al., 2002; Saleh et al. 2002b).

Oxidative stress is known to play a major role in the aetiology of defective sperm function via sperm membrane lipid peroxidation (lipoperoxidation)(Twigg et al. 1998). Unrestrained ROS react with the highly vulnerable cell membrane lipids, and the resultant generation of lipid peroxyl radicals propagates further oxidation (Bedaiwy et al. 2002). Lipoperoxidation associated accumulation of lyso- and truncated phospholipids leads to membrane instability, loss of membrane permeability with concomitant loss of cytosolic enzymes, substrates and cofactors. Ultimately, peroxidation can cause loss of motility, failures of sperm-oocyte fusion, and cell death (Aitken 1997; Storey 1997). In addition, ROS are able to induce damage to sperm chromatin, which severely compromises the integrity of sperm genome (Hughes et al., 1996; Twigg et al. 1998).

2.2.1.2.4 Spermatozoa Plasma Membrane

The plasma membrane of spermatozoa plays a vital role in fertilisation, and its lipid composition is of particular interest in sperm physiology and pathology (Lenzi et al. 1996). Of significance, the phospholipids within the sperm membrane possess high levels of polyunsaturated fatty acids (PUFA), especially arachidonic acid (C20:4 *n-6*) and docosaesaenoic acid (C22:6 *n-3*)(Lenzi et al., 1996). These long chain PUFA are derived from the essential di-unsaturated fatty acids, linoleic acid (C18:2 n-6) and α-linoleic acid (C18:3 n-3). Both of these PUFA are obtained through dietary intake (Lenzi et al. 1996).

High PUFA composition endows the sperm plasma membrane with fluidity, a feature consist with its roles in fertilisation (Aitken 1997; Lenzi et al. 1996; Ramos & Wetzels 2001). Increased fluidity facilitates membrane fusion, lateral mobility of proteins on the membrane surface during capacitation, and initiation and propagation of bending waves along the sperm tail during motility (Sastry & Janson 1983). During the acrosomal reaction, for example, a capacitation-associated increase in the

fluidity overlying the acrosomal vesicle proceeds fusion of this membrane with the outer acrosomal vesicle (Lenzi et al. 1996). With maturation and migration through the epididymides, total PUFA in the sperm plasma membrane increases, and total saturated fatty acid - cholesterol and desmosterol - decreases. The latter renders the sperm membrane less rigid and collectively there is a net increase in membrane fluidity (Ollero et al. 2001). Of all cellular components, lipids are the most vulnerable to ROS (Bedaiwy et al. 2002); therefore, the high PUFA levels in sperm plasma membranes render spermatozoa especially susceptible to oxidative stress (Aitken 1997; Lenzi et al. 1996).

2.2.1.2.5 Spermatozoa Oxidative Defense Mechanisms

Both intracellular and extracellular antioxidant defense systems act to prevent excessive ROS formation (Guérin, El Mouatassim & Ménézo 2001). Intracellular mechanisms are mainly antioxidant enzymes (Guérin, El Mouatassim & Ménézo 2001). Due to the small volume of cytoplasm, intracellular cytoplasmic antioxidant enzymes are minimal and offer only limited protection against ROS (Aitken 1997). Furthermore, the intracellular antioxidative enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail (Saleh et al. 2002a). In compensation, antioxidative enzymes and free radical scavengers are present at high concentrations in seminal plasma and the luminal fluid of the epididymides (Hinton et al. 1995; Lenzi et al. 1996; Aitken 1997). Antioxidants in these fluids tend to be present at significantly higher concentrations than other biological fluids (Lenzi et al. 1996), which highlights the vulnerability of spermatozoa to oxidative stress.

Spermatozoa utilise a number of antioxidant defense systems for protection against oxidative stress (Guérin, El Mouatassim & Ménézo 2001). In general, transitional metals are eliminated from the environment by metal-binding molecules (e.g. transferrin and albumin). Intracellularly, enzymatic antioxidants - SOD, GPX and catalase - 'mop-up' $O_2^{-}\cdot$ and H_2O_2 before they react with metal catalysts to form the highly reactive hydroxyl radicals ($OH\cdot$). However, the small volume and restricted location of the cytoplasm in spermatozoa limits the degree of protection afforded by these intracellular enzymatic antioxidants (Aitken 1997). ROS that escaped enzymatic degradation can be terminated by non-enzymatic antioxidants - vitamin C,

reduced glutathione (GSH), hypotaurine and vitamin E. Hypotaurine neutralises $OH^{\cdot}$, and its by-product, taurine, neutralises cytotoxic aldehydes, which are the end-product of peroxidation reactions. Molecules damaged via oxidative stress are repaired by intracellular enzymes (Halliwell 1994). Of note, phospholipase A_2 plays a significant role in membrane integrity by cleaving peroxidised fatty acids from the parent phospholipid in order to make them available to glutathione peroxidase for detoxification (Aitken 1997). Aitken (1997) states that this is an extremely important defense mechanism for spermatozoa.

2.2.1.2.6 Spermatozoa

The latter stage of spermiogenesis involves cytoplasm dehydration of the elongating spermatid (Gil-Guzman et al. 2001). Up to 70% of the cytoplasm is phagocytosed by the Sertoli cells before release into the seminiferous tubule (Barratt 1995). Because of this lack of cytoplasm, spermatozoa have limited intracellular enzymatic antioxidants and repair mechanisms (Zenzes 2000). Though in limited capacity, superoxide dismutase (SOD), glutathione peroxidase and catalase are present (Hinton et al. 1995). In compensation, thiol compounds (organic compounds similar to alcohol but with a sulphur atom replacing the oxygen atom) are found in high concentrations within the spermatozoa cytoplasm, as well as smaller amounts of vitamin C, vitamin E and uric acid (Potts, Jefferies & Notarianni 1999). Intracellular enzymes, however, cannot protect the outer plasma membrane (Hinton et al. 1995). Given this and the limited cytoplasmic organelles for the prevention against ROS formation and repair from oxidative stress, spermatozoa are highly reliant on extracellular protection mechanisms and antioxidants (Aitken 1999).

2.2.1.2.7 Seminal Plasma

Seminal plasma is rich in antioxidant enzymes; SOD and GPX are the most representative (Hinton et al. 1995). Vitamin C is the principal non-enzymatic antioxidant, and its concentrated in seminal plasma is some 10-fold higher than that in blood plasma; thus, highlighting its physiological importance (Donnelly, McClure & Lewis 1999). It is a potent antioxidant because it reacts quickly with many of the ROS metabolites, peroxyl radicals especially (Halliwell, Gutteridge & Cross 1992). Vitamin E is also present in seminal plasma and its ability to break the

lipoperoxidative chain reaction renders it a powerful antioxidant. Vitamin C and E work in a synergistic fashion with vitamin C regenerating vitamin E for added prevention against lipoperoxidation (Donnelly, McClure & Lewis 1999). During preparation for IVF treatment, spermatozoa are washed free from the protective environment provided by seminal plasma and resuspended in culture media, though most culture media is current use are supplemented with antioxidants (Aitken 1999).

2.2.1.2.8 Epididymides

Oxidative stress is a significant threat to spermatozoa within the epididymides, as it is here that they spend a high proportion of their lifespan. Based on the approximated duration of 22 days for spermiogenesis (Hamilton & Waites 1990 cited in Barratt 1995) and the commonly quoted duration of 72 for spermatogenesis, it is estimated that spermatozoa exist within the epididymides for about 50 days, being 70% of their lifespan *in vivo*. The significance of the epididymides role in protection has only recently been recognised (Hinton et al. 1995).

Evidence suggests that the predominant antioxidant in the epididymides is reduced glutathione (GSH). Glutathione (GSH, reduced; GSSG, oxidised) is a tripeptide (γ-glu-cys-gly) that is synthesised intracellularly via several enzymatic reactions, where it either remains in the cytoplasm or is exported (Hinton et al. 1995). GSH provides reducing equivalents for many roles: 1) scavenging of ROS and peroxides, detoxification of xenobiotics, 2) maintenance of sulfydryl groups in proteins, and 3) storage of cysteine (Hinton et al. 1995). The antioxidative role of vitamin C and uric acid within the epididymides appears minimal by comparison (Potts, Jefferies & Notarianni 1999).

The epididymides plays a significant role in the structural and changes that occur to spermatozoa as they transverse this organ. When spermatozoa are released from the seminiferous tubules they are structurally differentiated but functionally immature (Zamboni 1992). In order to fulfil their reproductive role, they must develop effective motility, penetrating capacity, fusogenic ability and fertilising competence (Zamboni 1992). At release from the seminiferous tubules, spermatozoa are incapable of motility, though at best may exhibit spasmodic, shivering like,

ineffective movements (Zamboni 1992). Gradually, they acquire the ability to move in a forward progressive direction during transit through the epididymis. By the time spermatozoa reach the cauda, they are fully motile (Zamboni 1992).

2.2.2 Nutritive Factors

In terms of male fertility, nutrition is the most neglected of the lifestyle factors (Wong et al. 2000). Deficient levels of antioxidants are increasingly being linked to male sub-fertility, and projected to account for as many as one-third of such cases (Marik 2000). It has been suggested that oxidative stress may be associated with impaired antioxidant protection due to dietary deficiencies (Aitken 1999). Yet, the impact of nutrition on male factor infertility has scarcely been studied (Wong et al. 2000).

2.2.2.1 Zinc

Zinc is present in seminal plasma at a concentration 100-fold higher than blood serum (Eggert-Kruse et al. 2002). Consequently, it is the nutritive factor that has been the most studied in terms of male reproduction. As a co-factor for several hundred metallo-enzymes, zinc is involved in DNA transcription, expression of steroid receptors and protein synthesis (Wong et al. 2000). Of significance, zinc plays an important role in maintaining the integrity of the spermatozoa plasma membrane by preventing lipoperoxidation through the action of phospholipases (Eggert-Kruse et al. 2002; Wong et al. 2000). It has been suggested that the removal of zinc from the sperm cell surface destabilise the plasma membrane in preparation for the completion of capacitation and the acrosome reaction (Eggert-Kruse et al. 2002). Zinc is also required for the intestinal absorption of folate (Wong et al. 2000).

2.2.2.2 Folate

Although it is well known that peri-conceptional folate supplementation by the female is beneficial in the prevention of offspring with neural tube defects, little research has been done on its role in male reproduction (Wallock et al. 2001; Wong et al. 2000). Folate is present in a wide range of foods, notably green leafy vegetables, liver, yeast and fruits (Wong et al. 2000, 2002). Folate is essential for DNA synthesis and repair (Wong et al. 2002). Inadequate 5-methyltetrahydrofolate

(5-methyl-THF) is known to cause massive mis-incorporation of uracil in DNA, and this is believed to lead to associated chromosome breaks (Wallock et al. 2001).

Folate concentrations are approximately 1.5 times higher in seminal plasma than in blood plasma, and strongly correlated. The biochemically active forms of folate are all derivates of the reduced form of folic acid, tetrahydrofolate (Wong et al. 2000). Whilst 5-methyl-THF is the predominant form of folate in blood plasma, non-methyltetrahydrofolate (non-methyl-THF) accounts for 26% of folate in seminal plasma (Wallock et al. 2001). This suggests that non-methyl-THF has a physiologically significant role in male fertility (Wallock et al. 2001).

2.2.2.3 Selenium

Selenium is an essential trace mineral that plays both structural and enzymic roles as a constituent of selenoproteins, including in antioxidant defense systems (Brown & Arthur 2001; Rayman & Rayman 2002). Almost the entire selenium content of the testis is found in the selenoprotein phospholipid hydroperoxide glutathione peroxidase (Foresta et al. 2002). This enzyme is abundant in spermatids as an active peroxidase, but transformed into an oxidatively inactived protein in the mitochondrial capsule mid-piece of spermatozoa, where it stabilises the flagella (Brown & Arthur, 2001; Foresta et al. 2002). Selenium has been long recognised as an essential nutrient in male fertility, in particular sperm maturation and motility (Brown & Arthur 2001; Foresta et al. 2002; Rayman & Rayman 2002).

2.2.3 Caffeine Consumption

The effect of female and male caffeine consumption on fertilisation *in vitro* has been examined in only one study to date (Klonoff-Cohen, Bleha & Lam-Kruglick 2002). Caffeine consumption was measured in detail and included coffee, tea and kola drinks (caffeinated and decaffeinated), cocoa drinks and chocolate. Caffeine consumption by neither the male nor female was shown to affect the number of oocytes fertilised.

2.2.4 Alcohol Consumption

Excessive and chronic alcohol consumption is associated with ejaculatory dysfunction and testicular atrophy with decreased spermatogenesis secondary to the endocrinological sequelae of hepatic compromise (Dunphy, Barratt & Cooke 1990). By contrast, males attending infertility clinics have relatively low to moderate levels of alcohol consumption. The majority of men (n=446) in the study by Marshburn, Sloan and Hammond (1989) consumed no more than three standard alcoholic beverages per day. Over 80% of the 258 men in the Dunphy, Barratt and Cooke (1990) study consumed, on average, less than three standard alcohol drinks per day. In the study by Gerhard et al. (1992), almost half of the males were non-drinkers (46.8%; n=225), and only 27.3% had a daily consumption that exceeded two standard drinks. None of these studies showed alcohol consumption to have an adverse effect on sperm quality. No study has yet specifically investigated the effect of alcohol consumption on rates of fertilisation *in vitro*.

2.2.5 Stress

Little is known about the impact of male stress on clinical outcomes of IVF, although overall anxiety experienced by these men is significant (Clarke et al. 1999). Contributing factors include worry about providing an adequate semen sample, concern for their female partner undergoing oocyte retrieval, and uncertainty of *in vitro* fertilisation rates (Boivin et al. 1998; Clarke et al. 1999). The mechanism by which psychological stress affects male reproductive function is unclear. Like that in females, it has been suggested that a stress-related alteration in the hypothalamic-pituitary-adrenal axis may suppress secretion of LH, and in turn, testosterone production (Clarke et al. 1999).

To date, the impact of male stress has mostly been assessed by comparing sperm quality in semen samples collected before and during IVF treatment. As such, Clarke et al. (1999) reported that semen samples collected during IVF treatment exhibited reduced total counts and motile counts of 61% and 65%, respectively. The findings from earlier studies are clinically less significant. Harrison, Callan and Hennessey (1987) showed differences in sperm parameters in only 4-8% of 500 semen samples. From more than 600 patients, Kentenich et al. (1992) reported a marginal, albeit

statistically significant, poorer morphology and lower sperm counts from semen samples collected during IVF treatment. It was noted that sperm counts showed substantial intra-individual variability (Kentenich et al. 1992). It is hypothesised that such intra-variability in sperm parameters reflect the susceptibility of spermatozoa to the epididymal milieu.

Pellicer and Ruiz (1989) investigated the effect of stress induced by IVF treatment on fertilisation by selecting males from among couples at risk of high stress during IVF treatment. At this clinic, IVF treatment was confined to women less than 40 years of age and restricted to six attempts. The sample comprised 26 normozoospermic men whose partners were aged ≥37 years and on the last permissible IVF treatment cycle. Sperm count, motility and morphology in semen samples collected during infertility work-up and at oocyte retrieval did not differ significantly. Cryopreserved samples from infertility work-up and fresh ejaculates were simultaneously and independently used for insemination. No difference in rates of *in vitro* fertilisation or cleavage from these two sperm samples was observed. Pellicer and Ruiz (1989) concluded that stress induced during IVF treatment does not affect the fertilising ability of spermatozoa among normozoospermic men. However, it should be mentioned that damage to the sperm caused by cryopreservation of the initial sample may compromise the validity of this finding; damage from cryopreservation is likely to counterbalance a potentially detrimental IVF-treatment stress-related effect.

2.3 Pregnancy

Implantation involves both maternal uterine and embryonic factors. Earlier studies suggested endometrial thickness was a significant factor in regard to pregnancy (Check et al. 1991; Gonen & Casper 1990); however, the findings from more recent studies implicate the age-related decline in pregnancy rates is associated with oocyte quality rather than senescence of the uterus (Abdalla et al. 1993; Check et al. 2000). Moreover, in studies that engaged multivariate statistical techniques of data analysis, the relative importance of uterine factors were overshadowed by factors with higher predicability. This is not that uterine factors are unimportant, but rather methods of measurement are not sensitive enough to account for differences in uterine quality. Female age is well recognised as a factor in terms of pregnancy prognosis. More recently, however, embryo quality has been reported to be the best predictor (Lundin, Berg & Hardarson 2001; Terriou et al. 2001). In order to derive valid findings and conclusions from studies investigating the effect of lifestyle factors on pregnancy, it is imperative that these factors are taken into account.

2.3.1 Smoking

Eighteen studies have examined smoking and pregnancy following IVF treatment (Table 2.1). Most studies used univariate statistical methods of data analysis, and as such reported crude pregnancy rates. The findings from these studies have been collated and presented by female, male and couple smoking status in Table 2.5. As shown, various pregnancy outcomes have been examined, including beta-human chorionic gonadotrophin (β-hCG), clinical and ongoing pregnancies. Although more so the case among earlier studies, the pregnancy outcome was not always specified or defined, and as such these studies are denoted accordingly in Table 2.5. Of note, few studies have investigated the effect of male smoking on pregnancy outcome, as reproductive problems are traditionally associated with women (Zenzes 2000).

Pregnancy was first assessed 15-20 days post-ET by plasma β-hCG, oestradiol and progesterone levels. Whilst in clinical practice β-hCG pregnancy is considered a clinical endpoint, representing an early positive pregnancy test outcome that failed to reach a clinically evident pregnancy by week 7, it was used as a statistical end-point

Table 2.5: Crude Pregnancy Rates from Studies involving IVF Patients

unit	study	smoking status[1]	no. preg.	preg. rate	*P*
pregnancy (not defined)					
female:	Elenbogen et al., 1991	non-smokers	4/21	19.0%	>0.05
		smokers	1/20	5.0%	
	Pattinson, Taylor & Pattinson 1991	non-smokers	50/236	21.2%	>0.05
		smokers	19/124	15.3%	
	Sharara et al., 1994	non-smokers	21/73	27.6%	>0.05
		smokers	8/29	28.8%	
	Maximovich & Beyler 1995	non-smokers	65/210	31.0%	>0.05
		smokers	15/43	34.9%	
	Weigert et al., 1999	non-smokers	?	30.6%	>0.05
		smokers	?	24.3%	
	Crha et al., 2001	non-smokers	26/90	28.8%	-
		ex-smokers	7/29	15.4%	
		occasional smokers	2/16	12.5%	
		smokers	0/24	0%	
β-hCG pregnancy					
female:	Trapp, Kemeter & Feichtinger 1986	non-smokers	12/76	15.8%	>0.05
		smokers	3/38	7.9%	
	Joesbury et al., 1998	non-smokers	118/391	30.2%	>0.05
		smokers	28/74	36.5%	
clinical pregnancy					
female:	Harrison, Breen & Hennessey 1990	non-smoker	119/542	22.0%	**<0.05**
		smokers	9/108	8.3%	
	Van Voorhis et al., 1992	non-smokers	16/36	39%[2]	>0.05
		smokers	7/18	44%	
	Hughes et al., 1994	non-smokers	25/182	13.7%	>0.05
		ex-smokers	23/165	13.9%	
		smokers	13/115	11.3%	

Table 2.5: Crude Pregnancy Rates from Studies involving IVF Patients (cont.)

unit	study	smoking status[1]	no. preg.	preg. rate	*P*
clinical pregnancy (cont.)					
female:	Gustafson, Nylund & Carlström 1996	non-smokers	?	36%[2]	**<0.01**
		smokers	?	10%	
	Sterzik et al., 1996	non-smokers	15/68	32.6%	>0.05
		passive smokers	5/26	33.3%	
		smokers	23/103	32.9%	
	Van Voorhis et al. 1996	non-smokers	141/351	40.2%	**<0.05**
		ex-smokers	42/111	37.8%	
		smokers	8/37	21.6%	
	Feichtinger et al., 1997	non-smokers	126/399	32%[2]	>0.05
		smokers	40/102	28%	
	El-Nemr et al., 1998	non-smokers	23/108	21.3%	>0.05
		smokers	11/65	16.9%	
	Klonoff-Cohen et al., 2001	never smoked	46/105	22%[2]	**0.002**
		ever smoked[1]	24/111	12%	
12 week pregnancy					
female	Joesbury et al., 1998	non-smokers	66/391	16.9%	>0.05
		smokers	10/74	13.5%	
ongoing pregnancy					
female:	Maximovich & Beyler 1995	non-smokers	49/210	23.3%	**<0.05**
		smokers	4/43	9.3%	
	Van Voorhis et al., 1996	non-smokers	124/351	35.3%	>0.05
		ex-smokers	37/111	33.3%	
		smokers	6/37	16.2%	
β-hCG pregnancy					
male:	Joesbury et al., 1998	non-smokers	102/321	31.8%	>0.05
		smokers	29/101	28.7%	

Table 2.5: Crude Pregnancy Rates from Studies involving IVF Patients (cont.)

unit	study	smoking status[1]	no. preg.	preg. rate	*P*
clinical pregnancy					
male:	Klonoff-Cohen et al., 2001	never smoked	29/89	16%[2]	>0.05
		ever smoked[1]	33/89	19%	
12 week pregnancy					
male:	Joesbury et al., 1998	non-smokers	59/321	18.4%	>0.05
		smokers	11/101	10.9%	
pregnancy (not defined)					
couple:	Pattinson, Taylor & Pattinson 1991	both non-smokers	?	≈20%[3]	>0.05
		female smoker only	?	≈13%[3]	
		male smoker only	?	≈29%[3]	
		both smokers	?	≈15%[3]	
β-hCG pregnancy					
couple:	Joesbury et al., 1998	both non-smokers	91/287	31.7%	>0.05
		female smoker only	11/30	36.7%	
		male smoker only	15/59	25.4%	
		both smoke	14/39	35.9%	
clinical pregnancy					
couple:	Rowlands, McDermott & Hull 1992	both non-smokers	11/30	37%[2]	-
		female smokes only	4/15	27%	
		male smokers only	2/13	15%	
		both smoke	4/13	31%	
couple:	Klonoff-Cohen et al., 2001	none or one smoked[4]	22/47	12%[2]	**<0.05**
		both ever smoked[5]	40/131	22%	
12 week ongoing pregnancy					
couple:	Joesbury et al., 1998	both non-smokers	53/287	18.5%	>0.05
		female smoker only	6/30	20.0%	
		male smoker only	7/59	11.9%	
		both smokers	4/39	10.3%	

preg. = pregnancy
? not reported by author(s)
[1] ever smoked = had smoked in their lifetime (past or present)
[2] not presented to 1 decimal place as only whole numbers were reported by author
[3] calculated from Pattinson, Taylor & Pattinson (1991), Figure 3: p.782
[4] neither or one member of the couple smoked in their lifetime (past or present)
[5] both partners smoked in their lifetime (past or present)

by Trapp, Kemeter and Feichtinger (1986) and Joesbury et al. (1998). A clinical pregnancy is ultrasound confirmed foetal cardiac activity at week 7 or conceptus products of a miscarriage. By convention, an ongoing pregnancy is one in which has progressed beyond week 20. In the former study, 12 week pregnancy was used as the dependent variable because it was deemed more clinically meaningful, as 90% of pregnancy losses occurred within the first trimester (Joesbury et al. 1998).

2.3.1.1 Female Smoking

Clinical pregnancy is the most studied pregnancy outcome, with nine studies reporting crude rates by female smoking status (Table 2.5). Four of these studies showed smokers had significantly lower rates of pregnancy than non-smokers. As previously discussed, female smokers tend to be younger than their counterparts. Therefore, age has the potential to confound the relationship between female smoking and pregnancy outcome by favouring the null hypothesis of no difference between the pregnancy rates of smokers and non-smokers.

The probability of pregnancy is related to female infertility aetiology. Female smokers and non-smokers differ in terms of infertility aetiologies (Table 2.6), with tubal factor more prevalent among smokers (Phipps et al. 1987). Conversely, smokers have lower rates of endometriosis (Cramer et al. 1986), which appears to be related to a smoking-induced inhibitory effect on the production of oestrogen (Zeyneloglu, Arici & Olive 1997). As compared to patients with endometriosis, those with tubal factor infertility have significantly better clinical outcomes, including better quality oocytes, and higher rates of fertilisation and pregnancy (Azem et al. 1999). Consequently, differences in the occurrence of tubal factor infertility and endometriosis among smokers and non-smokers also has the potential to confound the relationship between smoking and pregnancy. With smokers more likely to have tubal factor infertility and less likely to have endometriosis, the null hypothesis of no difference is again favoured.

The smoking status of the female patient is associated with that of her male partner (Crha et al. 2001; Hughes et al. 1994; Joesbury et al. 1998). Crha et al. (2001) reported that almost 70% of female smokers had a male partner that smoked as compared to only one-third of the male partners of female non-smokers. This

Table 2.6: Female Smoking and Infertility Aetiology

study	smoking status	n	rate	*P*
Tubal Factor				
Trapp, Kemeter & Feichtinger 1986	non-smokers	76	79.0%	?
	smokers	38	86.8%	
Harrison, Breen & Hennessey 1990	non-smokers	542	44%[1]	?
	smokers	108	49%	
Pattinson, Taylor & Pattinson 1991	non-smokers	236	80.0%	**<0.001**
	smokers	124	93.5%	
Van Voorhis et al., 1992	non-smokers	36	31%[1]	?
	smokers	18	55%	
Zenzes, Wang & Casper 1995	non-smokers		63.7%	**0.025**
	passive smokers		76.2%	
	light smokers		89.6%	
	heavy smokers		92.9%	
Van Voorhis et al. 1996	non-smokers	351	34%[1]	**0.001**
	ex-smokers	111	57%	
	smokers	37	62%	
Endometriosis				
Harrison, Breen & Hennessey 1990	non-smokers	542	15%[1]	?
	smokers	108	19%	
Male Factor				
Trapp, Kemeter & Feichtinger 1986	non-smokers	76	14.7%	?
	smokers	38	25.7%	
Harrison, Breen & Hennessey 1990	non-smokers	542	13%[1]	?
	smokers	108	14%	
Van Voorhis et al., 1992	non-smokers	36	36%[1]	?
	smokers	18	22%	

? not reported by author(s)
[1] not presented to 1 decimal place as only whole numbers were reported by author

association between the smoking habits of the male and female member of the couple introduces yet another potentially confounding factor: male smoking status. Therefore, studies reliant on crude rates of pregnancy are limited in their capacity to draw conclusions about the effect of female smoking on pregnancy. Without having controlled for the potentially confounding effects of female age and infertility aetiology, and the smoking status of the male partner, any observed differences in pregnancy rates between female smokers and non-smokers cannot, with confidence, be attributed to the effect of female smoking.

Only three studies (Hughes et al. 1994; Joesbury et al. 1998; Klonoff-Cohen et al. 2001) used multivariate statistical methods to control for multiple covariates, including male factors (Table 2.1). In addition, Van Voorhis et al. (1996) reported age-adjusted odds ratios (OR) for clinical pregnancy and ongoing pregnancy outcomes. Specifically, female smokers as compared to non-smokers (past and never) had a clinical pregnancy age-adjusted OR of 0.38 (95% CI 0.17-0.86) and an ongoing pregnancy age-adjusted OR of 0.32 (95% CI 0.13-0.79). However, the potentially confounding effect of male smoking status cannot be ruled out as the causal factor for differences in the rates of pregnancy in this study by Van Voorhis et al. (1996).

Multiple logistic regression analysis was used by Hughes et al. (1994) to examine the effect of both female and male smoking on clinical pregnancy. Covariates in the regression analysis included female age, female caffeine consumption, duration of infertility and treatment cycle attempt. Of all the variables investigated, including female and male smoking, only female age was shown to have a significant effect on clinical pregnancy ($P<0.05$), with rates declining with age.

Multiple logistic regression analysis on pregnancy was also undertaken by the Investigator in the former study (Joesbury et al. 1998). Unlike the Hughes et al. (1994) study, in which clinical pregnancy was the pregnancy outcome of interest, a 12 week pregnancy was investigated as the dependent variable in the previous study by Joesbury et al. (1998). Covariates included embryo quality, female and male age, intracytoplasmic sperm injection (ICSI), female tubal factor infertility and

endometrial grade and thickness. Embryo quality and female tubal factor infertility were both significant ($P<0.05$). Of significance, however, male smoking, but not female smoking, was shown to decrease the chance of a 12 week pregnancy ($P<0.05$). Furthermore, a significant interaction between male smoking and male age was found. The chance of a 12 week pregnancy among male smoking couples decreased by 2.4% for every one-year increase in male age. It was suggested that male age was a surrogate measure of duration of smoking exposure (Joesbury et al. 1998). In order to compare results with that of the Hughes et al. (1994) study, regression analysis was repeated using clinical pregnancy as the dependent variable instead of a 12 week pregnancy. Like that reported by Hughes et al. (1994), no adverse effect of smoking by either the male or female on clinical pregnancy was evident.

2.3.1.2 Male Smoking

This previous study by the Investigator was the first to demonstrate an adverse effect of male smoking on pregnancy following IVF treatment (Joesbury et al. 1998). It was inferred that this was due to smoking-related pre-conceptual damage to the spermatozoa DNA that impaired embryonic development *in utero*. No other study to date has replicated this finding. One possible reason why no confirmatory evidence exists, is the choice of pregnancy outcome. Like Hughes et al. (1994), Joesbury et al. (1998) showed no adverse effect of male smoking on clinical pregnancy. On average, spontaneous abortions occur at 7.1-8.0 weeks (Maximovich & Beyler 1995), which implies that at least one-half of pregnancy losses would not be detected by ultrasound at week 7. Furthermore, a clinical pregnancy by definition can include pregnancy loss cases - products of conceptus following a spontaneous abortion, as evidence of pregnancy. Therefore, if smoking by the male partner is related to early pregnancy losses, the inability to statistically demonstrate such an effect is not unexpected when clinical pregnancy is selected as the pregnancy outcome.

Another issue of concern is selection bias. In the study by Van Voorhis et al. (1992), female non-smokers were women who had a non-smoking male partner, whereas female smokers had a partner who was either a smoker or non-smoker. If male smoking is associated with pregnancy loss and in turn a decreased pregnancy rate,

higher female non-smoking pregnancy rates would be expected if female non-smokers with male smoking partners are excluded from among the total group of female non-smokers. Avoiding selection bias is paramount in observation studies.

2.3.1.3 Meta-Analyses

Meta-analyses of studies investigating the effect of smoking on pregnancy have been reported in two publications (Feichtinger et al. 1997; Hughes & Brennan 1996). The first by Hughes and Brennan (1996) examined pregnancy by female smoking status, and included seven of the 22 studies reviewed here (Table 2.1): Elenbogen et al. (1991), Harrison, Breen and Hennessey (1990), Hughes et al. (1994), Pattinson, Taylor and Pattinson (1991), Rowlands, McDermott and Hull (1992), Sharara et al. (1994) and Trapp, Kemeter and Feichtinger (1986). However, the studies were not uniform in regard to pregnancy outcome of interest, and included positive test β-hCG pregnancies, clinical pregnancies and 'pregnancy' not clearly defined. Notwithstanding, a common odds ratio of 0.57 (95% CI 0.42 to 0.78) for conception among smokers relative to non-smokers was shown by Hughes and Brennan (1996), suggesting that the chance of pregnancy following IVF treatment for smokers is almost only half that of non-smokers.

In the second meta-analysis, Feichtinger et al. (1997) included only data from couples undertaking their first IVF treatment cycle. This totalled 2,314 treatment cycles from seven studies, all of which have been reviewed here (Table 2.1): Elenbogen et al. (1991), Feichtinger et al. (1997), Harrison, Breen and Hennessey (1990), Hughes et al. (1994), Pattinson, Taylor and Pattinson (1991), Sterzik et al. (1996) and Trapp, Kemeter and Feichtinger (1986). Most of these were included in the meta-analysis by Hughes and Brennan (1996). Overall, female smokers had a significantly lower pregnancy rate than non-smokers, 14% vs 21% ($P<0.01$). The success quotient was 1.8 (95% CI 1.2 to 2.6), which is a measure of how many IVF cycles a female smoker, as compared to a non-smoker, has to undergo to achieve a clinical pregnancy.

Firm conclusions are difficult to draw from the majority of studies published to date because of flawed methodology (Hughes & Brennan 1996). Whilst a meta-analysis

may overcome inadequate statistical power by pooling the subjects from multiple studies, it cannot rectify study biases. In particular, meta-analyses cannot remedy the failure of studies to control for potentially confounding factors. Nor can meta-analyses compensate for incomplete or questionable validity of data.

2.3.2 Caffeine Consumption

It has been mentioned previously that there is only one study to date to investigate specifically the effect of male and female caffeine on the clinical outcomes of IVF, being that by Klonoff-Cohen, Bleha and Lam-Kruglick (2002). Despite caffeine consumption being measured in detail, neither male nor female caffeine intake had an effect on the likelihood of achieving a pregnancy. Nor did female caffeine consumption affect first trimester miscarriages. Hughes et al. (1994) included female caffeine intake as a covariate in their study on the effect of smoking on clinical pregnancy. Consistent with the study findings of Klonoff-Cohen, Bleda and Lam-Kruglick (2002), female caffeine consumption was not shown to affect pregnancy outcome.

2.3.3 Stress

The majority of studies that investigated the impact of stress did so in terms of pregnancy. Psychometric instruments were the most common method of measuring stress, especially the State-Trait Anxiety Inventory (STAI)(Speilberger, Gorsuch & Lushene 1983). This self-administered questionnaire is comprised of two separate sets of 20-item questions (state and trait), with scores ranging from 20 (low anxiety) to 80 (high anxiety). The 'state' scale purports to measure anxiety at the time of testing, and the 'trait' scale measures the tendency to respond to stressful circumstances with raised anxiety (Sanders & Bruce 1999); the latter being a more stable measure of long term levels of anxiety (Gallinelli et al. 2001).

As stress was most frequently measured using the STAI (Table 2.3), findings from studies that examined STAI and pregnancy outcome from a single IVF treatment cycle have been compiled and are shown in Table 2.7. One problem with comparing findings is the different points in time in relation to IVF treatment when the STAI was administered. Thiering et al. (1993) stated that the timing of the psychological

assessment is critical in the valid attainment of mood state and its possible impact on IVF outcome. In the eight studies listed in Table 2.7, 20 analyses were performed. Only three showed significantly higher anxiety scores among women who became pregnant as compared to those who did not (Facchinetti et al. 1997; Gallinelli et al. 2001; Merari et al. 1992). In all these studies, anxiety was measured prior to oocyte retrieval, which is consistently reported by patients to be an especially stressful time during treatment (Boivin & Takefman 1995).

Only one study to date has assessed stress and pregnancy loss among IVF patients (Milad et al. 1998). Specifically, the effect of anxiety on <20 week pregnancy loss was examined among a sample of 40 women who had a β-hCG pregnancy 11-13 days post-ET. The STAI was used to measure anxiety, and mean values from the women who did and those who did not experience a pregnancy loss were compared. The mean STAI scores were not significantly different ($P>0.05$). The authors concluded that high levels of stress and anxiety do not appear to contribute towards the loss of a pregnancy.

As expected, studies that compared stress levels based on pregnancy outcome found that pregnant women had significantly better intermediate clinical outcomes: more oocytes retrieved and fertilised, and more and better quality embryos transferred. To counter this, Boivin and Takefman (1995) included in the analysis only female patients who had a three-embryo transfer. Stress levels among these pregnant and non-pregnant women were comparable ($P>0.05$). By explanation, Boivin and Takefman (1995) proposed the negative feedback hypothesis; non-pregnant women received more negative feedback throughout treatment because of poorer clinical progress during the treatment cycle. As patients are "exquisitely sensitive" to comments made by medical staff about progress of their treatment, any negative feedback is an additional source of stress (Boivin & Takefman 1995).

In general, former studies on stress and IVF clinical outcomes have not adequately controlled for covariates of stress, including lifestyle factors, nor other factors known to impact significantly on pregnancy outcome. Therefore, it is difficult to draw a sound conclusion about the effect of stress on pregnancy from the studies published to date.

Table 2.7: Pregnancy Outcome by STAI Assessment Following IVF Treatment

authors	inclusion criteria	pregnancy outcome	STAI administered	pregnancy status	STAI state score mean (SD)	STAI trait score mean (SD)	STAI score[a] mean (SD)
Merari et al., 1992	-	+ve β-hCG	day 3-5 follicular phase	preg (n=90)	-	-	43.0 (15.5)
				not preg (n=23)	-	-	39.2 (10.7)
			before oocyte retrieval	preg (n=71)	-	-	**54.9 (10.5)[b]**
				not preg (n=23)			**47.3 (12.1)**
			before ET	preg (n=62)	-	-	41.1 (11.3)
				not preg (n=23)	-	-	39.5 (12.2)
			day of initial preg test	preg (n=62)	-	-	42.8 (11.1)
				not preg (n=23)	-	-	46.1 (12.8)
Boivin & Takefman, 1995	1st IVF cycle	+ve β-hCG	before-IVF cycle	preg. (n = 17)	34.1 (8.2)	35.0 (9.7)	-
				not preg (n = 23)	37.9 (12.4)	36.9 (10.1)	-
Harlow et al., 1996	only 1 cycle per patient	not defined	day before TVOA	preg (n=9)	41 (-)[c]	36 (-)[c]	-
				not preg (n=27)	40 (-)	40 (-)	-
Facchinetti et al., 1997		+ve β-hCG	evening pre-TVOA	preg (n=9)	**41.0 (8.7)[b]**	41.9 (6.7)	-
				not preg (n=20)	**48.6 (9.4)**	46.6 (7.8)	-

Table 2.7: Pregnancy Outcome by STAI Assessment Following IVF Treatment (cont.)[a]

authors	inclusion criteria	pregnancy outcome	STAI administered	pregnancy status	STAI state score mean (SD)	STAI trait score mean (SD)	STAI score[a] mean (SD)
Milad et al., 1998	+ve β-hCG	<20 week	post-ET	preg (n=20)	-	-	42.2 (9.0)
		preg loss		preg loss (n=18)	-	-	45.0 (9.6)
Ardenti et al., 1999		not defined	day before TVOA	preg (n=34)	41.1 (11.2)	37.9 (9.2)	-
				not preg (n=128)	43.0 (11.7)	38.6 (8.2)	-
			day of TVOA	preg (n=34)	35.8 (7.9)	37.8 (8.7)	-
				not preg (n=128)	38.2 (9.5)	38.3 (8.7)	-
			day after TVOA	preg (n=34)	40.6 (14.5)	40.6 (14.5)	-
				not preg (n=128)	39.9 (11.8)	37.7 (9.2)	-
Gallinelli et al., 2001		+ve β-hCG	day before TVOA	preg (n=11))	**38.5 (5.6)**[b]	41.5 (9.2)	-
				not preg (n=29)	**45.0 (8.3)**	42.2 (8.9)	-
Verhaak et al., 2001		not defined	before IVF cycle	preg (n=59)	-	-	35.6 (8.3)
				not preg (n=148)	-	-	38.0 (10.9)

[a] STAI state and STAI trait score combined
[b] pregnancy vs not pregnant; $P<0.05$
[c] not presented to 1 decimal place as only whole numbers were reported by author

Multivariate methods of data analysis have been engaged by only a few studies. In one such study, Facchinetti et al. (1997) used multiple regression analysis to assess the effect of stress on pregnancy, and included patient, demographic and clinical variables in the model. Cardiovascular changes during administration of the Stroop Color Word test were used as measures of stress. This test was administered just prior to the oocyte retrieval procedure. In relation to pregnancy, employment in a job outside of the home (P=0.015) and an increase in percent total change in heart rate during the Stroop Color Word test (P=0.021) were shown to be significant. The authors concluded that the elevated heart rate response to simulated stress reflects an individual's tendency toward reactivity to daily hassles.

Sanders and Bruce (1999) used Cox proportional hazards regression to determine significant factors on the number of cycles to (clinical) pregnancy, which included IVF, GIFT and frozen embryo transfers. Psychosocial stress was measured prior to commencement of the first treatment cycle. Both the STAI and the Bi-polar Profile of Mood States (POMS) were used. The POMS measures six bipolar subjective mood states: composed-anxious, agreeable-hostile, elated-depressed, confident-unsure, energetic-tired and clearheaded-confused. In an initial investigation of eighteen variables including patient, treatment and lifestyle factors, only 'history of previous pregnancy' was significant in relation to number of cycles to conceive (P<0.05). With the inclusion of this variable, the scores from the psychometric tests were regressed on 'cycles to conceive'. Of the psychosocial factors, 'trait' anxiety and POMS scores on the agreeable-hostile and elated-depressed scale were significant (P<0.05). The relationship between 'trait' anxiety was quadratic; the chance of pregnancy increased with high and low levels of anxiety. Contrary to the authors' expectations, rates of pregnancy increased with scores toward the depressive pole of the POMS elated-depressed scale.

A limitation of the study by Sanders and Bruce (1999) is the temporal relationship between stress and pregnancy outcome. Levels of stress ascertained prior to the first treatment cycle were linked to the outcome of subsequent treatment cycles up until conception prevailed. Theoretically, this period could span a maximum of three years (February 1990 to February 1993). The highest number of cycles undertaken

was 12, though the median was only two cycles per couple. Changes in individual psychosocial and lifestyle factors are inevitable following repeated IVF treatment cycles. 'State' (current) anxiety prior to treatment is unlikely to reflect anxiety during the first treatment cycle, nor that of subsequent cycles after a failed attempt. Therefore, it was not surprising that 'state' anxiety was not shown to be of significance in regard to 'cycles to conceive'. By contrast, 'trait' anxiety was significant, and less unexpected, as it is a measure of potential anxiety and more stable over the long term. Hence, its significance on this occasion, albeit showing pregnancy rates were higher among women with low and high tendencies to respond to stressful circumstances with raised anxiety.

2.4 Pregnancy Loss

2.4.1 Multigeneration View of Reproduction

Wyrobek (1993) highlighted the vulnerability of humans to abnormal reproductive outcomes in his *Multigeneration View of Reproduction*, in which reproduction is seen as the transition of the germ line from generation to generation. Viewed in this manner, one cycle of reproduction involves five individuals from three generations: 1) the offspring (in question), 2) its mother and father, and 3) its maternal grandmother and paternal grandmother. The latter were included as they carried the pregnancies during which the maternal and paternal germ cells, reproductive organs and supporting systems developed. This model emphasises that viability in *utero* and well-being after birth, and probably throughout later life, depends on the quality of the genome inherited from one's parents (Wyrobek 1993).

2.4.2 Smoking

2.4.2.1 Female Smoking

Five studies examined female smoking and pregnancy loss (Table 2.1). All utilised univariate methods of data analysis and reported crude rates of pregnancy loss (Table 2.8). The definition of pregnancy loss differed across studies. Harrison, Breen and Hennessey (1990), Hughes et al. (1994), and Pattinson, Taylor and Pattinson (1991) defined clinical pregnancy loss as that following confirmation of a clinical pregnancy. Due to the exclusion of early pregnancy losses (usually <7 weeks), under-reporting is a potential flaw when this definition of pregnancy loss is applied. Conversely, over-reporting is a potential shortcoming in β-hCG pregnancy losses (Joesbury et al. 1998; Maximovich & Beyler 1995), due to inclusion of cases with a false-positive β-hCG pregnancy test result. All of these studies were based on crude rates. Without having taken into account potentially confounding factors, they contribute little to understanding the effect of female smoking on pregnancy loss.

2.4.2.2 Cigarette Smoke

A comprehensive investigation of free-radical chemistry of cigarette smoke and its toxicological implication was reported by Church and Pryor (1985). Cigarette smoke is a complex mixture of reactive species and there are many pathways by which these species can interact with one another and other smoke constituents. Church and

Table 2.8: Crude Pregnancy Loss Rates from Studies involving IVF Patients

unit	study	smoking status	no. preg. losses	%	*P*
β-hCG pregnancy loss					
female:	Maximovich & Beyler, 1995	non-smokers	16/65	24.6%	**<0.001**
		smokers	11/15	73.3%	
	Joesbury et al., 1998	non-smokers	52/123	42.3%	>0.05
		smokers	17/28	60.7%	
β-hCG pregnancy loss					
female:	Harrison et al., 1990	non-smokers	24/119	20.2%	**<0.05**
		smokers	5/9	55.6%	
	Pattinson et al., 1991	non-smokers	10/50	20.0%	>0.05
		smokers	8/19	42.1%	
	Hughes et al., 1994	never smoked	3/25	12.0%	>0.05
		ex-smokers	6/23	26.1%	
		smokers	0/13	0%	
β-hCG pregnancy loss					
male:	Joesbury et al., 1998	non-smokers	43/106	40.6%	>0.05
		smokers	18/31	58.1%	
β-hCG pregnancy loss					
couple:	Joesbury et al., 1998	both non-smokers	38/95	40.0%	>0.05
		female only smokes	5/11	45.5%	
		male only smokes	8/16	50.0%	
		both smokers	10/15	66.7%	

preg. = pregnancy

Pryor (1985) identified at least three pathways: 1) Combustion produces both oxygen- and carbon-centred, yet these radicals are too reactive and short-lived to play a role in the toxicology of smoke. 2) Several relatively stable free radicals are formed in the tar, the principal one being the quinone/hydroquinone complex (Q/QH_2). Q/QH_2 reduces molecular oxygen to generate superoxide, potentially leading to the production of H_2O_2, and in turn, the highly reactive hydroxyl radicals. 3) Cigarette smoke contains high levels of both nitric oxide (NO) and nitrogen dioxide (NO_2). NO is oxidised to the more reactive NO_2, which reacts with other constituents of cigarette smoke and generates the carbon-centred, peroxyl, alkyl and alkoxyl radicals.

2.4.2.3 Vulnerability of DNA: Male vs Female Gametes

As early as 1947 and based on the premise that the major source of mutations arise from copying mistakes during cell replication, Haldane suggested that mutation rates are likely to be higher in male than female gametes because of the greater number of mitotic divisions during gametogenesis (Haldane 1947 cited in Lessells 1997). In humans, it is estimated that there are about 33 cell divisions from zygote formation to oogenesis, and 205 from that to spermatogenesis in a 20 year old male (Chang et al. 1994 cited in Lessells 1997), eliciting a risk ratio of gamete mutations in males of 6.2 (Lessells 1997). In fact, this value is close to the male versus female gamete mutation risk ratio observed in higher order primates (Chang et al. 1994 cited in Lessells 1997). Zenzes, Bielecki and Reed (1999) also stated that the risk of germ cell damage from cigarette smoke is higher in males than females.

Furthermore, spermatogenesis is continuous from puberty to advanced age. DNA damage in the spermatozoa has been shown to increase with age. Spanò et al. (1998) demonstrated that the load of genetically abnormal cells was almost double in men aged 55 years as compared to those aged 25 years. Morris et al. (2002) also showed nuclear DNA damage increased as a function of age. Studies on rodents have identified three main patterns of germ cell sensitivity in males: 1) in late-step spermatids, 2) in early spermatids, and 3) stem cells (Wyrobek 1993). Whilst males continue to produce sperm throughout their adult lives and, therefore, in theory remain fertile into advanced age, the evidence to date suggests that the quality of the

genetic material of male gametes declines with age. It is no wonder that research on the integrity of the sperm nuclear DNA is currently the focus of intense study (Saleh et al. 2002b).

In contrast to male gametes, oocytes are almost mature at birth and remain in an arrested state (the dictyate stage of meiosis) until resumption of maturation (Ashwood-Smith & Edwards 1996). Relative to maturing oocytes in metaphase I and anaphase I and at metaphase II, these diplotene oocytes are less sensitive to the effects of radiation and chemicals (Ashwood-Smith & Edwards 1996). Diplotene oocytes are also less susceptible to mutations than spermatogonia (Ashwood-Smith & Edwards 1996). The efficient repair mechanisms of oocytes and virtual lack of cytoplasm in spermatozoa may also account for differences in mutation rates in male and female gametes (Ashwood-Smith & Edwards 1996).

The study findings of Bos et al. (1989) support the contention that female germ cells have fewer opportunities for mutations than that of the male. Levels of mutagens in follicular fluid and urine were measured in 12 smokers and 12 non-smokers using the Salmonella/microsome assay (Ames test). This test was developed for the detection of DNA-reactive agents, but has since been used to detect mutagenic and carcinogenic agents in biological specimens. Bos et al. (1989) reported that mutagens were only detectable in the follicular fluid of one individual, a smoker, and at a concentration approximating only 0.0125 of a cigarette. However, smokers had significantly higher levels of mutagens in their urine than non-smokers. Bos et al. (1989) concluded that the relative absence of mutagens in follicular fluid is possibly due to the difficulty the mutagens have reaching or penetrating the ovarian follicle, in particular, the protective surrounding layers: theca interna, basement membrane and granulosa cells.

2.4.2.4 Male Smoking

Male smoking has been implicated as a cause of early pregnancy losses. In the Investigator's previous study, male smoking was shown to have a significant adverse affect on the likelihood of the female partner achieving a 12 week pregnancy (Joesbury et al. 1998). It inferred that this was due to an elevated rate of early

pregnancy losses among male smoking couples resulting from pre-conceptual smoking-related DNA damage of the fertilising spermatozoa. Despite the plethora of anecdotal evidence, no study has yet to demonstrate empirically that pre-conceptual cigarette smoking by the male impairs embryonic viability and development *in utero*.

2.4.2.5 Sperm DNA

The term chromosomal abnormalities tends to include aneuploidies and structural errors (e.g., unbalanced translocations). Aneuploidy, however, occurs at a substantially higher rate in the gametes of females than the males (Zenzes, Wang & Casper 1992). In the case of trisomy 21, non-disjunction resulting in an extra chromosome 21, 95% of cases are of maternal origin (Antonarakis et al. 1991 cited in Zenzes, Wang & Casper 1992). More subtle anomalies include de novo point mutations and epigenetic changes, which are poorly defined molecular, non-mutational alternations, and variably termed DNA nicks, adducts, strand breaks, loss, deletions, fragmentation and denaturation. Epigenetic changes have the potential to become mutations and chromosomal abnormalities (Wyrobek 1993). In a recent study of 125 karyotyped abortuses, only 29% had chromosome abnormalities (94% aneuploidy and 6% unbalanced translocations); however, only structural and numeric rearrangements were detectable through karyotyping (Carp et al. 2001). For the remaining 71% of abortuses, there was an alternative and unaccounted cause of pregnancy loss (Carp et al. 2001). Whereas aneuploidy tends to be maternal in origin, structural rearrangements and the more subtle, yet less detectable, point mutations are more frequent in male than female gametes (Fraga et al. 1996; Little & Vainio 1994).

Fragmentation of genomic DNA is thought to be indicative of apoptosis, programmed cell death (Donnelly et al. 2000). The final stage of apoptosis is DNA fragmentation resulting from activation of endogenous endonucleases that cause multiple DNA strand breaks in the chromatin (Donnelly et al. 2000; Ramos & Wetzels 2001). Apoptosis may be a means of eliminating spermatozoa carrying DNA mutations, so as to prevent the transmission to offspring (Donnelly et al. 2000). Failure in the mechanisms of apoptosis, however, may result in sperm with DNA strand breaks being present in the ejaculate (Morris et al. 2002).

ROS species are capable of attacking and causing damage to most cellular components, including DNA (Donnelly, McClure & Lewis 2000). Spermatozoa from semen samples containing high levels of ROS-producing spermatozoa exhibit the most extensive DNA damage (Ollero et al. 2001). Both $O_2^{-}\cdot$ and H_2O_2 are mutagenic and cause chromosome deletions, dicentrics and sister chromatid exchanges. They can also attack DNA at either the sugar causing fragmentation, base loss and strand breaks (Ollero et al. 2001). If generated in close proximity, the highly reactive OH· will attack the DNA purine and pyrimidine bases and cause mutations (Halliwell 1994).

The uniqueness of the sperm nucleus is a result of profound chemical and physical changes (Zamboni 1992), which include nuclear elongation, development of the acrosome and condensation of the chromatin. Of significance is the complex process of nuclear remodelling. The chromatin is condensed in a process that is believed to commence late in spermiogenesis and finalised in the epididymides (Potts et al. 1999; Seligman et al. 1994; Zamboni 1992). Specifically, histones are replaced by protamines, which are low molecular mass basic proteins that are rich in arginine and cysteine (Seligman et al. 1994). These form the cross-linking disulphide bridges and function to stabilise the chromatin (Lopes et al. 1998; Seligman et al. 1994). The result is tightly packaged and highly condensed sperm nuclear chromatin that renders the DNA more resilient to DNA damage by chemical and physical insults, inadvertent cleavage and *in situ* enzymatic degradation (Potts et al. 1999; Zamboni 1992). In fact, the mouse sperm nucleus is 40-fold smaller than the nucleus of the mouse somatic cell (Bianchi et al. 1996). Interestingly, it is only in the ooplasm of the activated oocyte that the sperm chromatin decondenses primarily as a result of cleavage of the disulfide bonds and substitution of protamines with oocyte-derived histones (Zamboni 1992).

2.4.2.6 Smoking and Sperm DNA

Among males attending an infertility clinic, several studies compared the occurrence of DNA damage in the spermatozoa of smokers and non-smokers. With the exception of one study by Sergerie et al. (2000), which involved non-infertile, male

volunteers, all showed DNA damage to be higher in the spermatozoa of smokers than non-smokers. Fraga et al. (1996) examined the guanosine oxidative lesion of DNA, 8-oxo-deoxyguanosine (oxo^{8}dG), in semen samples of non-infertile smokers (n=21) and non-smokers (n=22). The oxo^{8}dG lesion was significantly more prevalent in the smokers by a factor of two (P=0.005). Shen et al. (1997) measured the oxidative lesion, 8-hydroxydeoxguanosine (8-OHdG) in 60 non-infertile males, aged 22 to 35 years. The geometric mean was significantly higher in the smokers (n=28) than the non-smokers (n=32), 6.2 (geometric standard deviation (GSD) 1.7) vs 3.9 (GSD 1.3; P<0.001). Sun, Jurisicova and Casper (1997) showed 27% of semen samples contained spermatozoa with fragmented DNA, which ranged from 5% to 40% (n=298). Again, smokers (n=35) had a significantly higher percentage of DNA fragmented spermatozoa than non-smokers (n=78), 4.7%$\pm$1.2% vs 1.1$\pm$0.2% (P<0.01). In normozoospermic sperm samples, Potts et al. (1999) demonstrated that the DNA of smokers (n=35) was more sensitive to acid-induced denaturation, and had significantly more strand breaks than non-smokers (n=35; P<0.05). Zenzes, Bielecki and Reed (1999) observed another benzo(a)pyrene-induced guanosine adduct (BPDE-I-dG-DNA). Similarly, the frequency in smokers (n=11) was two-fold higher than non-smokers (n=12). Furthermore, BPDE-I-dG-DNA frequency correlated highly with seminal plasma levels of cotinine (r=0.76). Collectively, these studies provide compelling evidence that smokers have significantly higher levels of sperm DNA damage than non-smokers.

Parental transmission of the BPDE-I-dG-DNA adducts was investigated by Zenzes, Bielecki and Reed (1999) in 27 human 4-8 cell embryos. The frequency of BPDE-I-dG-DNA adducts was 3.7-fold higher in embryos originating from male smokers (P<0.01), regardless of female smoking status. It was inferred that transmission of adducts occurs mainly through the spermatozoon (Zenzes, Bielecki & Reed 1999). In recent confirmation, Tesarik, Mendoza and Greco (2002) demonstrated that impaired developmental potential was transmitted exclusively by the fertilising spermatozoon, and without any maternal contribution.

2.4.2.7 Sperm DNA and Fertilisation Potential

Attention is currently focused on the fertilisation potential of spermatozoa with abnormal DNA. In an earlier study by Peluso, Luciano and Nulsen (1992), the ability of spermatozoa with altered and intact DNA to bind heparin-coated beads was compared. Because heparin binds to the surface membrane and induces nuclear chromatin decondensation and DNA synthesis, this technique was used as an indirect measure of fertilisation potential. Spermatozoa with altered DNA had the same binding affinity as those with intact DNA. This suggests that spermatozoa carrying abnormal DNA can undergo heparin-induced acrosomal reaction and nuclear decondensation (Peluso, Luciano & Nulsen 1992). However, the applicability of this model to rates of fertilisation *in vitro* is not without question.

More recently, Twigg, Irvine and Aitken (1998) examined the fertilisation capacity of DNA damaged spermatozoa, which represent those that could potentially be used in the treatment of ICSI. Even spermatozoa with abnormal DNA were shown to undergo normal nuclear decondensation and pronucleus formation following intracytoplasmic injection into the oocyte. The evidence to date suggests that the events of fertilisation do not select in favour of genetically normal spermatozoa.

With the exception of viruses and spermatozoa, all cells have DNA repair mechanisms (Ashwood-Smith & Edwards 1996). These mechanisms involve DNA polymerases and ligases, and are induced into effect by base-pair mismatches or strand breaks. The damage is excised and replaced with new synthesised DNA in which the undamaged DNA on either side of the lesion is used as the template. Yet, not all DNA damage is perfectly repaired; hence, the term 'error prone repair' (Ashwood-Smith & Edwards 1996). Although spermatozoa do not have DNA repair mechanisms, those of the oocyte can repair damaged DNA introduced by the fertilising spermatozoon (Ashwood-Smith & Edwards 1996).

The degree to which mutations prevail between entry of the sperm into the oocyte and the first cleavage depends, to the most part, on the 1) extent of sperm DNA damage, and 2) capability of the oocyte's repair mechanisms (Wyrobek 1993). Poorer quality oocytes may have less efficient repair mechanisms with limited or no

capacity to repair damage DNA of the fertilising spermatozoa. It was suggested by Ollero et al. (2001) that this may be the case in oocytes from older women. Fertilisation by a spermatozoon with genetic abnormalities that are not repaired by the oocyte is likely to result in an abnormal reproductive outcome. This may become evident during embryo development (e.g. pregnancy loss, morphologic defect) or after birth (e.g. behavioural changes, cancer, disease)(Wyrobek 1993).

2.4.2.8 Male Smoking and Morbidity in Offspring

Malformation can occur of organs, cells, cell components (e.g. synapses) and molecules (e.g. haemoglobin), forming a continuum from gross to subtle molecular disturbances (Auroux 2000). Those not overtly evident at birth may manifest and present later in life. Paternal pre-conceptual smoking is associated with morbidity in offspring. Zhang et al. (1992) reported a higher occurrence of birth defect among offspring born to male smokers. From the records of the Oxford Survey of Childhood Cancers, Sorahan et al. (1997a, 1997b) showed that offspring of paternal smokers had an increased incidence of childhood cancers. Ji et al. (1997) also showed children born to fathers that smoked were four times more likely to develop blood-borne cancers within their first five years of life. Collectively, these findings add to the accumulating evidence that pre-conceptual paternal cigarette smoking jeopardises the health and well-being of offspring.

Chapter 3

Materials and Methods

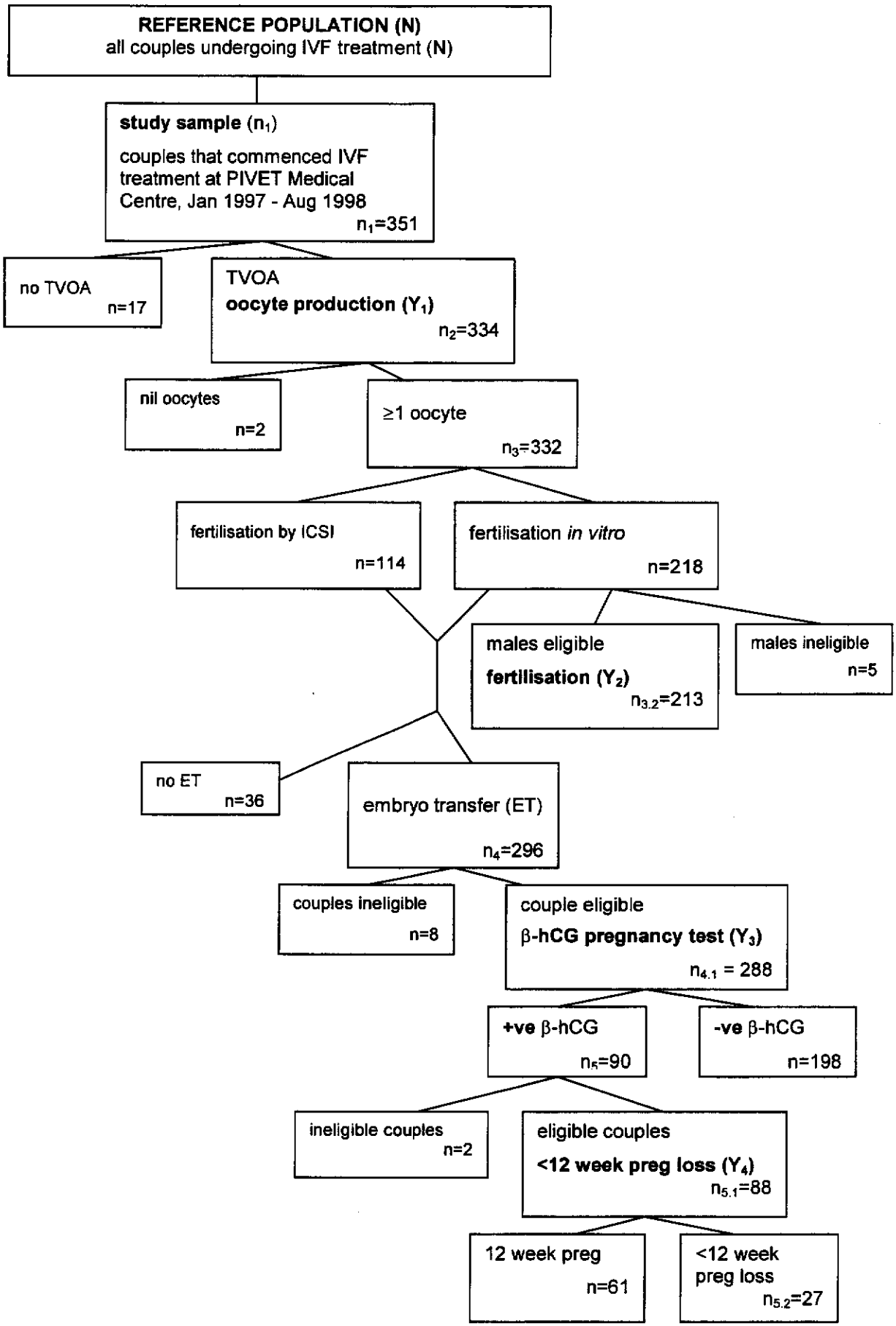
3.1 Study Design

By design, this study is a prospective cohort. In brief, it involved the recruitment and follow-up of couples undergoing IVF treatment. Early in the course of IVF treatment, measures of lifestyle were ascertained from the female and male member of each couple. The lifestyle variables included 1) years of smoking exposure, 2) tobacco and caffeine consumption, and 3) dietary and stress indicators. As couples proceeded through the treatment stages of IVF, the intermediate clinical outcomes of oocyte production (Figure 3.1; Y_1), fertilisation rate (Y_2) and ET were documented. Sixteen days post-ET, pregnancy status was determined (Y_3) by hormonal analysis. Couples having achieved a positive pregnancy test at this stage were monitored until week 12 of pregnancy (Y_4). Multivariate statistical methods were used to determine the impact of lifestyle factors on these clinical outcomes of IVF treatment.

3.2 Ethics Approval

Data collection for this study was undertaken at PIVET Medical Centre, Perth, Western Australia. PIVET has national accreditation by the Reproductive Technology Accreditation Committee and state licensing under the Human Reproductive Technology Act, 1991, which is enforced by the Western Australian Reproductive Technology Council (WARTC). Ethical approval to conduct the proposed study was sought from WARTC, and obtained on 29 February 1995. Ethical approval was also sought from PIVET Medical Centre's institutional ethics committee, Cambridge Private Hospital Ethics Committee, and from the Curtin University Human Research Ethics Committee (CUHREC). Both of the ethics committees act under the National Health and Medical Research Council guidelines, which embrace the principles of the Helsinki Declaration of 1975, as revised in 1983. Ethical approval was obtained from CUHREC on 15 December 1994, and from CPHEC, on 27 September 1994. CPHEC was subsequently disbanded in 1996; thereafter, CUHREC operated as PIVET Medical Centre's institutional ethics committee, of which ethical approval had previously been obtained.

Figure 3.1: Flowchart of Reference Population, Study Sample and Sub-Samples



3.3 Study Sample and Sub-Samples

The study sample comprised of 351 couples (n_1) who commenced IVF treatment at PIVET Medical Centre between January 1997 and August 1998. Couples whose treatment involved donor oocytes were ineligible for inclusion. Only the female was recruited among couples whose treatment involved donor sperm. Individuals whose command of the English language precluded an adequate understanding of the study, as determined by the Head of Nursing, were not approached for inclusion. Completion and return of the study questionnaires was assumed consent.

As shown in Figure 3.1, a different sub-sample of the study sample was used to examine each of the clinical outcomes: oocyte production (n_2=334), fertilisation rate ($n_{3.2}$=213), β-hCG pregnancy (n_4=288) and <12 week pregnancy loss ($n_{5.1}$=88). Each sub-sample will be discussed further within the respective section of the *Results* chapter.

3.4 IVF Treatment Protocol

PIVET Medical Centre has a standard protocol for IVF treatment (PIVET Laboratory Manual). However, IVF treatment tends to be modified, within clinical and legislative guidelines, to cater for prognostic differences in infertility between couples. At the time of the study, the standard treatment protocol at PIVET Medical Centre will be described, with exceptions to this protocol noted accordingly.

Couples were monitored for the duration of one IVF treatment cycle. In brief, an IVF treatment cycle involved multiple ovarian follicle stimulation, oocyte collection, oocyte-sperm insemination, ET and the determination of pregnancy status 16 days post-ET (approximately 4 weeks after the commencement of the IVF treatment cycle). Couples whose treatment resulted in a positive pregnancy outcome at this stage were monitored for an additional eight weeks, that is, until week 12 of gestation.

IVF treatment entails a sequence of treatment events that coincide with the female menstrual cycle. Menses signalled Day 1 of the treatment cycle, or in the event menses commenced post-midday, the following day was designated Day 1. During

the first two weeks of the menstrual cycle (follicular phase), exogenous FSH and/or other gonadotrophins were administered to stimulate the development of multiple ovarian follicles and concomitant oocytes. At approximately the mid-point of the menstrual cycle (≈Day 14), oocyte maturation and ovulation were induced by a single injection of human chorionic gonadotrophin (hCG). Thereafter, the oocytes are surgically retrieved from the ovaries, and inseminated with spermatozoa. Two or three days post-insemination, one to four resultant embryos are transferred to the uterus. Sixteen days post-ET, serum β-hCG levels are measured to determine pregnancy status.

3.4.1 Ovarian Follicle Stimulation

In the majority of patients, stimulation of multiple ovarian follicles was achieved by the flare regimen, which is a combination of GnRH agonist and FSH. The release of pituitary gonadotrophins was suppressed by GnRH, leuprorelin acetate (Lucrin, Abbott Australasia Pty Ltd, Kurnell, New South Wales (NSW), Australia). Commencing on Day 2, Lucrin was administered subcutaneously (10-20IU/l), which continued daily until pituitary gonadotrophin suppression was evident, that being serum LH levels <5IU/l and oestradiol (E_2) levels <200pmol/l, usually around Day 6. Most patients received a purified form of FSH (Metrodin HP, Serono, Aubonne, Switzerland), acquired from the urine of menopausal women, although two other forms of FSH were also used: Humegon (Organon, Australia Pty Ltd, NSW, Australia) and Puregon (Organon, Australia Pty Ltd, NSW, Australia). Humegon contains both human menopausal gonadotrophin (hMG) and human chorionic gonadotrophin. The latter is standardised with hMG to achieve a ratio of luteinizing to follicle stimulating hormone activity of approximately 1. The origin of the luteinizing activity is approximately two-thirds of placental and one-third pituitary. The origin of FSH in Humegon is two-thirds placental and one-third pituitary. Puregon contains recombinant human FSH, produced by the Chinese hamster ovary cell line transfected with the human FSH subunit gene. The flare regimen is so named after the *flare phenomenon,* that being the transient increase in pituitary gonadotrophins that proceeds the inhibition of gonadotrophin production.

A minority of female patients, approximately 10%, were prescribed the down regulation regimen. Instead of receiving GnRH from Day 2 to Day 6 as per the flare regimen, the down regulation regimen involved a prolonged period of GnRH suppression with Lucrin, from Day 21 of the pre-treatment menstrual cycle to Day 6 of the treatment cycle. The protocol for FSH administration was identical to the flare regimen. An exception was one patient who did not receive any GnRH for pituitary suppression, but instead received only FSH (Metrodin HP, Serono, Aubonne, Switzerland), as per the flare regimen.

The quantity of FSH administered was patient-specific. The age of the female was the principal factor governing the initial dose, as was a clinical history among those who had previously attempted an IVF treatment cycle. Each female's response to FSH was monitored, and if necessary, the dose amended accordingly. On Day 8, transvaginal ultrasound was used to determine ovarian follicle development, and was performed periodically thereafter, or as required. Upon satisfactory follicular development, usually the presence of at least three follicles ≥18mm in diameter, ovulation was triggered by a single intramuscular injection of hCG (10 000IU; Pregnyl; Organon, Australia Pty Ltd, Lane Cove, New South Wales, Australia). This activates the oocytes to undergo their second meiotic division, thus rendering them capable of fertilisation.

3.4.2 Oocyte Retrieval

Oocytes were retrieved by the ultrasound-directed, transvaginal oocyte aspiration (TVOA) method under minimal anaesthetic, 36±2 hours after the hCG injection. This technique entailed puncturing each ovarian follicle by needle (PIVET-Cook Laparoscopic/Ultrasound Double-Lumen Ovum Pickup Needle) and the subsequent aspiration of its contents. The follicle was flushed with a fine spray of flushing medium. Systematically, the embryologist used a stereomicroscope (X50) to locate the cumulus-corona-oocyte complex within each aspirate, from which the oocyte was isolated. Each oocyte was twice rinsed with flushing medium, and then placed in a single well of a multi-well dish.

3.4.3 Sperm Preparation and Insemination

A sample of semen ejaculate was produced by masturbation 1-2 hours post-TVOA. It was then processed, and the spermatozoa prepared for insemination. Typically, the swim-up method was used for normozoospermic samples, and the gradient separation method for oligozoospermic, asthenozoospermic and/or teratozoospermic samples. The spermatozoa classification systems was that according to the World Health Organization (WHO) guidelines; a sperm count of <20 x 106/mL (oligozoospermia), <50% of sperm with progressive motility and/or <30% of the sperm with normal morphology (teratozoospermia) in the ejaculate was regarded as abnormal (WHO, 1993).

Oocyte insemination was either *in vitro* or by the micromanipulation technique, ICSI. For insemination *in vitro*, oocytes were incubated in single wells for 4-6 hours, after which 50 000 to 100 000 spermatozoa were added to each well. Oocytes were examined 18-20 hours post-insemination for evidence of fertilisation, that being the presence of two pronuclei (2PN).

ICSI was the oocyte insemination method of choice for poor quality semen samples and/or previously poor *in vitro* fertilisation. ICSI was also used when spermatozoa were surgically acquired by testicular sperm aspiration (TESA) or micro-epididymal sperm aspiration (MESA). Following sperm preparation, a single sperm was aspirated into a micropipette. A mature oocyte (metaphase II) was immobilised by a holding pipette. The micropipette was then inserted through the zona pellucida and oolemma into the cytoplasm, with the spermatozoon being injected head-first into the ooplasm, and evidence of fertilisation observed 14-18 hours thereafter.

3.4.4 Embryo Quality

An embryologist graded all embryos (PIVET Laboratory Manual) on a scale from 1 to 4, with increments of 0.5. Grade 4 embryos had symmetrical, spherical blastomeres and no extracellular fragmentation. Grade 3 embryos had symmetrical, spherical blastomeres but some extracellular fragmentation. Grade 2 embryos had irregular blastomeres, either in size and/or shape, with considerable fragmentation. Grade 1 embryos had poorly defined blastomeres and considerable extracellular

fragmentation. The best quality embryos were selected for embryo transfer, and excess embryos of acceptable quality were made available for cryopreservation.

The modified Cumulative Embryo Score (mCES)(Joesbury et al., 1998) was used as a single measure of the collective quality of the embryos that were transferred into the uterus. The Cumulative Embryo Score (CES) was devised by Steer et al. (1992), and was modified by Joesbury et al. (1998). For each embryo selected for transfer, the grade was multiplied by the number (no.) of blastomeres (cells). The CES was the summation of these values, as shown in Figure 3.2.

Figure 3.2: Calculation of the Cumulative Embryo Score (CES)

		embryo grade	X	no. of blastomeres		
transferred embryos:	1	_____	X	_____	=	_____
	2	_____	X	_____	=	_____
	3	_____	X	_____	=	_____
	4	_____	X	_____	=	_____
					Σ	_____

Evidence indicates that an embryo developing at a normal rate has four blastomeres at 18-20 hours post-*in vitro* insemination and at 14-18 hours post-ICSI insemination. A >4 blastomere-embryo is considered to have a rate of development faster than that deemed to be normal. As the CES does not take into account the inferiority of fast developing embryos, it was modified accordingly by Joesbury et al. (1998). Specifically, embryos with 5, 6, 7 or 8 blastomeres were treated as having 3, 3, 2 or 2 blastomeres, respectively. For example, a purportedly inferior embryo of 8 blastomeres on day two post-insemination, hypothetically graded 2, had an embryo transfer value of 4 based on the mCES (2 x 2), in contrast to 16 as would of resulted using the CES system (8 x 2).

Approximately one-quarter of couples had a uterine ET on the third day post-insemination (3-day ET). An embryo developing at an optimal rate is expected to

have eight cells on day 3. In order to apply the mCES to 3-day ETs, an 8-cell embryo was treated as a 4-cell embryo, which hypothetically would have been the stage of development if transferred on the second day post-insemination. As such, a 4-cell embryo at day 3 was treated as a 2-cell embryo at day 2, with all other exceptions treated accordingly.

3.4.5 Embryo Transfer

Female patients had the embryos placed (transferred) into the uterus by catheter whilst under light sedation. The number of embryos transferred was determined by both the number of available embryos and the female patient's clinical prognosis of pregnancy. The transfer of only one embryo (1-ET) was usually the result of there only being one embryo available for transfer, although one exception was a couple who had a 1-ET by choice, despite having multiple embryos available for ET.

A two-embryo transfer (2-ET) was the result of either 1) the availability of only two embryos or 2) that the couple electing to have only two of a pool of available embryos transferred. The latter being at the recommendation of the clinician and/or embryologist in order to minimise the risk of a multiple pregnancy when the probability was perceived to be above average. The WARTC stipulates that a maximum of three embryos can be transferred. In exceptional circumstances, the WARTC grants approval to couples for a four-embryo transfer (4-ET), mostly because of advanced female age and/or a history of unsuccessful attempts at IVF treatment. Less than 5% of couples had a 4-ET in this study.

3.4.6 Pregnancy Assessment

Serum β-hCG levels 16 days post-ET were measured to determine pregnancy status. A β-hCG level of >25IU/L, with E_2 and progesterone (P_4) levels consistent the 4^{th} week of gestation, was confirmation of pregnancy. Thereafter, serum β-hCG, E_2, and P_4 levels were measured at weeks 5, 6, 7 and 8. At week 7, a clinical pregnancy was confirmed by transvaginal ultrasound detection of a heart-beating foetus.

3.5 Data Collection

The *Lifestyle Questionnaire/Diary* (LQ/D), designed and compiled by the Investigator, was used to collected lifestyle data from study participants. The female LQ/D is presented in Appendix C, and the male LQ/D in Appendix D. The female LQ/D was printed on mauve A4 paper, and the male LQ/D on pale green A4 paper. Before despatch, an identification (ID) no. was recorded in the LQ/D. This ID number was used to match the returned LQ/Ds with the respective couples.

The LQ/D comprised two parts: 1) questionnaire and 2) diary. The first part was a four page questionnaire that was used to obtain data on demographic information not available from patient records, including birth place and education. It was also used to acquire data on smoking history, including years of tobacco consumption, as well as information on *usual* tea and coffee beverages, and for smokers, *usual* cigarette brand. Patients were instructed to complete the questionnaire on Day 4 of the female patient's treatment cycle. The second part of the LQ/D was a 7-day, questionnaire-style diary. Patients were requested to commence the diary on Day 4, after completing the questionnaire, and continue through to Day 10.

Tobacco consumption and caffeine intake were included in the data collected in the diary each day. Data on caffeine consumption included intake from coffee, tea, kola and iced coffee beverages. In accord with the recommendations of Schreiber et al. (1988), the diary was devised so as caffeine intake took account of 1) decaffeinated beverages, 2) brewing methods of coffee, 3) the proportion of the beverage consumed, and 4) caffeine-containing prescription and 'over-the-counter' medication. For smokers, the number of *usual* brand cigarettes smoked and other tobacco products was also recorded. The specific calculations for nicotine and caffeine intake are described later in Section 3.8.2 and 3.8.3 of this Chapter, respectively.

Collection of data on other dietary factors included alcohol intake and fruit and vegetable consumption. Each day patients were asked to record alcohol intake in terms of the number of standard 10gms alcohol beverages consumed. Patients also documented the number of serves of fruit and vegetables consumed daily. To

facilitate completion of these questions, examples of standard alcoholic beverages and standard serves of fruit and vegetables were illustrated on the back, outer page of the LQ/D (Appendices C & D). Although the question was devised to account for caffeine intake from 'over-the-counter' medications, patients also recorded oral vitamin and mineral supplementation. Due to study limitation, only vitamin E supplementation was selected for detailed calculation and subsequent inclusion in the data analysis on fertilisation, which at the time of data analysis was regarded as the one of importance in terms of fertilisation. The specific manner in which total weekly vitamin E supplementation was calculated is detail later in Section 3.8.4.

Two general appraisal questions were devised to separate the stress experienced due to 1) daily living in general and 2) IVF treatment. Each required the respondent to indicate their stress level on a scale from 0 ('absolutely no stress') to 10 ('most stress possible'). For each criteria of stress, total weekly levels were calculated by simply summing the seven daily scores, creating a possible value range from 0 to 70.

Further data on demographics and infertility aetiology were obtained from the patients' files. Laboratory and clinical records were used to acquire data on clinical outcomes.

3.6 Patient Recruitment

IVF treatment is relatively time-intensive, especially for female patients, and tends to be an emotional period for the couple, which necessitated that each patient was treated with professional sensitivity. As quality of patient care was the priority, patient recruitment was organised so as not to interfere with treatment and with the aim of minimising any disruption to the work of staff members.

Patients for potential participation in the study were identified through PIVET's *Appointment Book*. Prior to IVF treatment, couples had an appointment with a clinician, which was scheduled for the female patient's Day 21 of the pre-IVF treatment menstrual cycle. At this *Day 21 Appointment* patients received written information sheets regarding IVF treatment, including an information sheet about the study, which stated that they might be asked to participate.

One week before the anticipated commencement date of IVF treatment, each couple was sent a male and female LQ/D by mail. A covering letter was also enclosed, individually signed by the Medical Director. The covering letter detailed the expectations of study participation, the issue of confidentiality, and instructions on how and when to complete the LQ/D. Strategically mailed to coincide with a Friday-delivery, it was envisaged that the recipients' moods would be more positive at the end of a working week, and that the weekend additionally provided leisure time in order to read the covering letter and review the contents of the LQ/D.

A *Study Follow-Up Sheet* was used to monitor the recruitment of eligible couples, and the follow-up of participants. On the first day of menses (Day 1), the female contacted the clinic to register commencement of an IVF treatment cycle, which was documented in the *Day One Register.* The *Day One Register* was reviewed daily in order to ascertain if any of the eligible couples were to due to commence treatment. If so, details were recorded on the *Study Follow-Up Sheet,* and arrangements were made for their anticipated attendance at the clinic the following day (Day 2).

Patients who attended PIVET Medical Centre for blood sampling and/or drug administration placed their names on a list upon arrival and awaited consultation with a nurse and/or the phlebotomist. This patient list was used to identify the attendance at the clinic of couples eligible for study inclusion. First the couple had a consultation with the phletobomist and then with a nurse. Thereafter, a meeting was arranged between the Study Investigator and the couple, or at least with the female in the event that the male partner did not attend the clinic.

During this first meeting with eligible couples, patients were advised that they were not obligated to participate, but their involvement would be valued. They were informed that their participation would be treated with the strictest of confidentiality. Couples were instructed on how and when to complete and return the LQ/Ds. Any questions or concerns were addressed at this meeting.

Over one-fifth of the eligible couples lived in a rural region of Western Australia. Due to problems concerning geographical distance, not all of the couples were able to attend PIVET Medical Centre on Day 2. Alternative options for blood sampling and hormonal analyses were utilised by couples who resided in remote regions. As these couples did not attend the clinic on Day 2, the Study Investigator was unable to meet with them until Day 8 when they first attended the clinic for ultrasonography of the ovaries. Nevertheless, these couples should have received the LQ/Ds prior to the commencement of IVF treatment (Day 1), with instructions on how and when to complete them.

3.7 Patient Follow-up

Couples who had not returned the LQ/Ds by the day of the pregnancy test, 16 days post-ET, were sent a letter requesting that they return the LQ/Ds by mail, incomplete or otherwise. A prepaid, addressed envelope was enclosed for convenience and also to encourage the return of the LQ/Ds.

3.8 Data Preparation

Values of the lifestyle variables that represent weekly measures were derived from data acquired from the LQ/D. The data required extensive re-coding and computing functions, which were performed with the programming facility of SPSS for Windows. An assessment of the accuracy of the computed weekly values was undertaken among a random sample of 10% of the couples. The value of each lifestyle variable was calculated manually and cross-referenced with the respective value in the database. As the manual values were equivalent to the computer-derived values, confirmation of the accurate computation of the lifestyle variables was substantiated.

3.8.1 Smoking Status Reclassification

Within the questionnaire of the LQ/D, patients were asked to report their smoking status, that being either 1) never smoked tobacco, 2) smoked tobacco in the past, 3) smoke tobacco occasionally, or 4) smoke tobacco regularly. Each respondent's self-reported smoking status was cross-referenced with their total weekly consumption of nicotine. If there was a discrepancy between these two variables, the respondent's

smoking status was reclassified accordingly (Table 3.1). Specifically, one female who reported to be a regular smoker (ID no. 222) indicated that she had quit smoking just days prior to commencing the IVF treatment cycle. She did, however, smoke during the week of the diary, but only 2.5 cigarettes, and therefore was reclassified as an occasional smoker. One male reported to have never smoked (ID no. 238), but smoked two cigars during the week of the diary and, therefore, was reclassified as an occasional smoker. Two females (ID no. 85 and 288) and one male (ID no. 136) reported that they were ex-smokers, but each had reported to having had smoked at least one cigarette. Similarly, all were reclassified as occasional smokers.

Table 3.1: Re-classification of Self-Reported Smoking Status

gender	ID	self-reported smoke status[a]	no. of days smoked[b]	no. of cigs/wk[b]	nicotine mg/wk	reclassified smoke status
female	85	ex-smoker	1	1	1	occasional
	222	regular	2	2.5	2	occasional
	288	ex-smoker	1	3	3	occasional
male	136	ex-smoker	4	30	18	occasional
	238	never	1	2[b]	-	occasional

[a] as reported by the respondent in the LQ/D
[b] during the one week diary

Among the occasional smokers (self-reported and reclassified), there was considerable variability in nicotine consumption. A profile of the smoking behaviour of the male and female occasional smokers is displayed in Table 3.2. An occasional smoker was reclassified as a regular smoker if the respondent 1) consumed tobacco on at least four of the seven days of the diary, and 2) total consumption was ≥10 cigarettes. As shown in Table 3.2, seven female and one male occasional smokers were re-classified as regular smokers.

Smoking status and history were re-examined among the males who had spermatozoa collected and cryopreserved prior to IVF treatment. As self-reported in the LQ/D, the time since quitting smoking was used among the male ex-smokers to confirm that smoking status at the time of spermatozoa collection was consistent with that reported at the time of IVF treatment. An inconsistency applied to only one of the

male ex-smokers (ID no. 21). Although this male ex-smoker had quit smoking two months prior to IVF treatment, he was a regular smoker at the time his spermatozoa were collected and cryopreserved, that being 18 months before treatment. As smoking data at the time that his spermatozoa were collected was not available, male ID no. 21 was deemed ineligible for study inclusion.

Smoke years was also adjusted among the male smokers to account for any difference in time between spermatozoa cryopreservation and the administration of the LQ/D (Table 3.3).

3.8.2 Nicotine Quantification

3.8.2.1 Cigarettes

The Smoke Yield Table (1992), as shown in Appendix E, was utilised to establish the nicotine yield of each smoker's usual cigarette brand. This table lists the nicotine yield (in milligrams) of different brands of cigarettes available in Australia. Weekly nicotine consumption (mg) was calculated by multiplying the nicotine yield of the usual cigarette brand by the number of cigarettes smoked for the week.

Occasionally, a cigarette brand reported by a respondent was not listed on the Smoke Yield Table (1992). The median value for nicotine yield, 0.7mg, was calculated from all of the 170 non-imported cigarette brands listed and was applied to the unlisted brands.

The usual cigarette brand of 14 smokers was indeterminable. Two females and four males consumed cigarettes of different brands, with the two female smokers providing insufficient information to identify their usual cigarette brand. One female and five male occasional smokers did not report a usual brand of cigarette. Consequently, the previously determined median nicotine yield of 0.7mg from non-imported cigarettes was applied as the nicotine yield per cigarette reported by these 14 smokers.

Table 3.2: Reclassification of Occasional Smokers

gender	ID	no. of days smoked (per/week)[a]	no. of cigs (per/week)[a]	nicotine (mg/week)[a]	reclassified smoke status
female	23	0	0	0	-
	40	4	13	3	regular
	76	1	2	1	-
	85	1	1	1	-
	99	0	0	0	-
	104	5	18.5	17	regular
	136	7	33	20	regular
	145	3	9	4	-
	165	6	10.5	9	regular
	179	0	0	0	-
	200	2	8	2	-
	217	4	12	12	regular
	222	2	2.5	2	-
	277	3	4	3	-
	288	1	3	3	-
	303	3	3	2	-
	315	7	17	9	regular
	322	7	67	20	regular
	342	0	0	0	-
					-
male	16	0	0	0	-
	63	0	0	0	-
	76	0	0	0	-
	84	3	14		-
	85	1	1	1	-
	136	4	30	18	regular
	144	0	0	0	-
	149	0	0	0	-
	179	0	0	0	-
	186	1	2	1	-
	223	2	4	4	-
	238	1	2[b]	-	-
	308	2	3	-	-

[a] during the week of the diary

Table 3.3: Adjustments to Smoke Years for Males with Cryopreserved Spermatozoa

ID no.	male smoking status	no. of days cryopreserved	self-reported smoke years	adjusted smoke years
079	never	125	-	-
119	ex-smoker	289	-	-
131	never	158	-	-
137	smoker	57	16	15.8
138	smoker	54	7	6.9
142	smoker	111	16	15.7
145	ex-smoker	39	-	-
169	ex-smoker	59	-	-
194	ex-smoker	154	-	-
207	smoker	343	20	19.1
208	ex-smoker	160	-	-
217	smoker	11	27	27.0
297	never	32	-	-
335	smoker	62	20	19.8
360	never	62	-	-

3.8.2.2 'Roll-Your-Own' Cigarettes

Nine of the male smokers consumed 'roll-your-own' (RYO) cigarettes. A British study based on 26 regular smokers of RYO cigarettes (Darrall & Figgins, 1998) reported that the overall average weight of tobacco used per cigarette was 0.5gms (range 0.2 to 0.9gms) yielding a mean of 1.3mg of nicotine per cigarette. Therefore, 1.3mg was used as the standardised nicotine yield of RYO cigarettes in this study. Weekly nicotine consumption from RYO cigarettes was calculated by multiplying the number of RYO cigarettes smoked over the week of the diary by a value of 1.3 (average nicotine yield per RYO cigarette).

3.8.2.3 Marijuana Cigarettes

One male, who was a regular smoker, reported that he consumed five marijuana cigarettes during the week of the diary. As the question in the diary read *'Did you consume any other tobacco products today?'*, it was inferred that the marijuana cigarettes contained tobacco. For the purpose of quantifying nicotine consumption, the five marijuana cigarettes consumed by this male respondent were added to the number of cigarettes smoked for the week using the RYO nicotine yields.

3.8.2.4 Cigars

Three males reported smoking a least one cigar during the week of the diary. One male consumed 15 cigars, another consumed two cigars, and the other male reported the consumption of one cigar. Unlike manufactured cigarettes, the nicotine yield per cigar is not listed on the packaging. Following an extensive literature search, it was found that data on the nicotine yield of cigars was lacking. The only relevant study was reported by Rickert et al. (1985) of Canada, in which the nicotine yield of 16 international cigars was listed (Table 3.4). The cigar brand and type of one of the three male respondents was listed, and therefore used as the nicotine yield per cigar smoked. The brand names and type of cigars smoked by the other two males were not detailed. Based on the 16 cigar brands in the Rickert et al. (1985) study, the mean nicotine yield per cigar was 2.0mg, which was used as the nicotine yield per cigar for these two males. The weekly nicotine consumption from cigars was calculated in accordance with the other tobacco products.

3.8.3 Caffeine Quantification

The Caffeine Survey (1995) was used to convert self-reported lifestyle data obtained by the LQ/D into total weekly caffeine consumption in milligrams (mg). Between June and December 1994, the Health Dept of WA submitted 107 food samples to the Chemistry Centre of WA for caffeine analysis. The caffeine content of these 107 food samples are listed in the Caffeine Survey (1995) and included beverages made from specified brands of coffee and tea and commercially available kola and iced coffee drinks, all of which are readily available in Western Australia.

If the respondent specified the brand name of their caffeinated product, and if the product was listed in the Caffeine Survey (1995), the corresponding caffeine value was used. If the brand was not specified or not listed in the Caffeine Survey (1995) then an estimated average (mean) value was used. The manner in which these estimated average values were calculated is detailed below.

Table 3.4: Cigar Brands Included in the Study by Ricket et al. (1985)

	brand and type	length (mm)	diameter (mm)	weight (mg)	puffs (no.)	nicotine yield per cigar (mg)
	small cigars					
1	O'Sherry (filter)	95	8	1215	11.8	1.44
2	O'Sherry (non-filter)	94	8	1349	11.0	1.69
3	Old Port Colts Mild Tipped (non-filtered)	98	9	1545	13.9	1.38
4	Planter Sprint (non-filtered)	74	7	1081	8.9	1.44
5	Panter Brasil Cigarillo (non-filtered)	74	7	1126	10.6	1.59
6	Witterman Café Creme (non-filtered)	74	7	980	8.8	1.68
7	Meadowbrooke (non-filtered)	-	-	1369	14.2	1.02
8	Old Port Colts Reg. Tipped (non-filtered)	98	9	1494	12.6	2.26
9	Old Port Cigarillos Tipped (non-filtered)	133	10	3068	28.1	4.06
10	Old Port Mild Cigarillos (non-filtered)	110	10	2909	24.0	4.15
	large cigars					
11	White Owl Invincibles (non-filtered)	120	16	7963	74.0	6.44
12	Pedro Montero (non-filtered)	147	16	9157	129.0	0.72
13	It's A Boy (non-filtered)	136	16	7724	114.0	1.13
14	El Producto Corona Extra (non-filtered)	122	16	7497	116.0	0.82
15	Muriel Magnum (non-filtered)	123	17	8083	108.0	0.86
16	House of Lords Panatellas (non-filtered)	128	13	6556	108.0	1.37
	mean	108.4	11.3	3945	49.6	2.00
	SD	23.8	3.9	3199	48.3	1.56

3.8.3.1 Coffee Beverages

The caffeine content of beverages made from instant coffee beverages, listed in the Caffeine Survey (1995), was based on a serve size of 2 grams (gm). Within the LQ/D instant coffee beverages varied by teaspoon measures: ½, ¾, 1 level, 1½, 1 heaped and 2 teaspoons. In order to use the caffeine values of the instant coffee beverages listed in the Caffeine Survey (1995), it was necessary to convert the various teaspoon measures into grams of instant coffee. Consequently, a project was undertaken to determine the average grams of instant coffee in a level and heaped teaspoon.

The laboratory work for this project was conducted at Curtin University of Technology, WA, in the School of Public Health food science laboratory. A standard kitchen teaspoon was randomly selected from among a range of teaspoons that were currently representative of those in domestic use. Based upon granule size, three relatively common brands of instant coffee were selected: International Roast Coffee (fine granules), Nescafe 43 Blend (medium granules) and Moccona Indulgence (large granules, which result from a freeze-dried process). For each of these three coffee brands, the average grams of instant coffee per level teaspoon were calculated by weighing the contents five times (Table 3.5). The grand mean weight of instant coffee in a level and heaped teaspoon was 1.26gm and 2.10gm, respectively.

A level teaspoon of instant coffee weighed 60% that of a heaped teaspoon. Whereas a level teaspoon of the fine granule instant coffee (International Roast) was the lightest of the three instant coffee brands, it weighed the most on average per heaped teaspoon. The fine structure of the International Roast coffee granules resembled that of powder, which facilitated a larger quantity of instant coffee being placed (heaped) upon the teaspoon.

The Caffeine Survey (1995) listed the caffeine content of ten brands of caffeinated instant coffee beverages (Table 3.6), each being made from a serve size of 2gm. The mean caffeine content of these ten brands was 72mg per 2gm serve.

As established previously, a level teaspoon of instant coffee has an average weight of 1.26gms, which is 63% of a serve size of 2gm, whereas a heaped teaspoon of instant coffee weighs an average of 2.10gm, being 105% of a 2gm serve. These values, in conjunction with that of a 2gm serve of instant coffee containing an average of 72mg of caffeine, were used to estimate the milligrams of caffeine in each of the teaspoon measures (Table 3.7).

Listed in the Caffeine Survey (1995) is the caffeine content of four brands of decaffeinated instant coffee, each being made from a serve size of 2gm (Table 3.8). The average caffeine content of these four brands of decaffeinated instant coffee is 3mg. Using the previously estimated teaspoon conversion factors, the estimated average caffeine content per teaspoon serve size is shown in Table 3.9.

The percolated caffeine content of coffee beverages obtained from seven different brands of roasted ground coffee is presented in the Caffeine Survey (1995)(Table 3.10). An investigation of these seven brands showed that the mean caffeine content of a percolated coffee beverage made with a serve size of 2gm of roasted ground coffee was estimated to be 28mg. However, most brands of roasted ground coffee are purchased with a standard 5gm scoop, which is the recommended amount of coffee per beverage. The scoop approximates the size of a dessert spoon. Based on an estimated 28mg of caffeine in percolated coffee beverage made from a 2gm serve of roasted ground coffee, a percolated coffee beverage made with serve size of 5gm would contain approximately 70mg of caffeine.

Respondents reported their usual percolated coffee beverages made from roasted ground coffee with serve sizes measured by the standard scoop, dessert spoon or teaspoons. Shown in Table 3.11 are the conversion factors that were used for the various serve sizes of roasted ground coffee, and the estimated average caffeine content of the respective percolated coffee beverage.

In a comprehensive study of the quantification of caffeine consumption from self-reported data, Schreiber et al. (1988) showed that the drip/filter method is more efficient at extracting caffeine than percolation. Specifically, the caffeine content of

drip/filter coffee beverages is 135% greater than that produced by percolated coffee beverages. Therefore, a conversion factor of 1.35 was used to convert the caffeine content of percolated coffee beverages into Table 3.12.

In the Caffeine Survey (1995), the caffeine contents of four decaffeinated roasted ground coffee brands were reported, of which the caffeine content of each made from a serve size of 2gm serve is presented in Table 3.13. The mean caffeine content of 2mg per 2gm was used to estimate the caffeine content by using the amount of decaffeinated roasted coffee used per coffee beverage. Presented in Table 3.14 is the conversion factor for each serve size of decaffeinated roasted ground coffee, and the caffeine content of the respective beverages. As the drip/filter method of brewing is 135% more efficient at extracting caffeine from the roasted coffee than the percolation method, adjustments to the caffeine content for drip/filter coffee beverages were made accordingly (Table 3.15).

Schreiber et al. (1988) stated that in the quantification of caffeine intake from self-reported coffee consumption, account should be taken of whether or not an individual consumed the full contents of their usual beverages. Approximately 15% of the respondents in the study by Schreiber et al. (1988) did not consume about 20% of their coffee beverage. The LQ/D was used to ascertain whether or not respondents usually consumed the full contents of their coffee beverages. If they did not usually drink (most) of the coffee in their cup/mug, the caffeine content of their usual coffee beverage was adjusted by a factor of 0.8. This was in accordance with Schreiber et al. (1988) who estimated that among individuals who habitually left a proportion of their beverages that only 80% of each beverage was consumed.

The caffeine content of two brands of iced coffee were documented in the Caffeine Survey (1995). The caffeine content of the *Brownes Coffee Chill* was 186mg per 600mL serve, and the *Masters Ice Coffee* was 126mg per 600mL. From these two products, the average caffeine content of a 600mL iced coffee beverage was 156mg.

Total weekly caffeine consumption from coffee beverages was calculated. Firstly, weekly caffeine consumption from *usual* coffee beverages was obtained by

multiplying the caffeine content of each respondent's *usual* coffee beverage with the actual number of *usual* coffee beverages consumed during the week of the diary. Caffeine consumption from coffee beverages other than their *usual* coffee beverage was estimated based on values listed in Table 3.16. The addition of these two values to caffeine consumption from iced coffee beverages resulted in the weekly caffeine consumption from coffee beverages.

Table 3.5: Estimated Average Grams of Instant Coffee in a Teaspoon

instant coffee brand	weight (gms)					
	first	second	third	fourth	fifth	mean
level teaspoon						
International Roast	1.23	1.27	1.00	1.19	1.22	1.18
Nescafe 43 Blend	1.29	1.47	1.50	1.31	1.24	1.36
Moccona Indulgence	1.31	1.19	1.26	1.20	1.30	1.25
					grand mean	**1.26**
heaped teaspoon						
International Roast	2.49	2.23	2.26	2.20	2.06	2.25
Nescafe 43 Blend	2.00	2.07	2.09	2.11	1.92	2.04
Moccona Indulgence	1.94	2.08	2.01	2.05	1.97	2.01
					grand mean	**2.10**

Table 3.6: Caffeine Content of Caffeinated Instant Coffee

	brand of instant coffee	product label %m/m[a,b]	caffeine (mg) per 2gm serve[c]
1	Bushells	3.8	76
2	Bushells Pablo	4.0	80
3	Home Brand	3.8	76
4	International Roast	4.0	80
5	Maxwell House Special Cup	3.1	62
6	Moccona freeze dried	3.2	64
7	Nescafe Blend 43	3.5	70
8	Nescafe Gold Blend freeze dried	3.1	62
9	Nescafe Espresso	3.6	72
10	Savings	3.8	76
		mean	71.8

[a] stated on product label, as reported in the Caffeine Survey (1995)
[b] 1% m/m is equal to 10,000mg/kg
[c] value obtained from Caffeine Survey (1995)

Table 3.7: Estimated Average Caffeine Content in Instant Coffee by Teaspoon Measurement

teaspoon measure	conversion calculation	conversion factor	caffeine (mg) per serve
1/8 teaspoon	72 x (0.63 x 0.125)	0.08	6
½ teaspoon	72 x (0.63 x 0.5)	0.315	23
¾ teaspoon	72 x (0.63 x 0.75)	0.47	34
1 level teaspoon	72 x 0.63	0.63	45
1 heaped teaspoon	72 x 1.05	1.05	76
1½ teaspoons	72 x (0.63 + 0.315)	0.945	68
2 teaspoons	72 x (0.63 + 0.63)	1.26	91

Table 3.8: Caffeine Content of Decaffeinated Instant Coffee

	brand of decaffeinated instant coffee	product label %m/m	caffeine (mg) per 2gm serve
1	Café Hag instant coffee	2400 mg/kg	5
2	International Roast instant coffee	0.09% m/m	2
3	Moccona instant coffee granules	1600 mg/kg	3
4	Nescafe Decaf	0.14% m/m	3
		mean	3

Table 3.9: Estimated Average Caffeine Content in Decaffeinated Instant Coffee by Teaspoon Measurement

teaspoon measure	conversion calculation	conversion factor	caffeine (mg) per serve
½ teaspoon	3 x (0.63 x 0.5)	0.315	1
¾ teaspoon	3 x (0.63 x 0.75)	0.47	1.5
1 level teaspoon	3 x 0.63	0.63	2
1 heaped teaspoon	3 x 1.05	1.05	3
1½ teaspoons	3 x (0.63 + 0.315)	2.84	3
2 teaspoons	3 x (0.63 x 2)	3.78	4

Table 3.10: Caffeine Content of Percolated Coffee Beverages

	brand of roasted, ground coffee	product label %m/m	caffeine (mg) per 2gm serve
1	Bewleys dark roast filter coffee	1.3% m/m	26
2	Eduscho Gala No. 1 premium filter coffee	1.3% m/m	26
3	Gala Arabica ground coffee	1.5% m/m	30
4	Harris Italian Style expresso filter coffee	1.9% m/m	38
5	Hazelnut coffee beans	1.2% m/m	24
6	Peerless Hazelnut ground coffee	1.3% m/m	26
7	Vittoria ground coffee	1.3% m/m	26
		mean	28

Table 3.11: Estimated Average Caffeine Content in Percolated Coffee Beverages by Serve Size

roasted coffee serve size	conversion calculation[a]	conversion factor	caffeine (mg) per serve
½ teaspoon	28 x (1.625 x 0.5)	0.8125	23
1 teaspoon	28 x (1.625)	1.625	46
2 teaspoons	28 x (1.625 x 2)	3.25	91
1 dessert spoon	28 x (2.5)	2.5	70
2 dessert spoons	28 x (2.5 x 2)	5	140
recommended scoop	28 x (2.5)	2.5	70

[a] based on an average of 28mg of caffeine in 2gm of percolated roasted coffee

Table 3.12: Caffeine Adjustment for Drip/Filter Brewed Coffee Beverages

roasted coffee serve size	caffeine (mg) in percolated beverage	conversion calculation[a]	caffeine (mg) per serve
½ teaspoon	23	23 x (1.35)	31
1 teaspoon	46	46 x (1.35)	62
2 teaspoons	91	91 x (1.35)	123
1 dessert spoon	70	70 x (1.35)	95
2 dessert spoons	140	140 x (1.35)	189
recommended scoop	70	70 x (1.35)	95

[a] based on the estimated average caffeine content in percolated coffee beverages by serve size (see Table 3.11)

Table 3.13: Caffeine Content of Decaffeinated Percolated Coffee Beverages

	roasted coffee sample	product label %m/m	caffeine (mg) per 2gm serve
1	Gala special decaffeinated ground coffee	0.05% m/m	2
2	Melitta Coffee perfection lite - decaff style	0.12% m/m	2
3	Peerless ground coffee - Irish cream - decaf	0.05% m/m	2
4	Vittoria natural decaffeinated expresso ground	0.06% m/m	2
		mean	2

Table 3.14: Estimated Average Caffeine Content in Decaffeinated Percolated Coffee Beverages by Serve Size

roasted ground coffee serve size	conversion calculation[a]	conversion factor	caffeine (mg) per serve
½ teaspoon	2 x (1.625 x 0.5)	0.8125	2
1 teaspoon	2 x (1.625)	1.625	3
2 teaspoons	2 x (1.625 x 2)	3.25	6.5
1 dessert spoon	2 x (2.5)	2.5	5
2 dessert spoons	2 x (2.5 x 2)	5	10
recommended scoop	2 x (2.5)	2.5	5

[a] based on an average of 2mg of caffeine in 2gm of decaffeinated roasted coffee

Table 3.15: Caffeine Adjustment for Decaffeinated Drip/Filter Brewed Coffee Beverages

roasted coffee serve size	caffeine (mg) in percolated beverage	conversion calculation[a]	caffeine (mg) per serve
½ teaspoon	2	2 x (1.35)	3
1 teaspoon	3	3 x (1.35)	4
2 teaspoons	6.5	6.5 x (1.35)	9
1 dessert spoon	5	5 x (1.35)	7
2 dessert spoons	10	10 x (1.35)	13.5
recommended scoop	5	5 x (1.35)	7

[a] based on the estimated average caffeine content in decaffeinated percolated coffee beverages by serve size (see Table 3.14)

Table 3.16: Estimated Average Caffeine Content of Other Coffee Beverages

brewing method	caffeine (mg)[a] regular	decaff.	brewing method	caffeine (mg)[a] regular	decaff.
Instant	45	2	Turkish coffee	70	5
ground	70	5	filter/drip	95	7
flat white	70	5	filter/express	95	7
cappuccino	70	5	brewed	70	5
unknown	70	5	Jarrah Swiss coffee	24	-
plunge	70	5	Jarrah mocha	24	-
percolated	70	5	Caro	0	0
expresso	70	5	Italian coffee	70	5
caffe latte	70	5	Nature's Cuppa	0	0
Ecco	0	0	coffee bag	52	-
long black	70	5			

[a] value obtained from biochemical analysis of food sample by the Chemistry Centre of WA, as reported in the *1995 Caffeine Survey*

3.8.3.2 Tea Beverages

The Caffeine Survey (1995) was also used to convert self-reported tea consumption into caffeine intake. Initially, the caffeine content of each respondent's *usual* tea beverage was established. The weekly caffeine consumption from *usual* tea beverages was calculated by the multiplication of the caffeine content of the *usual* tea beverage by the actual number of *usual* tea beverages consumed during the week of the diary. A caffeine value of 52mg was applied to beverages made from tea bags, which was the mean calculated from the nine brands of tea bags listed in Table 3.17. For tea beverages made from loose tea leaves, a caffeine value of 51mg was used, being the mean caffeine value from the four loose tea brands listed in Table 3.18.

The caffeine content of tea beverages made from decaffeinated tea bags varied considerably. Although the Australian Food Standards Code 1992 does not specify a maximum caffeine level for products that naturally contain caffeine (e.g. tea and coffee), it does prescribe a standard for products that caffeine has been added or extracted (Caffeine Survey 1995). Decaffeinated instant coffee and roast coffee, for example, must not contain more than 0.3% and 0.1% of caffeine, respectively. There is, however, no standard for decaffeinated tea in the code, as is reflected in caffeine values of the three brands of decaffeinated tea bags (Table 3.17). As a result, 17mg

was the mean caffeine content applied to tea beverages made from decaffeinated tea bags. As no decaffeinated brands of loose tea were reported in the Caffeine Survey (1995), 17mg was also applied to tea beverages made from decaffeinated loose tea.

Other tea beverages and their respective caffeine content reported by respondents are detailed in Table 3.19. As with coffee consumption, an adjustment for caffeine intake was made for respondents who habitually left a proportion of their tea beverages. Specifically, the caffeine content of each beverage was adjusted by a factor of 0.8, representing consumption of only 80% of beverages. Weekly caffeine consumption from tea beverages was calculated by adding the weekly caffeine consumption from *usual* tea beverages and the weekly caffeine consumption from all other tea beverages.

Table 3.17: Estimated Average Caffeine Content of Tea Bags

	tea bag sample	product label %m/m[a,b]	caffeine (mg) per 2gm teabag[c]
	caffeinated		
1	Brooke Bond PG tips two cup size tea bag	3.1	62
2	Bushells blue label tea cup bags	2.8	56
3	Bushells extra strong round tea bags	2.8	56
4	Dilmah 100% tea cup bags	1.5	30
5	Earl Grey - Twinings tea bags	3.0	60
6	English Breakfast - Twinings tea bags	3.4	68
7	Nerada tea bags	2.0	40
8	Tetley tea chest tea bags	2.4	48
9	Tetley tea cup bags	2.5	50
		mean	52
	decaffeinated		
1	Lipton naturally decaf teabag (<4.0% caffeine)	0.38	8
2	Tetley decaffeinated tea bag (<0.3% caffeine)	2.1	42
3	Tetley decaffeinated tea bag (<0.3% caffeine)	0.05	1
		mean	17

[a] stated on product label, as reported in the *1995 Caffeine Survey*

[b] 1% m/m is equal to 10,000mg/kg

[c] value reported in the *1995 Caffeine Survey*

Table 3.18: Estimated Average Caffeine Content of Tea Made From Tea Leaves

	loose tea leaves sample	product label %m/m	caffeine (mg) per 2gm serve
1	Highfield tea	2.6	52
2	Home brand packet tea	2.2	44
3	Jasmine Tea - Ceylon	2.8	56
4	Tea leaves - Coles plain label	2.6	52
		mean	51

Table 3.19: Caffeine Content of Other Tea Beverages

tea beverage	caffeine (mg)	tea beverage	caffeine (mg)
herbal (not specified)	0	green barley leaf	0
tea bag	52	chinese tea	68
instant tea	108	chamomile & lemon myrtle	0
jasmine tea	51	japanese roasted tea	68
Madura (decaffeinated)	17	alfafa & red clover leaves	0
chamomile	0	japanese green tea	68
green tea	68	fennel herb tea	0
dandelion root tea	0	ginger tea	0
peppermint tea	0	Lipton's lemon & ginger tea	0
honey & wildflower	0	iced tea/Liptonice	41
Natures Cuppa tea bag	0	lemon tea	0
Lipton yellow tea bag	52	raspberry/ginseng tea	0
Twining's blackcurrant/lemon	0	lemon soother	0
Lipton's golden honey lemon tea	0	orange tea	0

3.8.3.3 Kola Drinks

The Caffeine Survey (1995) was also used to quantify the respondents' LQ/D data into total weekly caffeine intake from the consumption of kola drinks. The caffeine content of each kola beverage was calculated by multiplying the amount of caffeine per 1 mL of the product by the total mL consumed. Three brands of kola drinks reported by the respondents were not listed in the Caffeine Survey (1995): *Schweppes Kola, Western Country Kola and Farmland Kola.* A caffeine value of 10.2mg/100mL was applied for each of these kola drinks, which was the mean caffeine value of the three kola drinks listed in Table 3.20. Two brands of caffeine-

free kola were listed, each with <1mg of caffeine per 375ml serving. Therefore, the consumption of any caffeine-free kola beverage was treated as zero caffeine intake.

Table 3.20: Caffeine Content of Kola Drinks

	Kola Drinks	caffeine mg/L[a]	serving size (mL)	caffeine (mg) per serve	caffeine (mg) per 100mL
1	Coca Cola	96	375	36	9.6
2	Dr Pepper	110	354	39	11.0
3	Pepsi Cola	100	375	38	10.1
				mean	10.2
	Diet				
1	Diet Coca Cola	140	375	53	14.1
2	Diet Pepsi	110	375	41	10.9
				mean	12.5
	Caffeine Free				
1	Diet Pepsi	<1	375	<1	0
2	Diet Coke	<1	375	<1	0
				mean	0

[a] as reported on the product label

3.8.3.4 Total Weekly Caffeine Consumption

Weekly caffeine consumption was calculated by summing caffeine consumption from the three main dietary sources: 1) total weekly caffeine consumption from coffee beverages, 2) total weekly caffeine consumption from tea beverages and 3) total weekly caffeine consumption from kola drinks.

Data were also collected on caffeine contained in prescription or 'over-the-counter' medications. Caffeine intake from this source was also added to weekly caffeine consumption. The collective sum represents total weekly caffeine consumption.

3.8.4 Vitamin E Quantification

Weekly consumption of vitamin and/or mineral supplements was used to quantify weekly intake of supplemented vitamin E (IU) among the males. Occasionally, neither the brand nor composition of the multivitamin supplement was specified. Therefore, an average vitamin E content of multivitamin supplements was estimated and applied to these cases. Vitamin E in composition is tocopherol and the recommended daily intake is 10mg. All of the 12 brands of multivitamin supplements listed in the MIMS [Over The Counter] OTC (1998)(Table 3.21) contain tocopherol. Only eleven were used to calculate an average quantity of tocopherol in multivitamin supplements; *Nature's Way Mega Multi* (Roche Consumer) was excluded from this calculation as it contained 100mg of tocopherol, which was well in excess of the other 11 brands. The mean quantity of tocopherol in the eleven multivitamin supplements was 13 mg, which was used as the average quantity of tocopherol in non-specified multivitamin supplements.

Table 3.21: Tocopherol Composition of Multivitamin Tablets[a]

	brand name	manufacturer	tocopherol (mg)
1	Accomin Centrum Capsules	Whitehall	15
2	Bioglan Daily Plus	Bioglan	8.5
3	Macro M	Whitehall	15
4	Myadec	Warner Lambert	5
5	Nature's Way All-in-one	Roche	10
6	Nature's Way Executive Formula	Roche	15
7	Naturetime Multi Vitamins & Minerals	Blackmores	41.3
8	Penta-Vite Chewable MultiVitamins + Minerals	Roche	6
9	Pluravit	Bayer	10
10	Supradyn Effervescent	Roche	10
11	Vita-minis Multivitamin and Mineral	Herron	8.3
		mean	13
12	Nature's Way Mega Multi	Roche	100

[a] listed in MIMS OTC (1998, pp. 89-98)

3.9 Statistical Methods

Within the *Results* chapter, a section has been dedicated to each clinical outcome, with the format of each section being similar. Initially, descriptive statistics are presented of the dependent and independent variables, followed by univariate analysis of the data. Thereafter, a detailed account of the multivariate analysis process is presented, culminating in the final regression model, being followed by an assessment of the adequacy of the model. An interpretation of the final regression model concludes each section.

Data were analysed using 1) Statistical Program for Social Sciences (SPSS) for Windows, Version 9 (SPSS Inc, Chicago, USA), 2) Statistical Package for Interactive Data Analysis (SPIDA), Version 6 (Statistical Computing Laboratory Pty Ltd, New South Wales, Australia) and 3) Statistix Analytical Software, Version 4.0 (St Pauls, MN, USA). Tests of statistical significance were set at the 0.05 alpha level, and 95% confidence intervals were presented for the coefficients of the final regression models.

Histograms were used to examine the distribution of values for variables with a continuous data format. If the distribution was relatively normal, the mean was presented as the measure of central tendency and the standard deviation (SD) as the measure of dispersion; otherwise, the median and inter-quartile range (IQR) are reported. Bar charts and grouped percentages are used to report categorical variables.

Univariate analyses commenced with an examination of the dependent variable. For dependent variables of continuous data format, the distribution was assessed for normality. Non-normal distributions were transformed accordingly. The Pearson's correlation coefficient (r) and scatterplot were used to examine the linear association between a continuous dependent variable and continuous independent variable. The t-test and one-way analysis of variance (ANOVA) were used to ascertain whether group means differed, and the chi-square (χ^2) was used to assess associations between categorical variables.

Multiple linear regression was used for the analysis of factors in relation to a dependent variable of a continuous data format. Multiple logistic regression was used when the dependent variable was binary. For both, the significance of each independent variable was examined first using three different model fitting approaches: stepwise forward, backward elimination and the full model approach (forcing all of the independent variables into the model). Independent variables significant in all three model fitting approaches ($P<0.05$) were deemed to be significant main effects. In the event that an independent variable was significant in at least one model fitting approach but not in all three, hierarchical regression analysis was performed to determine whether its inclusion improved the fit of the model. At its completion, a first-order model of linear main effects was achieved. Meaningful cross-products or two-way interaction terms between main effects were then created and assessed in relation to the main effects model. Hierarchical regression analysis was employed to determine whether any given two-way interaction term was significant and improved the model fit.

Once having determined the final model, including significant interaction terms, the test assumptions and adequacy of the fitted model were examined. The methods used are detailed and commentated throughout each section of the *Results* chapter. Methods were also employed to identify potentially influential cases, which included outlying cases (outliers). The potential influence of each was examined in relation to the final model. Each section of the *Results* chapter concluded with an interpretation of the final model, the discussion taking into account potentially influential cases.

3.10 Variables

Each clinical outcome of interest was treated as a dependent variable, being oocyte production, fertilisation, pregnancy and pregnancy loss. Basal serum FSH level was also considered as a dependent variable, as it was shown to significantly affect oocyte production. The independent variables examined in relation to each of these clinical outcomes are listed in Table 3.22. Independent variables collected but not examined in any multivariate analysis are reported in the first section of the *Results* chapter (Table 3.23).

Table 3.22: Dependent and Independent Variables in Multivariate Analyses

dependent variable	independent variables		
no. of retrieved oocytes (oocyte production)	female:	patient:	age
			infertility aetiology
			basal FSH (IU/l)
		treatment:	eFSH (ampoules)
		lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)
			alcohol (std drinks/wk)
			daily stress
			IVF stress
			fruit and vegetable (serves/wk)
basal FSH (IU/l)	female:	patient:	age
			infertility aetiology
		lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)
			alcohol (std drinks/wk)
			fruit and vegetable (serves/wk)
			daily stress
fertilisation	female:	patient:	age
		lifestyle:	nicotine (mg/wk)
			proportion of life smoked
			caffeine (mg/wk)
			alcohol (std drinks/wk)
			fruit and vegetable (serves/wk)
			daily stress
			IVF stress
	male:	patient:	age
			oligozoospermia
			asthenozoospermia
			teratozoospermia
		lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)

Table 3.22: Dependent and Independent Variables in Multivariate Analyses (cont.)

dependent variables	independent variables		
	male:	lifestyle:	alcohol (std drinks/wk)
			fruit and vegetable (serves/wk)
			vitamin E supplementation
			daily stress
			IVF stress
β-hCG pregnancy	couple:		embryo quality (mCES)
16 days post-ET		treatment:	two-embryo transfer (2-ET)
	female:	patient:	age
			infertility aetiology
		lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)
			alcohol (std drinks/wk)
			fruit and vegetable (serves/wk)
			daily stress
			IVF stress
	male:	lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)
			alcohol (std drinks/wk)
			fruit and vegetable (serves/wk)
			daily stress
			IVF stress
<12 wk non-ectopic	couple:		embryo quality (mCES)
pregnancy loss	female:	patient:	age
		lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)
			fruit and vegetable (serves/wk)
	male:	patient:	age
		lifestyle:	nicotine (mg/wk)
			smoke years
			caffeine (mg/wk)
			alcohol (std drinks/wk)
			fruit and vegetable (serves/wk)

Table 3.23: Independent Variables Used To Describe the Study Sample (n_1)

independent variables		
demographics	female:	place of birth
		years in Australia
		education
	male:	place of birth
		years in Australia
		education
	couple:	health insurance
		geographical residence
clinical features	female:	gravidity
		parity
		body mass index
	male:	gravidity
		parity
		body mass index
	couple:	children in current relationship
		duration of infertility
treatment characteristics:	female:	basal oestradiol level (pmol/L)
		basal progesterone level (nmol/L)
		basal luteinizing hormone level (IU/l)
		ovarian follicle stimulation drug regime
		thickness of endometrium
		grade of endometrium
	male:	sperm source
		total sperm count ($x10^6$)
		motile sperm count ($x10^6$)
		progressive sperm count ($x10^6$)
		sperm activity
	couple:	treatment cycle attempt
lifestyle:	female:	smoking status
		vegetarian diet
	male:	smoking status
		vegetarian diet

3.11 Cotinine Validation of Self-Reported Tobacco Consumption

Biochemical validation of the self-reported smoking status was undertaken among a sub-sample of the study participants. Cotinine levels in samples of blood plasma from the females and semen from the males were measured by high performance liquid chromatography (HPLC).

3.11.1 Patient Recruitment

The Study Investigator asked a sub-sample of the couples who had undertaken IVF treatment between November 1997 and April 1998 and who had returned the LQ/Ds prior to TVOA for their permission to use previous collected blood plasma and semen samples. Informed consent was obtained in writing.

3.11.2 Samples

From each consenting male, the semen sample left-over after insemination was frozen. From the consenting females, plasma remaining after hormonal analysis of blood samples collected on Day 6 (or closest thereto) were also frozen. Periodically, cotinine analysis was undertaken on batches of these frozen samples of semen and plasma.

3.11.3 Instrumentation

The HPLC system was comprised of a pump, Chromato-Integrator, automated sample injector, and a printer for the plotting of the chromatograms. For separation a 25cm x 4.0mm column (i.d: Merck Lichrespher) was used, with 5μm particle size.

3.11.4 Mobile Phase

The mobile phase consisted of 270ml of water, 35ml of acetonitrile, 10ml sodium acetate and 200μL of triethylamine. This mixture was adjusted to pH 4.3 with acetic acid at room temperature and degassed in a ultrasonic water bath before use. The chromatographic column was equilibrated with the mobile phase prior to analysis of samples.

3.11.5 Laboratory Procedure

To each 1mL sample of plasma or semen, 15μL of the internal standard, trimethoprin (IS) was added. The assay protocol commenced with acidifying the sample with 70μL of 1.5mol/L H_2SO_4. Extraction was with 6ml of dichloromethane in a 15mL conical screw-capped tube by manual agitation for 3 minutes. Separation of the aqueous and organic phases was by centrifugation (5 minutes x 3500rpm). The organic phase (lower layer) was then discarded.

To the aqueous phase remaining after aspiration, 70 μL of 3.6mol/L of potassium hydroxide (KOH) was added, and then extracted with 6mL of dichloromethane and was agitated for five minutes. After centrifugation at 3500rpm for five minutes, the aqueous phase (upper layer) was aspirated and discarded. The remaining organic phase was transferred to a conical screw-capped tube, and 200μL of methanolic HCL 0.02M was added. The resultant solvent was evaporated to dryness under a gentle stream of nitrogen whilst positioned in a water bath at 45°C.

The sample residue was reconstituted with 200μL of the chromatographic mobile phase. Following degassing in an ultrasonic water bath, the reconstituted sample was agitated on a vortex mixer for 2 minutes, and then allowed to stand for 10 minutes. A batch of samples were then placed in an auto-sampler vial from which 80μL was injected into the chromatographic system. The flow rate was 1.5mL/min, and cotinine was detected by absorbance at 254nm, with sensitivity set at 0.01aufs.

3.11.6 Cotinine Determination

Prior to the analysis of each sample batch, a calibration curve was compiled by plotting the peak area ratios (cotinine/IS) against cotinine standards of 5, 10, 15, 20μL. Cotinine levels per sample were then calculated from the calibration curve.

3.11.7 Results

Consent for cotinine analysis of blood plasma was obtained from 46 female patients, and 20 male patients.

Of the 46 female patients, 22 had never smoked, 17 were ex-smokers, five were occasional smokers and two were regular smokers. Cotinine was detectable in the plasma of the two female smokers, but undetectable in the plasma of all of the other female patients, including the five occasional smokers.

Of the 20 male patients, 11 had never smoked, six were ex-smokers, two smoked occasionally and one was a regular smoker. Cotinine was detectable in the plasma of the smoker, and in the plasma of one of the occasional smokers. A cotinine level of 7.7ng/ml was also detected in the semen of a male who reported to have never smoked. Cotinine levels in blood and semen are similar magnitude within each individual (Vine et al. 1993), and 13.7ng/ml of cotinine in blood was suggested by Gilbert (1993) as the optimum cut-off point to discriminate between true smokers and non-smokers (Gilbert, 1993). Therefore, it is not improbable that this low level of cotinine (7.7ng/ml) detected in the semen of this non-smoker was due to passive smoke exposure. This is consistent with the findings of Pacifici et al. (1995), in which the mean cotinine levels in the semen of 43 non-smokers was 4.4 (SD 5.1). Furthermore, the median (95% CI) cotinine level in semen of smokers of 1-19 cigarettes per day was 220.7 (95% 61.6 - 381.6), and 20+ cigarettes per day was 602.3 (95% 322.4 - 904.6).

Chapter 4

Results

4.1 Study Participation

Rates of study participation for males and females are shown in Table 4.1. From within the study sample, specific groups were used to investigate each clinical outcome: oocyte production, fertilisation, pregnancy and pregnancy loss. For each group, male and female rates of study participation are also presented in Table 4.1.

Individuals were classified by level of study participation: total-, part- or non-participation. Total-participation refers to completion of the lifestyle questionnaire/diary in full, and part-participation to the return of a lifestyle questionnaire/diary that was completed but not in full (missing data). Non-return or return of a blank lifestyle questionnaire/diary is referred to as non-participation.

Table 4.1 Rates of Study Participation

group	n[a]	participation		
		total (%)	part (%)	non (%)
females				
study sample	351	281 (80.1)	5 (1.4)	65 (18.5)
oocyte production group	290	235 (81.0)	3 (1.0)	52 (17.9)
fertilisation group	213	175 (82.2)	1 (0.5)	37 (17.4)
pregnancy group	288	238 (82.6)	3 (1.0)	47 (16.3)
pregnancy loss group	88	76 (86.4)	2 (2.3)	10 (11.4)
males				
study sample	351	247 (70.4)	14 (4.0)	90 (25.6)
fertilisation group	213	152 (71.4)	11 (5.2)	50 (23.5)
pregnancy group	288	210 (72.9)	11 (3.8)	67 (23.3)
pregnancy loss group	88	69 (78.4)	3 (3.4)	16 (18.2)

[a] n varied due to response rate; patients' completion and return of LQ/D.

4.2 Study Sample Descriptives

In describing the study sample, data from 351 females (n_1) and 341 eligible males ($n_{1.1}$) have been used. Only couples of the study-sample that have included an eligible male have been described, $n_{1.1}$=341 couples.

Presented in Section 4.2.1 is a description of the study sample by variables not examined in the subsequent sections of this *Results* chapter.

4.2.1 Demographics

4.2.1.1 Place of Birth

The place of birth was ascertained for 81.2% of the 351 females (n_1), and for 74.8% of the 341 males ($n_{1.1}$). Over two-third of these females were born in Australia (68.1%), as were 64.7% of the males.

Of the 91 women born outside of Australia, 30.8% were born in United Kingdom and 17.6% in New Zealand. Almost one-thirds of the 90 males born outside of Australia (31.1%) were born in England (United Kingdom), with an additional 11.1% born in New Zealand and 8.9% born in Ireland. The birthplace of the remaining non-Australian born women and men is listed in Table 4.2.

4.2.1.2 Years in Australia

Data on the number of years that the 91 non-Australian born females had lived in Australia was provided by 85 of these women (93.4%). Of these 85 women, 29.4% had lived in Australia for 20+ years, 29.4% for 10≤20 years, 23.3% for 5≤10 years and 18.6% for <5 years. Of the 90 non-Australian born males, the number of years in Australia was available for 84 of these men (93.3%). Over one-half of these 84 men had lived in Australia for 20+ years (54.8%), with 22.6% having lived in Australia for 10≤20 years, 9.5% for 5≤10 years and 13.1% for <5 years.

Table 4.2: Birthplace of Non-Australian Born Individuals

Place of Birth	females n	males n	Place of Birth	females n	males n
England/UK	28	28	Lebanon	1	-
New Zealand	16	10	Liberia	1	-
Ireland	7	8	Macedonia	1	-
Mauritius	3	1	Philippines	1	1
South Africa	3	2	Romania	1	-
Croatia	2	1	Sri Lanka	1	2
Czechoslovakia	2	1	Thailand	1	1
Greece	2	-	Trinidad	1	-
Indonesia	2	2	USA	1	1
Italy	2	1	Yugoslavia	1	2
Malaysia	2	4	Algeria	-	1
Rhodes (Zimbabwe)	2	-	Canada	-	2
Singapore	2	1	Malta	-	1
Brunei	1	1	Netherlands	-	1
Burma	1	2	Norway	-	1
Hong Kong	1	1	Saudi Arabia	-	1
India	1	2	Scotland	-	4
Iran	1	-	Sweden	-	1
Iraq	1	1	Switzerland	-	1
Jamaica	1	-	Wales	-	3
Jordan	1	-	West Germany	-	1
	-	-	total	91	90

4.2.1.3 Education

Level of education was reported by 282 of the 351 females (n_1, 80.3%) and by 249 of the 341 males ($n_{1.1}$, 73.0%). A relatively high proportion of these individuals were tertiary educated, 29.8% and 36.9% of the women and men, respectively (Table 4.3).

Table 4.3: Distribution by Level of Education

level of education	females n (%)	males n (%)
<year 10	7 (2.5)	19 (7.6)
completed year 10	68 (24.1)	44 (17.7)
completed year 12	47 (16.7)	31 (12.4)
certificate, diploma or TAFE* qualification	76 (27.0)	63 (25.3)
tertiary qualification	84 (29.8)	92 (36.9)
total	282	249

* Technical and Further Education

4.2.1.4 Health Insurance

Over one-half of the 341 couples ($n_{1.1}$, 53.1%) had private health insurance that covered both the female and male. An additional 17.9% of the couples had private health insurance that covered the female but not the male. Only one couple had private health insurance that covered the male but not the female. Among 28.7% of the couples, neither the male nor female were covered by private health insurance.

4.2.1.5 Geographical Residence

More than three-quarters of the 341 couples ($n_{1.1}$, 77.8%) resided within the metropolitan area. The remaining 22.2% of couples lived within a country region of Western Australia.

4.2.2 Clinical Features

4.2.2.1 Gravidity and Parity

Of the 351 females (n_1), 222 had been pregnant prior to this IVF treatment cycle (63.2%), and 112 of the 351 women (n_1) had delivered (31.9%)(Table 4.2.3). Among the 341 males (n_1), gravidity and parity of their partner(s), past and present, was available for 326 (95.6%) and 331 (97.1%) of these men, respectively. Prior to this IVF treatment cycle, almost one-half of the males (47.9%) had had a partner that had been pregnant, and 29.0% had fathered a child.

Table 4.4: Distribution by Gravidity and Parity

	number of previous pregnancies				
	0 n (%)	1 n (%)	2 n (%)	3+ n (%)	total n
females					
gravidity	129 (36.8)	94 (26.8)	53 (15.1)	75 (21.4)	351
parity	239 (68.1)	73 (20.8)	17 (4.8)	22 (6.3)	351
males					
gravidity	170 (52.1)	69 (21.2)	45 (13.8)	42 (12.9)	326
parity	235 (71.0)	61 (18.4)	23 (6.9)	12 (3.6)	331

4.2.2.2 Children in Relationship

Sixty of the 341 couples ($n_{1.1}$) had a child within the current relationship (17.6%). Of these 60 couples, the majority had one child (88.3%), four couples had two children, two couples had three children and one couple had four children.

4.2.2.3 Duration of Infertility

Data on the duration of infertility was available for 318 of the 341 couples ($n_{1.1}$, 90.6%). The median years of infertility was 3.0 (inter-quartile range (IQR) 3.0). Of the 341 couples ($n_{1.1}$), 41.7% had been infertile for ≤2 years, 34.6% for 2≥5 years and 23.6% for >5 years.

4.2.2.4 Body Mass Index

Body mass index (BMI) was ascertained for 279 of the 351 females (n_1) in the study sample (79.5%), and for 227 of the 341 males ($n_{1.1}$, 66.6%). Among the females BMI ranged from 17 to 45, with a mean value of 24.2 (SD 4.9). Of these 279 females, 15.1% had a BMI of <20 (underweight), 53.8% a BMI of 20≤25 (optimal weight), 18.6% a BMI of 25≤30 (overweight) and 12.5% a BMI of 30+ (obese). Among the males, BMI ranged from 18 to 38, with a mean value of 26.3 (SD 3.4).

4.2.3 Treatment Characteristics

4.2.3.1 Treatment Cycle Attempt

It was the first IVF treatment cycle for 63.0% of the 341 couples ($n_{1.1}$). For 16.1% of the 341 couples, it was the second attempt at an IVF treatment cycle, and at least the third for 20.8% of the couples.

4.2.3.2 Female Basal Hormone Levels

Of the 351 females (n_1) serum basal E_2, P_4 and LH levels were available for 348 of the women (96.7%)(Table 4.5 and Figure 4.1).

Table 4.5: Descriptive Statistics for Female Basal Serum Hormone Levels

basal serum	n	min	max	mean	median	IQR
E_2 (pmol/L)	348	5	794	158	153	109
P_4 (nmol/L)	348	0.3	12.4	2.4	2.2	1.1
LH (IU/l)	348	0.1	37.4	5.1	4.9	3.1

4.2.3.3 Female Drug Regime

Of the 351 women in the study sample (n_1), 317 (90.3%) undertook the flare regimen for ovarian follicle stimulation. Of these 317 women, most received a combination of Lucrin and Metrodin (91.5%). Ovarian follicle stimulation was by the down-regulation regimen for 34 of the 351 women (n_1, 9.7%).

4.2.3.4 Endometrium

Measurements of the endometrium on the day of the hCG injection were available for 212 of the 351 females (n_1, 60.4%). Endometrial thickness ranged from 6mm to 19mm (Figure 4.2), with a mean value of 11.4mm (SD 2.2). Of the 212 women, the endometrium was graded B among 51.4% and graded C among 47.2%. Only three women had a grade A endometrium (1.4%).

Figure 4.1: Histograms of Female Basal Hormones

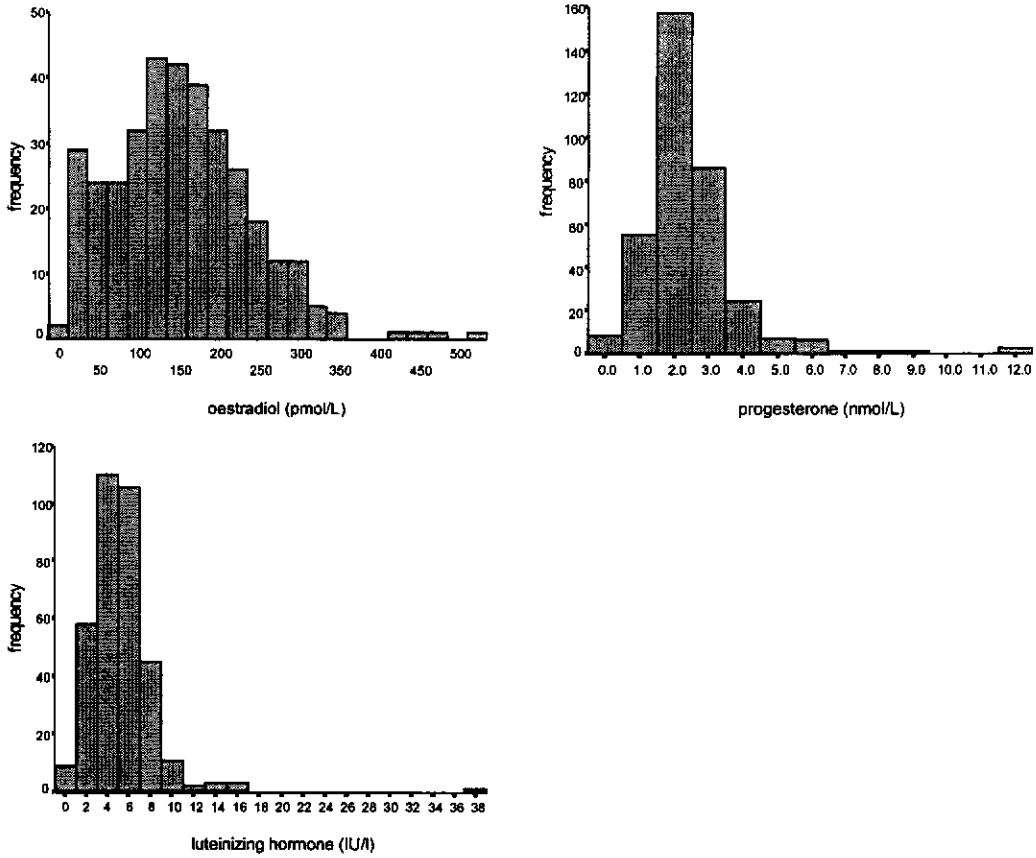
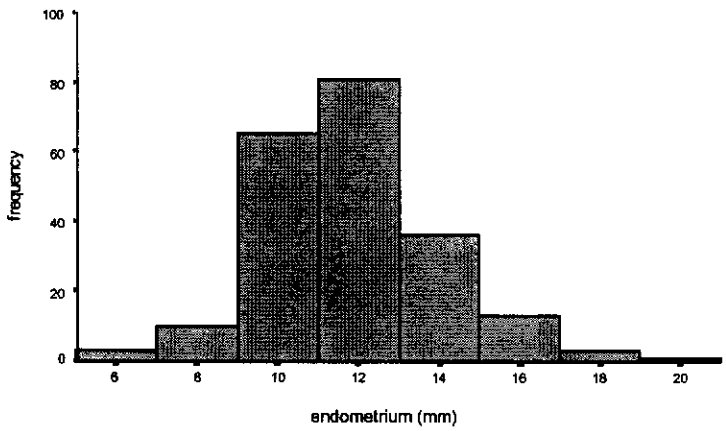


Figure 4.2: Histogram of Endometrial Thickness



4.2.3.5 Sperm Source

Of the 341 couples in the study sample (n_1), 324 couples underwent TVOA that resulted in the retrieval of at least one oocyte (95.0%). The majority of these 324 couples (91.7%) had a fresh ejaculate for insemination. Five couples (1.7%) used a thawed cryopreserved semen sample. Surgical retrieval of sperm was undertaken in the remaining 19 couples (6.6%): 12 MESA and seven TESA. With the exception of two of these 19 couples, whereby fresh sperm aspirates were used, retrieved sperm had been cryopreserved, and then thawed prior to insemination.

4.2.3.6 Semen Sample Characteristics

Data on the semen sample were available for 300 of the 324 couples who had oocyte insemination (92.6%). Descriptive statistics for the semen sample characteristics are presented in Table 4.6, and their respective distributions presented in Figure 4.3. As shown, sperm counts were highly variable, with semen samples containing less than 1 million sperm per millilitre (10^6/ml) to the extreme of one sample that contained 358 $\times 10^6$/ml.

Table 4.6: Descriptives of Semen Samples

	spermatozoa ($\times 10^6$/ml)					
	n	min	max	mean	median	IQR
total count	300	<1	358	72	60	79
motile count	300	<1	308	47	39	62
progressive motile count	300	<1	308	34	20	45

4.2.3.7 Sperm Activity

The bar chart in Figure 4.4 shows the frequency of couples by sperm activity.

Figure 4.3: Histograms of Semen Sample Characteristics

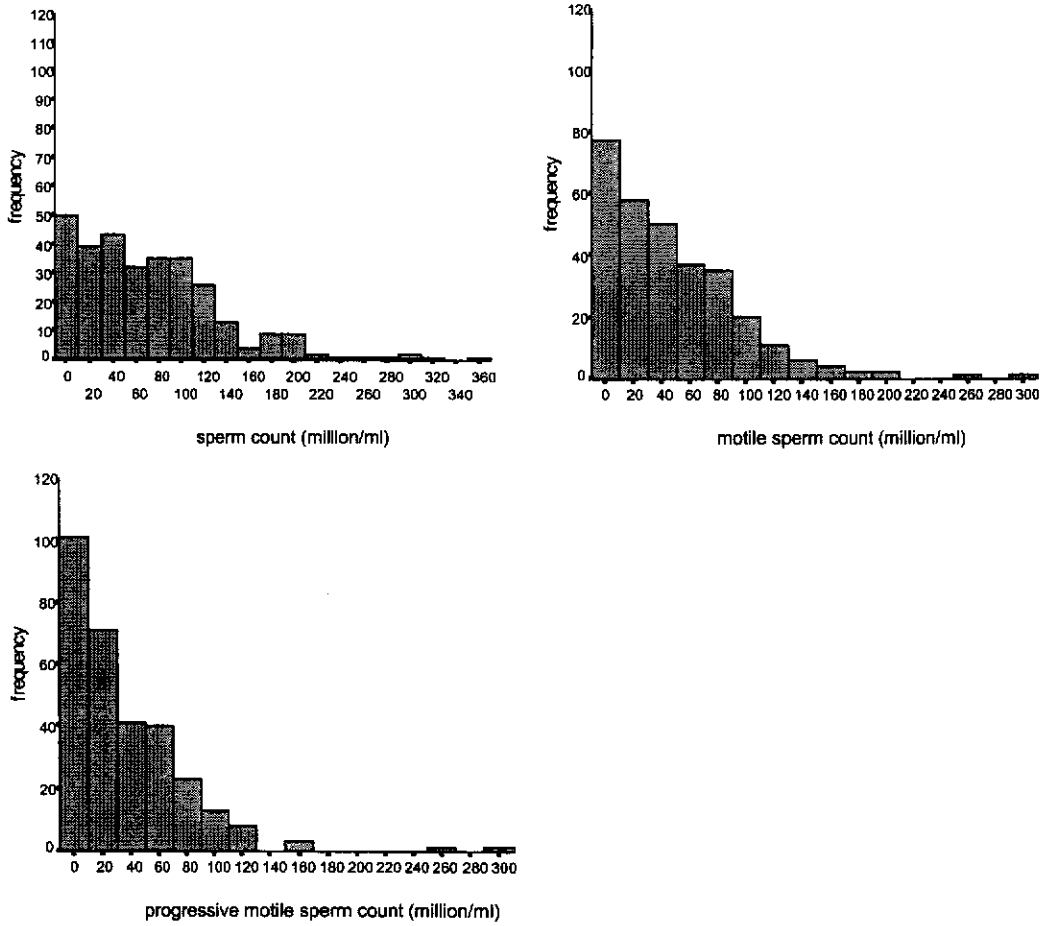
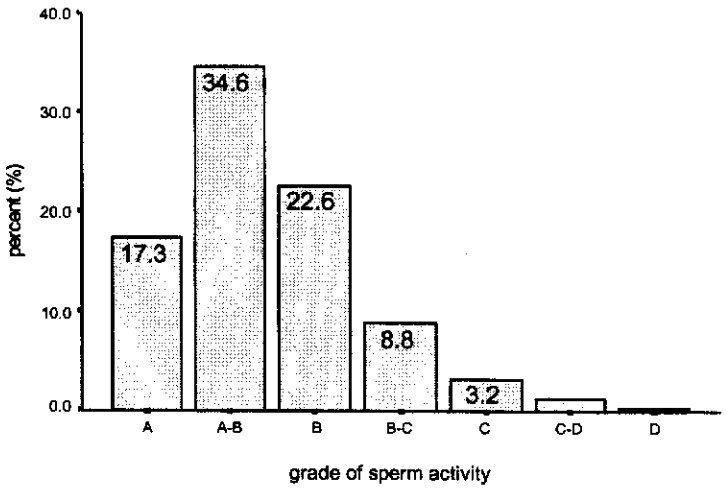


Figure 4.4: Bar Graph of Sperm Activity

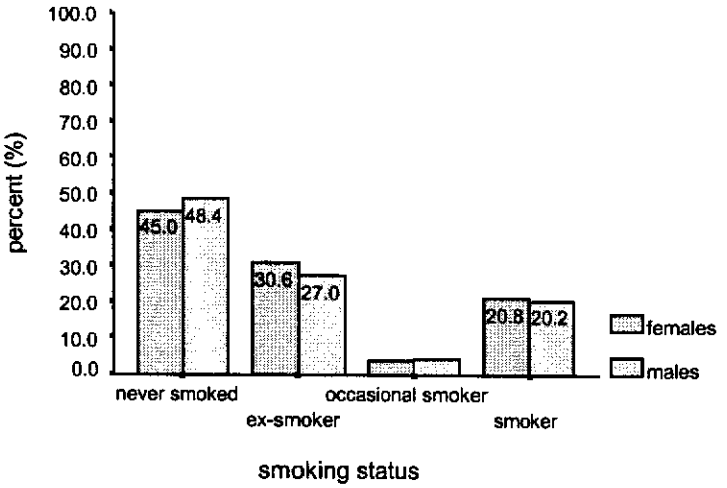


4.2.4 Lifestyle

4.2.4.1 Smoking Status

Of the 351 females in the study sample (n_1), the smoking status of 307 was established (87.5%). As shown in Figure 4.5, 20.8% were smokers and 3.6% smoked occasionally. Of the 341 males ($n_{1.1}$), the smoking status was known among 252 (73.9%). Male smokers comprised 20.2% of the male study sample, and 4.4% were occasional smokers.

Figure 4.5: Bar Chart of Female and Male Smoking Status



4.2.4.2 Vegetarian Diet

Of the 351 females (n_1) and 341 males ($n_{1.1}$) in the study sample, data on diet was available for 286 (81.5%) and 255 (74.7%), respectively. Of these 286 women, 15 reported to have a vegetarian diet (4.3%). Nine of these 15 women described themselves as a semi-vegetarian (no red meat but consume poultry and fish), four as a lacto-ovo vegetarian (no animal flesh but consume eggs and dairy products) and two as lacto-vegetarians (no animal flesh or egg but consume dairy products). Of the 255 males ($n_{1.1}$), only four reported to have a vegetarian diet (1.2%), of which, one described himself as a semi-vegetarian, two as lacto-ovo vegetarians and one an lacto-vegetarian.

4.3 Oocyte Production

Of the 351 women who commenced IVF treatment (n_1), 334 underwent TVOA (n_2, 95.2%)(Figure 4.6). Of these 334 women (n_2), 300 received the flare regimen ($n_{2.1}$) and 34 received the down-regulation regimen. As the down-regulation regimen affects basal FSH levels, the 34 women who received such treatment were excluded from the data analysis of oocyte production. Of the 300 women who had received the flare regimen ($n_{2.1}$), seven had only one ovary, and as this was likely to impact on the number of oocytes retrieved, these seven women were also excluded. Among the remaining 293 women ($n_{2.2}$), three women were receiving medication that was also known to affect FSH levels; two due to the use of oral contraceptives and one patient due to the administration of exogenous progesterone. Following the exclusion of these three women, data from a total of 290 women ($n_{2.3}$) were used to examine variables that affect oocyte production, the total number of oocytes retrieved through TVOA.

4.3.1 Descriptive Statistics

4.3.1.1 Oocyte Production

The distribution of the total number of oocytes retrieved is displayed in Figure 4.7. As evident, this distribution was right-skewed. Of the 290 women ($n_{2.3}$), three had in excess of 40 oocytes retrieved. Of note, no oocytes were retrieved from two of the 290 women ($n_{2.3}$). The number of oocytes retrieved from the remaining 288 women ranged from one to 69, with a median value of 10.0 (IQR 11.0).

Figure 4.7: Histogram and Boxplot of Oocyte Production

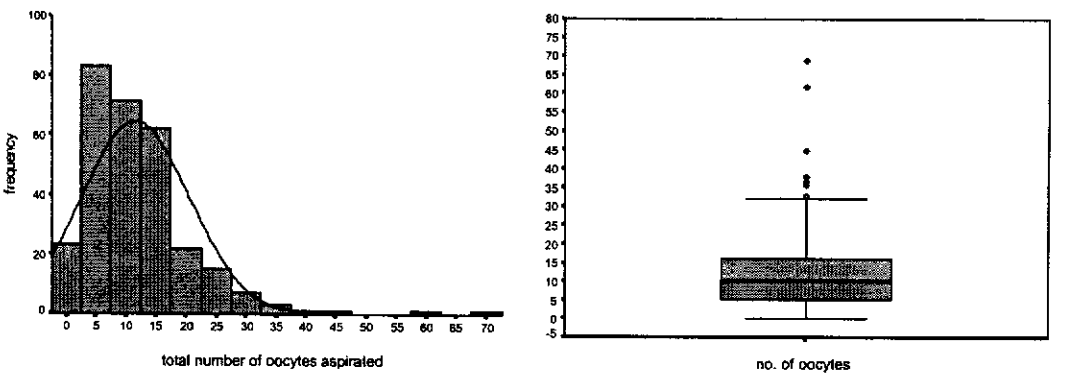
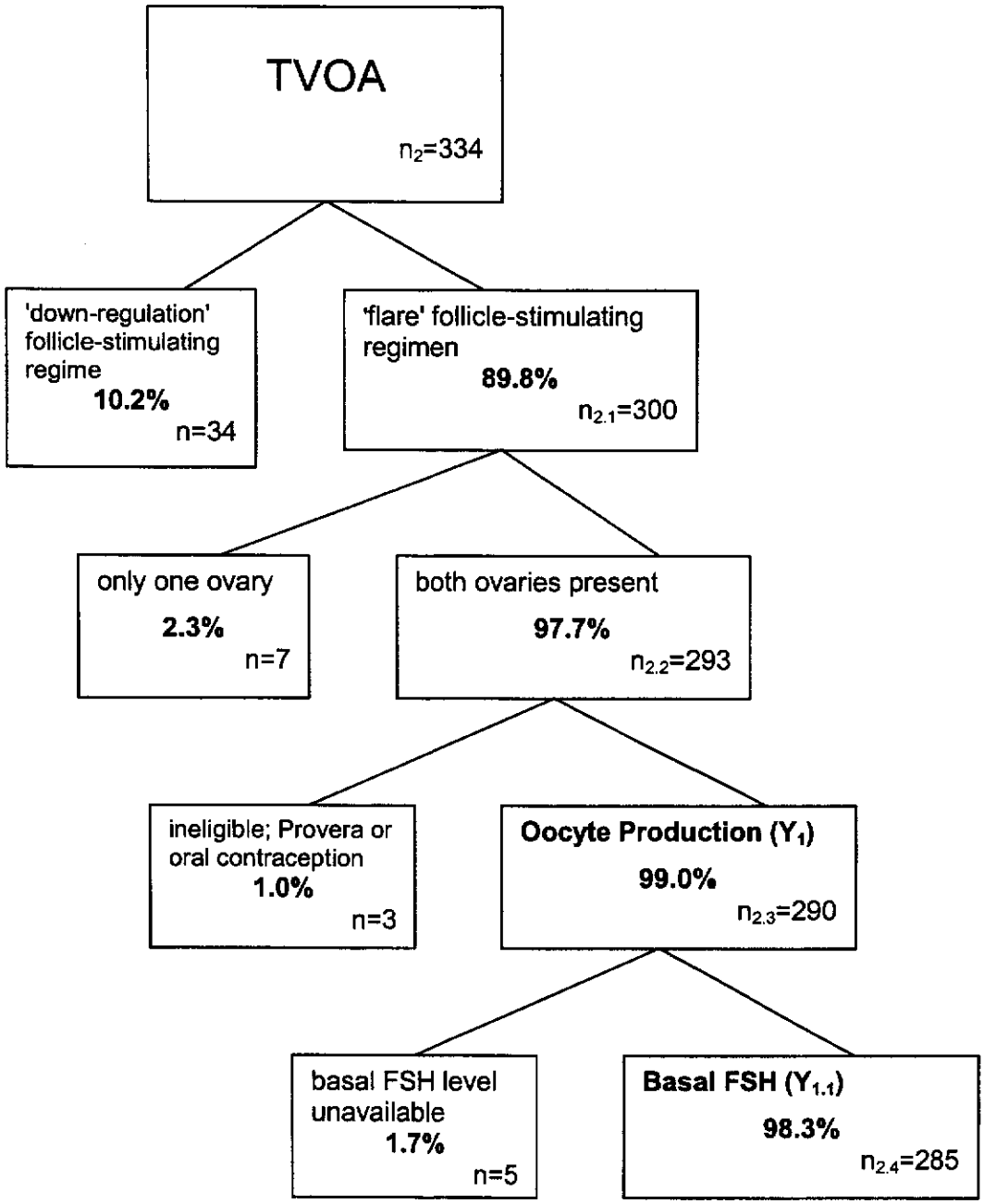


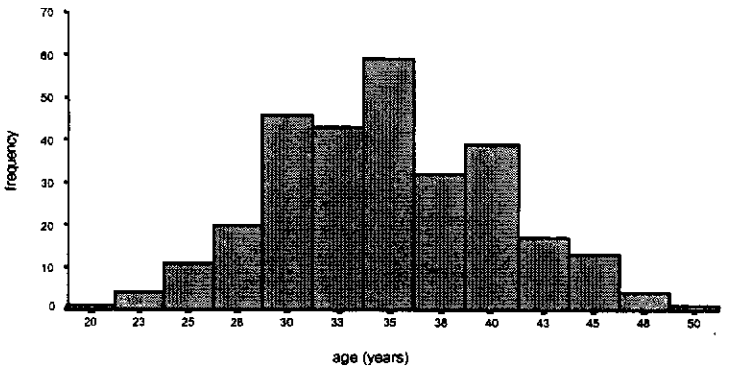
Figure 4.6: Flowchart of Oocyte Production and Basal FSH Sub-Samples



4.3.1.2 Patient and Clinical Features

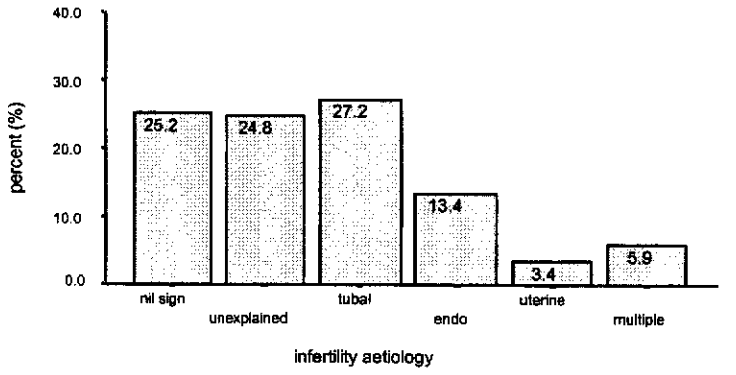
The 290 women ($n_{2.3}$) ranged in age from 21 to 49 years, with a mean age of 34.7 (SD 5.4)(Figure 4.8). One-fifth of the women were 40+ years of age (20.3%).

Figure 4.8: Histogram of Age



Distribution of the 290 women ($n_{2.3}$) by infertility aetiology is presented in Figure 4.9.

Figure 4.9: Bar Chart of Infertility Aetiology

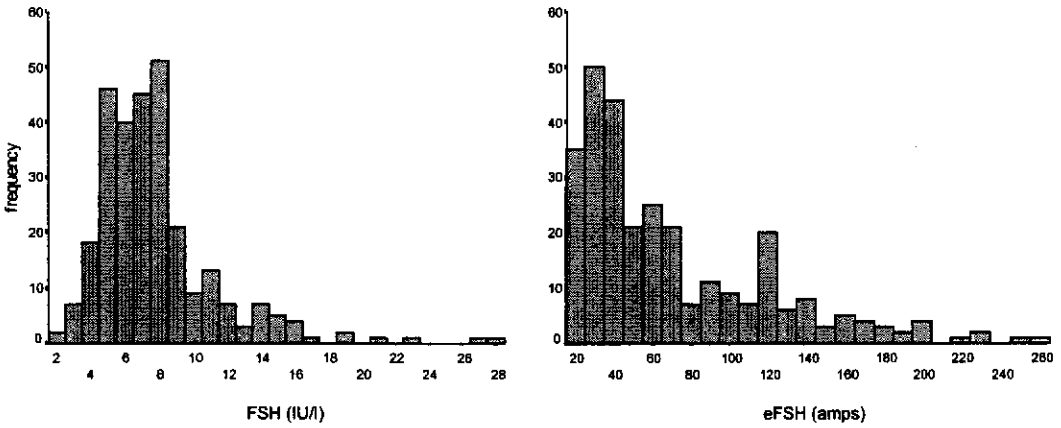


nil sign = infertility of nil significance
endo = endometriosis

Of the 290 women ($n_{2.3}$), 285 had their basal FSH level measured ($n_{2.4}$, 98.3%). As shown in Figure 4.10, the distribution of basal FSH values is right-skewed. Of the 285 women ($n_{2.4}$), basal FSH levels ranged from 2.0 to 28.3IU/l, with a median value of 7.1IU/l (IQR 3.2).

The total number of ampoules of exogenous follicle stimulating hormone (eFSH) or other gonadotrophins administered for ovarian follicle stimulation was examined. Dosage varied considerably among the 290 women, as values ranged from 15 to 264 ampoules. Although the median number of ampoules was 51.5 (IQR 64.5). As evident from Figure 4.10, the distribution of eFSH is right-skewed.

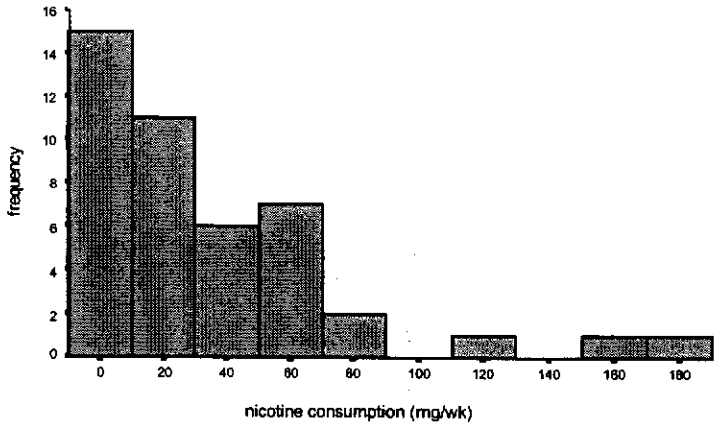
Figure 4.10: Histograms of FSH and eFSH



4.3.1.3 Lifestyle Factors

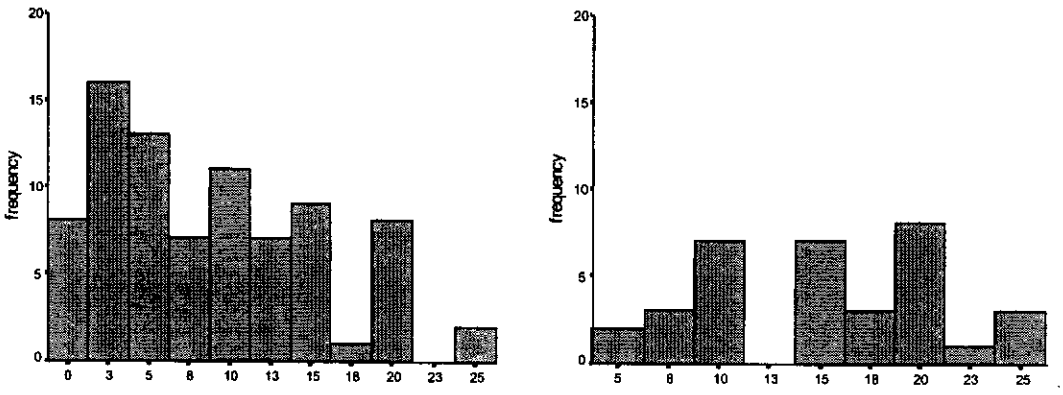
Nicotine consumption was ascertained for 236 of the 290 women ($n_{2.3}$, 81.4%;). The distribution of nicotine consumption among the occasional and regular smokers is presented in Figure 4.11. Three of the ten occasional smokers did not consume any nicotine over the week in question. Among the other seven occasional smokers and the regular smokers, nicotine consumption ranged from 0.7 to 175.0mg per week (mg/wk), and the median value was 24.0 (IQR 46.0).

Figure 4.11: Histogram of Nicotine Consumption Among Occasional and Regular Smokers



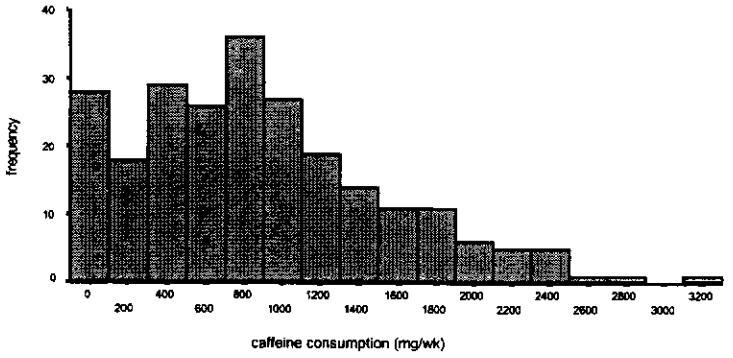
The total number of years of tobacco consumption (smoke years) was reported by 236 of the 290 women ($n_{2.3}$, 81.4%;). The distribution of smoke years among the female ex-smokers is displayed in Figure 4.12, as is that among the female regular smokers. Among the female ex-smokers, years of tobacco consumption ranged from one week to 25 years (median 7.0, IQR 10.2), and between six and 25 years among the female regular smokers (mean 15.2, SD 5.7).

Figure 4.12: Histograms of Smoke Years



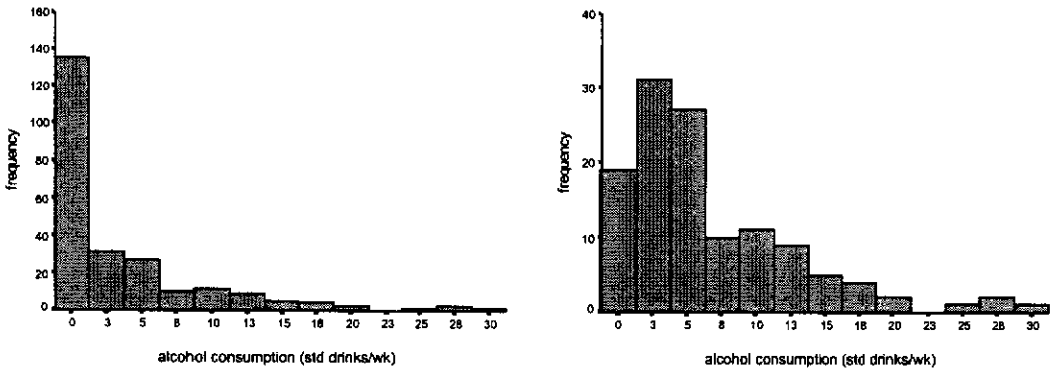
For 238 of the 290 women ($n_{2.3}$, 82.1%), the weekly levels of caffeine consumption were obtained; the distribution of values is displayed in Figure 4.13. Of these 238 females, 227 consumed caffeine during the week of the diary (95.4%), with consumption ranging from four to 3186mg/wk. Median caffeine consumption was 800 mg/wk (IQR 857).

Figure 4.13: Histogram of Caffeine Consumption



Of the 290 women ($n_{2.3}$), alcohol consumption was ascertained for 238 (82.1%). During the week of the diary, almost half of the women did not consume alcohol (48.7%). A further 32.4% of the women consumed less than seven standard drinks over the week. As a consequence, the distribution of weekly alcohol consumption is severely right-skewed (Figure 4.14). Of the 122 female alcohol consumers, consumption ranged from 0.3 to 30 standard drinks per week (std dks/wk), and the median was 4.8 (IQR 7.0).

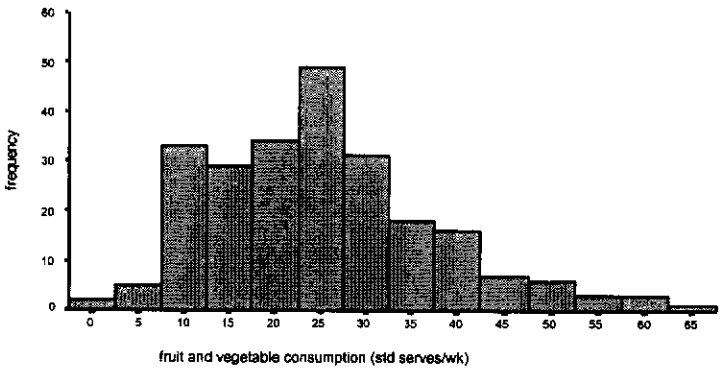
Figure 4.14: Histograms of Alcohol Consumption



all respondents (n=238) alcohol consumers (n=122)

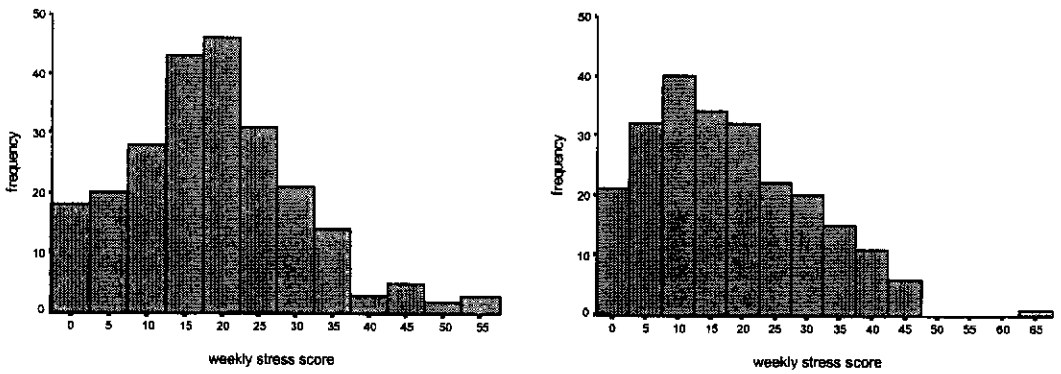
Of the 290 women ($n_{2.3}$), fruit and vegetable consumption was known for 237 (81.7%); the distribution of values is displayed Figure 4.15. The mean serves of fruit and/or vegetable consumed per week was 24.8 (SD 12.1), an average of 3.5 serves per day.

Figure 4.15: Histogram of Fruit and Vegetable Consumption



Weekly stress scores were available for 234 of the 290 women ($n_{2.3}$, 80.7%). As shown in Figure 4.16, stress scores from daily living and from IVF treatment varied considerably. Eleven of the 234 females reported zero daily stress for the week (4.7%). Although 57 was the highest daily stress score, the mean value was 19.0 (SD 11.5). The distribution of IVF stress is right-skewed (Figure 4.16). Nine of the 234 females reported zero IVF stress (3.8%), five of which also reported zero daily stress. Although an IVF stress score of 65 was reported by one woman, the median was 16.8 (IQR 17.0).

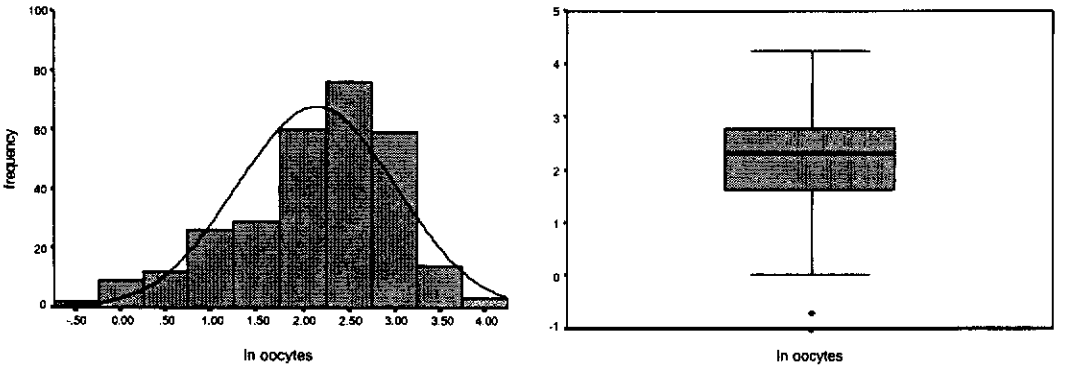
Figure 4.16: Histograms of Weekly Stress Scores



4.3.2 Univariate Analysis

As a preliminary investigation of oocyte production, univariate analysis was undertaken. Firstly, normality of the oocyte distribution was examined (Figure 4.7). As this distribution was right skewed, the oocyte variable was transformed into that of the natural logarithm. The natural logarithm (ln) of the number of oocytes, ln(oocytes), had a relatively normal distribution (Figure 4.17). Consequently, ln(oocytes) was used in the subsequent data analysis of oocyte production.

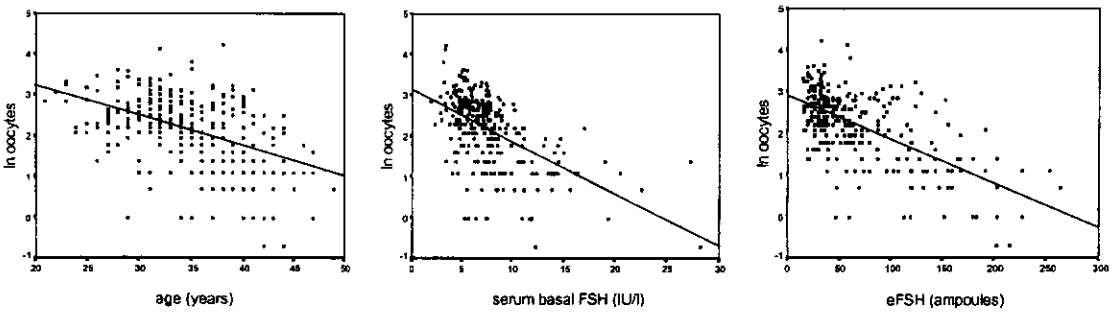
Figure 4.17: Histogram and Boxplot of ln(oocytes)



Each independent variable was examined separately in relation to oocyte production (ln(oocytes)). Scatterplots and Pearson's correlation coefficients (r) were used to investigate linear associations between continuous independent variables and ln(oocytes) (Figure 4.18).

A moderate negative association was evident between ln(oocytes) and age (r=-0.47, P<0.001). More specifically, ln(oocytes) decreased with age. In a negative manner, FSH and eFSH were also moderately associated with ln(oocytes), r=-0.54 (P<0.001) and r=-0.62 (P<0.001), respectively. Oocyte production decreased with increasing basal FSH levels, and with an increasing quantity of eFSH.

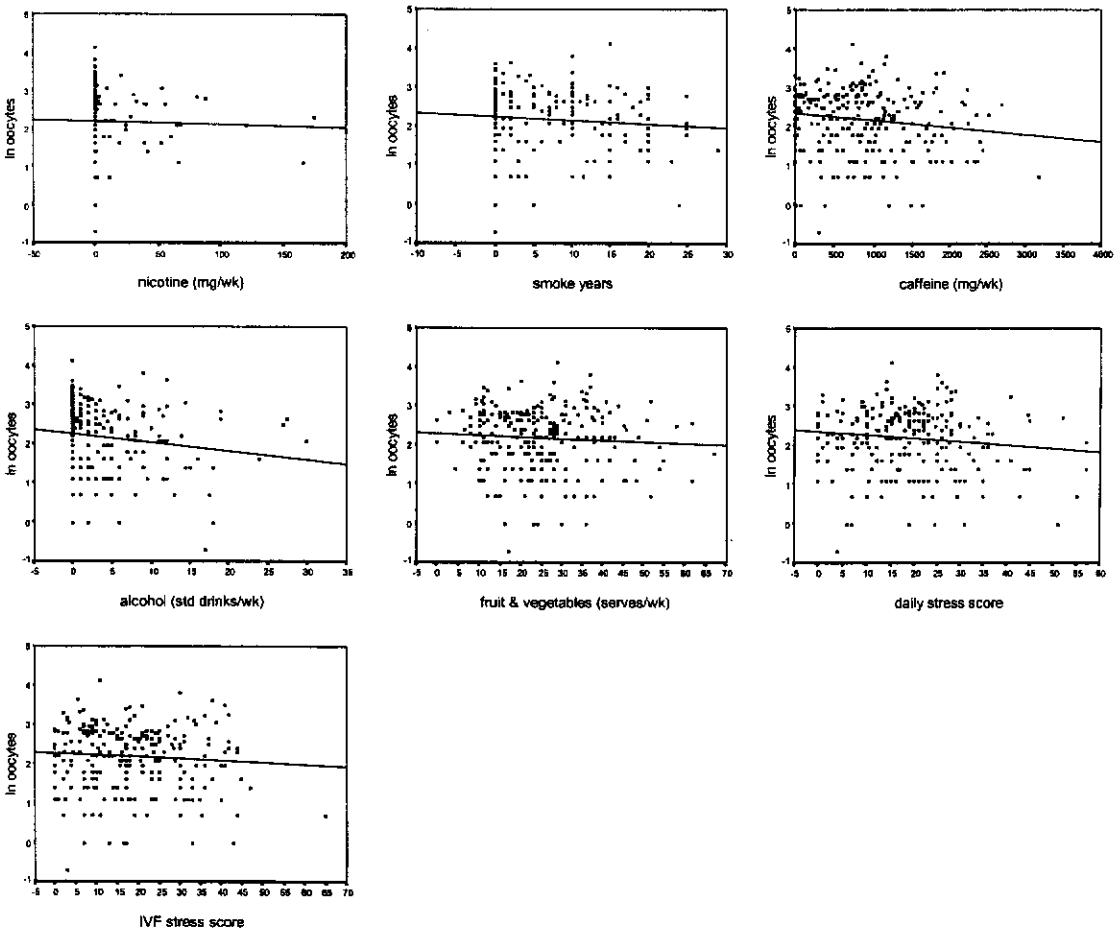
Figure 4.18: Scatterplots of ln(oocytes) vs Patient and Treatment Variables



As infertility aetiology is an ordinal variable with six levels, one-way ANOVA was used to compare the group means. A significant F statistic resulted ($F_{5, 284}$=3.07, P=0.010), indicating that the mean ln(oocytes) of at least two infertility groups was significantly different. Tukey's multiple comparison test revealed that women with unexplained infertility had significantly fewer oocytes aspirated, on average, than women whose infertility was of nil significance (P<0.05).

A scatterplot of ln(oocytes) and each of the lifestyle variables is presented in Figure 4.19. No linear association was apparent between ln(oocytes) and nicotine consumption (r=-0.02, P=0.74), nor between ln(oocytes) and smoke years (r=-0.08, P=0.19). Negative linear associations were suggested, albeit weak, between ln(oocytes) and caffeine consumption (r=-0.15, P=0.02), and alcohol consumption (r=-0.15, P=0.02). No linear association was evident between ln(oocytes) and fruit and vegetable consumption (r=0.07, P=0.31). Neither daily stress nor IVF stress was linearly associated with ln(oocytes), r=-0.12 (P=0.07) and r=-0.07 (P=0.24), respectively.

Figure 4.19: Scatterplots of ln(oocytes) and Lifestyle Variables



4.3.3 Multiple Linear Regression Analysis

Multiple linear regression (MLR) was used to simultaneously examine the effects of patient, treatment and lifestyle variables on ln(oocytes). The initial goal was to obtain a first-order model, a model of linear main effects. Three model fitting strategies were used: stepwise forward, backward elimination and the full model approach, which involved forcing all of the independent variables into the model. As shown in Table 4.7, age, basal FSH and eFSH were significant in all three models, and daily stress was significant in two of the three models: stepwise forward and backward elimination.

Table 4.7: MLR Model Fitting Strategies on ln(oocytes)[a]

	model fitting strategy		
variable	full model *P* value	stepwise forward *P* value	backward elimination *P* value
age	0.004	0.008	0.008
basal FSH (IU/l)	<0.001	<0.001	<0.001
eFSH (ampoules)	<0.001	<0.001	<0.001
daily stress	-	0.022	0.022

[a] only variables with a $P<0.05$ are displayed

Age, basal FSH and eFSH were significant all three models derived from the different model fitting strategies; therefore, all were deemed to be significant main effects. Daily stress was significant in only two of the three models. Therefore, hierarchical regression analysis was undertaken to determine whether the addition of daily stress improved the fit of the model comprised of the significant main effects: age, basal FSH and eFSH. The F change statistic was used to ascertain whether daily stress significantly improved the model fit. The addition of daily stress to the main effects model generated an F change statistic of 4.773, with 1 and 227 degrees of freedom (df)(P=0.030); evidence that daily stress was significant in relation to oocyte production, and therefore included in the final model of main effects (Table 4.8).

Table 4.8: MLR Model of Main Effects on ln(oocytes)[a]

	coefficient	std error	*P* value
constant	4.129	0.309	<0.001
age	-0.025	0.009	0.009
basal FSH (IU/l)	-0.069	0.013	<0.001
eFSH (ampoules)	-0.059	0.001	<0.001
daily stress	-0.008	0.003	0.030

[a] R^2=0.456, adjusted R^2=0.446, $F_{4, 227}$=47.54, $P<0.001$

Next, an investigation of interrelationships between the main effects on oocyte production was conducted. A two-way interaction term was created for each dual combination of the main effect variables (Table 4.9).

Table 4.9: Two-Way Interaction Terms

	age	**basal FSH**	**eFSH**	**daily stress**
age	-	age*bFSH	age*eFSH	age*stdl
basal FSH	-	-	bFSH*eFSH	bFSH*stdl
eFSH	-	-	-	eFSH*stdl
daily stress	-	-	-	-

A full model comprising the main effects and all of the two-way interaction terms was generated. Of the interaction terms, only age*eFSH was significant (P=0.028). Hierarchical regression analysis was then performed; each interaction term in isolation was examined to assess whether it improved the fit of the main effects model. In each analysis, the F change and its corresponding P value was documented (Table 4.10). As before, age*eFSH was shown to significantly improved the fit of the model (P<0.025). As a result, age*eFSH was added to the main effects model of age, basal FSH and eFSH.

The final MLR ln(oocytes) model is presented in Table 4.11.

Table 4.10: F-test of Two-Way Interaction Terms in the ln(oocytes) Main Effects Model

variables in the model[a]					F change	P value
age	**bFSH**	**eFSH**	**dl stress**		-	-
age	bFSH	eFSH	dl stress	age*bFSH	0.001	0.979
age	**bFSH**	**eFSH**	**dl stress**	**age*eFSH**	**5.103**	**0.025**
age	bFSH	eFSH	dl stress	age*stdl	0.889	0.347
age	bFSH	eFSH	dl stress	bFSH*eFSH	1.971	0.162
age	bFSH	eFSH	dl stress	bFSH*stdl	1.770	0.185
age	bFSH	eFSH	dl stress	eFSH*stdl	<0.001	0.986

[a] model includes constant

Table 4.11: Final MLR ln(oocytes) Model[a]

	coefficient	95% CI lower, upper	std error	*P* value
constant	3.338	2.422, 4.254	0.465	<0.001
age	-0.002	-0.029, 0.025	0.014	0.859
basal FSH (IU/l)	-0.072	-0.098, -0.047	0.013	<0.001
eFSH (ampoules)	-0.007	-0.004, 0.019	0.006	0.226
daily stress	-0.007	-0.014, <0.001	0.003	0.039
age*eFSH	<0.001	-0.001, <0.001	<0.001	0.025

[a] R^2=0.468, adjusted R^2=0.456, ANOVA $F_{5, 226}$=39.74, P<0.001

4.3.4 Model Diagnostics

With regard to the main assumptions of MLR, the goodness-of-fit and sensitivity of the ln(oocytes) model were examined.

4.3.4.1 Overall Fit of the Model

The adjusted coefficient of multiple determination (adjusted R^2) is a measure of the fit of the model. Specifically, it is the variability of the dependent variable that is explained by the independent variables after taking into account the number of independent variables in the model. The adjusted R^2 for the ln(oocytes) model was 0.456. Therefore, 45.6% of the variation in the number of oocytes was explained by age, basal FSH, eFSH and daily stress.

4.3.4.2 Non-Linear Regression Function

Non-linearity of a regression model is evident when the residuals vary in a systematic manner portraying a curvilinear relationship. As the residuals shown in Figure 4.20 are randomly scattered, there is no evidence that the regression function of the ln(oocytes) model is non-linear. The standardised residuals were also plotted for each main effect (Figure 4.21). There is no evidence that any of the relationships are non-linear.

Figure 4.20: Scatterplot of Standardised Residuals vs Standardised Predicted Values

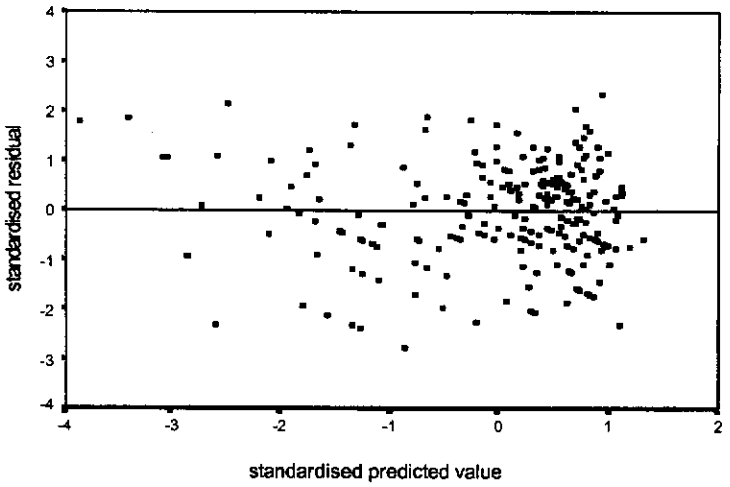
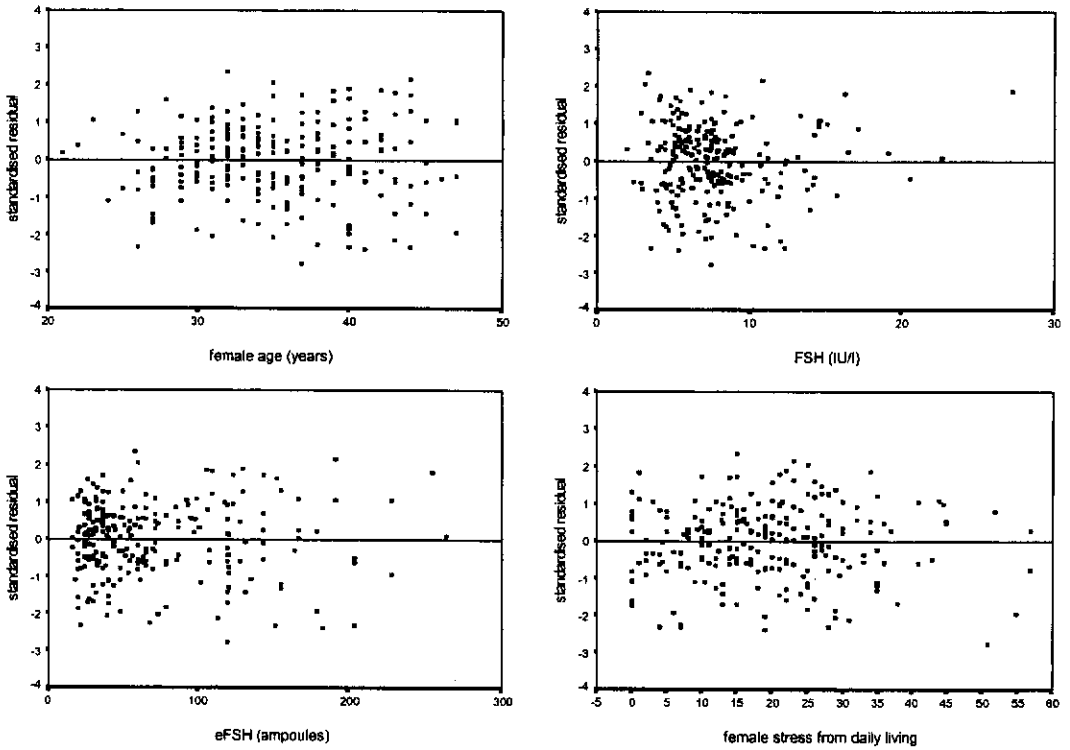


Figure 4.21: Scatterplots of Standardised Residuals vs Main Effects



4.3.4.3 Heteroscedasticity

Heteroscedasticity, non-constant error variance, was investigated (Figure 4.20). The scatter of points was relatively random, implicating a relatively constant variance of ln(oocytes).

4.3.4.4 Non-Normality

The assumption of non-normality was investigated. There was no systematic departure of the plotted regression standardised residuals from the linear trend (Figure 4.22). The distribution of the standardised residuals was relatively normal (Figure 4.23). Therefore, relative normality of the dependent variable was satisfied.

Figure 4.22: Normal P-P Plot of Regression Standardised Residuals

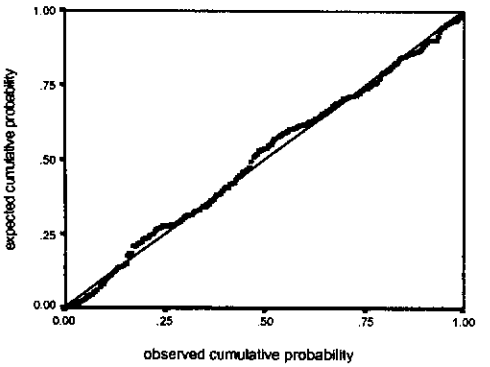
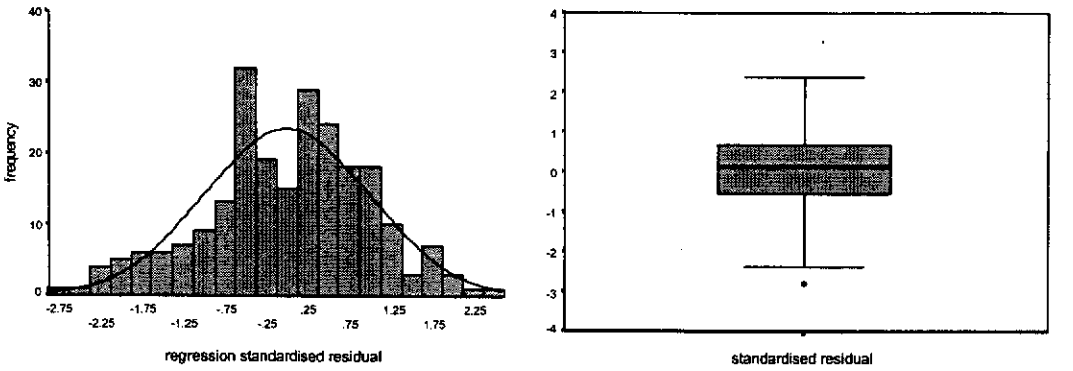


Figure 4.23: Histogram and Boxplot of Regression Standardised Residuals



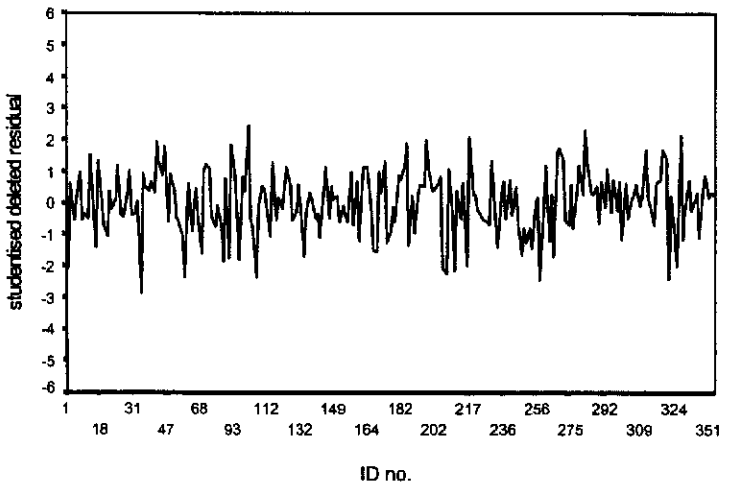
4.3.4.5 Collinearity

The variance inflation factor (VIF) for each main effect term was examined for collinearity. Collinearity results from strong correlations between main effects, which produce imprecise coefficients due to inflated standard errors. A VIF value of ≥7.0 has been suggested as a cut-off point of what constitutes a 'high' value (Statistix Analytical Software Manual, 2nd edn, Version 4.0, 1992). For age, FSH, eFSH and daily stress the VIF values were 1.6, 1.3, 1.8 and 1.0, respectively. As all of these VIF values are below 7.0, collinearity among the main effects was not suspected.

4.3.4.6 Outliers

A case is termed an outlier if its value of the dependent variable is extreme relative to other values of the model fit. The studentised deleted residual (SDR) values were used to identify outliers (Figure 4.24). A case was considered to be an outlier if its SDR exceeded ±3.0. As all SDR values were within this range, no outliers were identified.

Figure 4.24: Sequence Plot of Studentised Deleted Residuals



4.3.4.7 Leverage

In terms of the independent variables, leverage identifies outlying cases. For a given case, it is a measure of the distance between the values of the independent variables and the sample mean value of the independent variables. The more extreme the values of the independent variables are to that of the average values, the higher the leverage. A high leverage, therefore, is exhibited by a case with an 'extreme' value of one or more of the independent variables. For each case, the centred leverage (CL) value was plotted (Figure 4.25). One female was identified as having a CL value that was high relative to that of the other females: ID no. 24 . Two other women, ID no. 218 and 308, also had an elevated CL value. A summary of these three leverage cases is presented in Table 4.12.

Figure 4.25: Sequence Plot of Centred Leverage

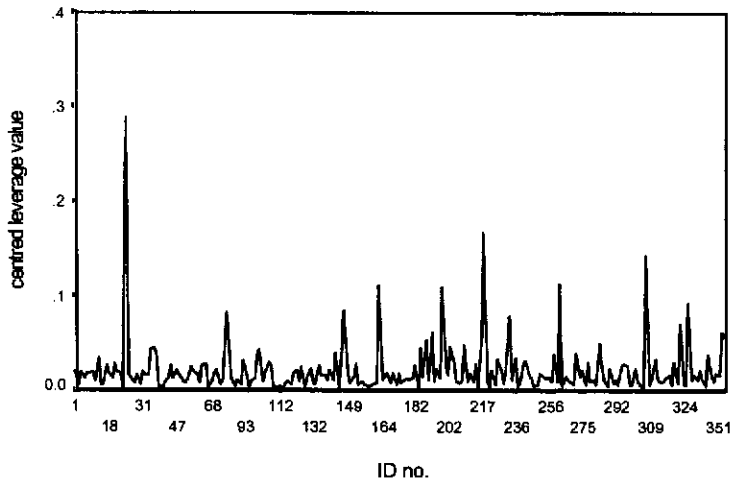


Table 4.12: Leverage Cases

ID no.	CL	age	FSH (IU/l)	eFSH (ampoules)	daily stress	no. of oocytes
24	0.287	31	22.7	264	10	2
218	0.166	42	27.3	107	34.0	4
308	0.143	30	19.1	180	11.0	4

4.3.4.8 Influential Cases

A case is considered to be influential when its exclusion from the model results in a significant change in the fitted regression function. In other words, the fitted MLR model is sensitive to such cases in the sample. Three measures of influence were used to identify potentially influential cases: the Cook's distance, the standardised change in the fitted value and the standardised change in the regression coefficients.

For each case, the Cook's distance (CD) is presented in a sequence plot (Figure 4.26). One woman in particular, ID no. 218, was shown to have an elevated CD value. Although to a lesser extent, the CD value was also elevated for four other females: ID no. 35, 199, 323 and 329. A case summary is presented for each of these potentially influential cases (Table 4.13).

Figure 4.3.21: Sequence Plot of Cook's Distance

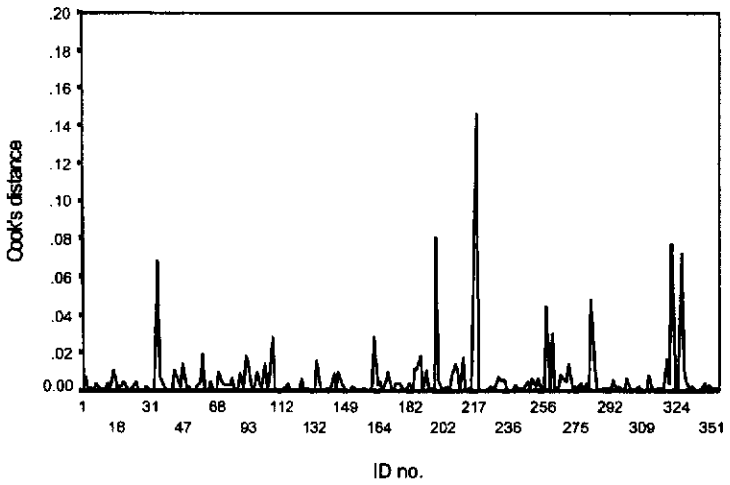
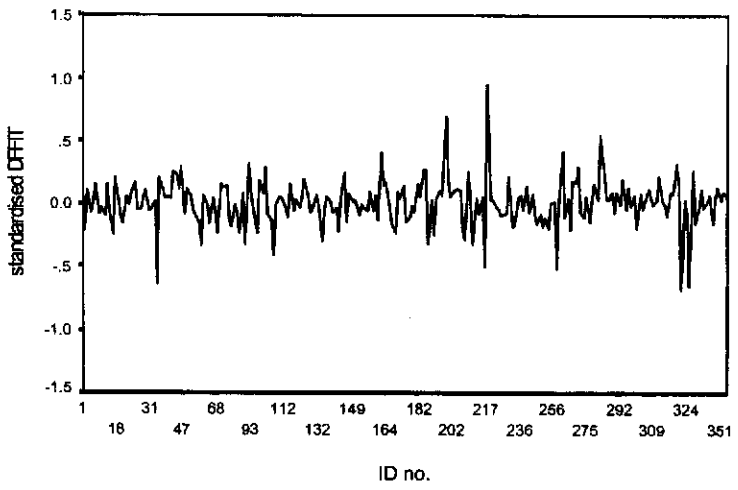


Table 4.13: Cases with Elevated Cook's Distance Values

ID no.	CD	age	FSH (IU/l)	eFSH (ampoules)	daily stress	no. of oocytes
35	0.068	37	7.5	120	51.0	1
199	0.081	43	16.2	254	21.0	3
218	0.147	42	27.3	107	34.0	4
323	0.077	44	12.3	204	4.0	0
329	0.073	47	5.7	180	6.0	1

The influence of each case on the fit of the model was assessed using the standardised change in the fitted value (SDFFITS). For a given case, it is a measure of the change in the fitted value for the omission of that case from the model. For a sample size such as this, a SDFFITS value of ±1.0 was used as a guide to the identification of potentially influential cases. As shown in Figure 4.27, no case had a SDFFITS value that exceed ±1.0. Therefore, no potential influential cases were evident from the SDFFITS values.

Figure 4.27: Sequence Plot of Standardised Change in the Fitted Value



The standardised change in the regression coefficient (SDFBETAS) was plotted for each of the main effects: age, basal FSH, eFSH and daily stress (Figure 4.28). This exercise is useful, as it can help explain the manner whereby a case was identified as potentially influential. For example, the influence for female ID no. 35 was predominantly due to daily stress. Extreme FSH and daily stress values were shown for female ID no. 218. For females ID no. 199, 323 and 329, identification as potentially influential was due mainly to the eFSH value.

The concluding stage of the diagnosis of the ln model adequacy was an examination of the influence of each case that had previously been identified as potentially influential on the significance of the variables in the final model (Table 4.14). For each variable, the coefficient *P* value is listed following the exclusion of the respective case from the ln(oocytes) model. The independent omission of female ID no. 199, 218, 323 and 329 did not alter the significance of the variables in the final model; changing the *P* value from <0.05 to ≥ 0.05, or vice versa. The exception was female ID no. 35, whereby her exclusion from the ln(oocytes) model rendered the daily stress non-significant ($P=0.129$).

Figure 4.28: Sequence Plots of SDFBETAS for the Main Effects

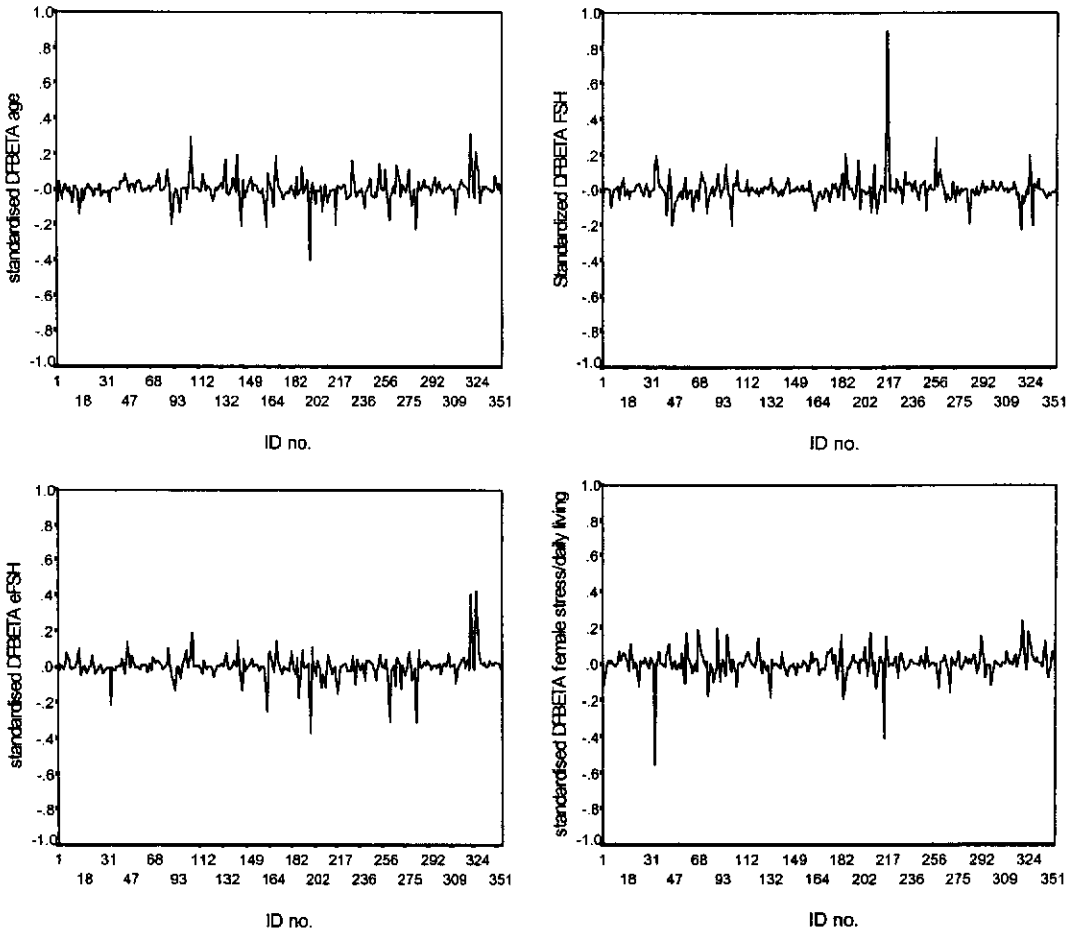


Table 4.14: Influence of Cases in the ln(oocytes) Model

	adjusted R^2	coefficient *P* value					SDR	CL	CD	SDFITTS
		age	basal FSH	eFSH	daily stress	age*eFSH				
	0.456	**0.859**	**<0.001**	**0.226**	**0.039**	**0.025**	-	-	-	-
ID no. of deleted case										
24	0.448	0.851	<0.001	0.296	0.040	0.043	-	0.287	-	-
35	0.459	0.920	<0.001	0.149	**0.129**	0.014	-	-	0.068	-
199	0.461	0.823	<0.001	0.119	0.037	0.009	-	-	0.081	-
218	0.464	0.842	<0.001	0.171	0.026	0.019	-	0.166	0.147	-
308	0.454	0.850	<0.001	0.275	0.040	0.035	-	0.143	-	-
323	0.440	0.626	<0.001	0.417	0.021	**0.075**	-	-	0.077	-
329	0.449	0.698	<0.001	0.433	0.025	**0.079**	-	-	0.073	-

4.3.5 Interpretation of the Regression Model

The ln(oocytes) regression equation is:

$$\ln(\text{oocytes}) = 3.338 - 2.431\times10^{-3}(\text{age}) - 7.231\times10^{-2}(\text{basal FSH}) + 7.078\times10^{-3}(\text{eFSH}) - 7.203\times10^{-3}(\text{daily stress score}) - 3.408\times10^{-4}(\text{age*eFSH})$$

The natural logarithm of the number of oocytes retrieved, ln(oocytes), declined with increasing basal FSH levels ($P<0.001$). The significant interaction term between age and eFSH administration (age*eFSH, $P=0.025$) showed that the effect of age on ln(oocytes) was dependent on the total dose of eFSH administered, and vice versa. The negative coefficient indicates that ln(oocytes) decreased with the cross product of these two variables: age and eFSH. In effect, oocyte production was lowest when the woman was of advanced reproductive age and had been administered a large quantity of eFSH. Conversely, oocyte production was highest among young patients with low eFSH administration. In the presence of this interaction, neither age nor eFSH was significant as a main effect, indicating that the combined effect of age and eFSH is more relevant than the effect of either in isolation.

Daily stress was the only lifestyle factor shown to be of significance in relation to oocyte production ($P=0.039$). Specifically, ln(oocytes) decreased with increasing levels of daily stress. This adverse effect of daily stress on oocyte production was evident even after adjustment for basal FSH, age and eFSH. However, this finding is not without question, as the datum of one female was influential on the overall significance of the relationship between daily stress and ln(oocytes).

Based on univariate statistical analysis, women with unexplained infertility had significantly fewer oocytes retrieved than women whose infertility was classified as 'nil significance' ($F_{5,\ 284}=3.07$, $P=0.010$). Infertility aetiology, however, was not among the

significant factors in the ln(oocytes) model. On further examination, it was revealed that women with unexplained infertility had significantly higher basal FSH levels than women with an infertility status of nil significance ($F_{5, 279}$=2.52, P=0.030). As shown in the ln(oocytes) model, elevated basal FSH levels are associated with reduced oocyte production. In light of this, it was deemed of value to investigate variables that affect basal FSH levels, as such factors, in theory, would have an indirect effect on oocyte production. An investigation of factors affecting basal FSH levels is presented in the following chapter.

4.4 Basal Follicle Stimulating Hormone Level (FSH)

In the previous *Results* Section 4.3, it was shown that oocyte production decreased with increasing basal FSH levels. Therefore, factors affecting basal FSH levels act indirectly on oocyte production. Furthermore, basal FSH levels are an indirect measure of ovarian reserve. As a consequence, an investigation into variables affecting basal FSH levels was deemed to be of value.

Of the 290 women who were included in the investigation on oocyte production ($n_{2.3}$), the basal FSH level was measured among 285 ($n_{2.4}$, 98.3%)(Figure 4.6 [Oocyte Production]). With a median of 7.1 (IQR 3.2), basal FSH levels ranged from 2.0 to 28.3 IU/l (Figure 4.29). The skewness of the basal FSH levels distribution necessitated a logarithm transformation. As shown in Figure 4.30, the natural logarithm of basal FSH levels, ln(FSH), resulted in a relatively normal distribution, and as such was used in the subsequent data analysis of basal FSH levels.

Figure 4.29: Histogram and Boxplot of Basal FSH Levels

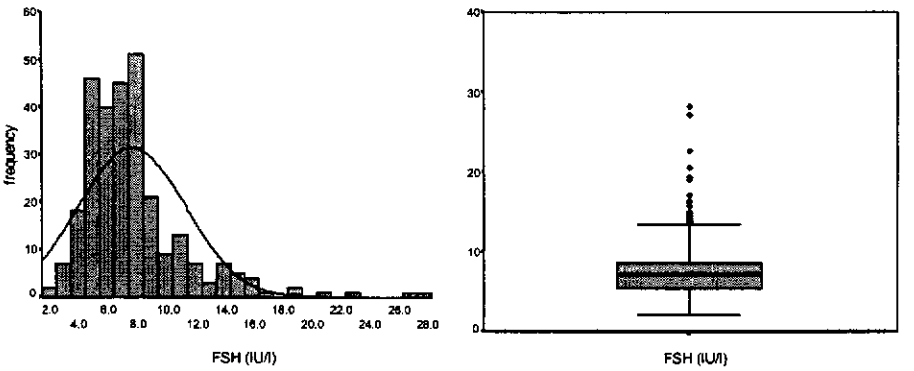
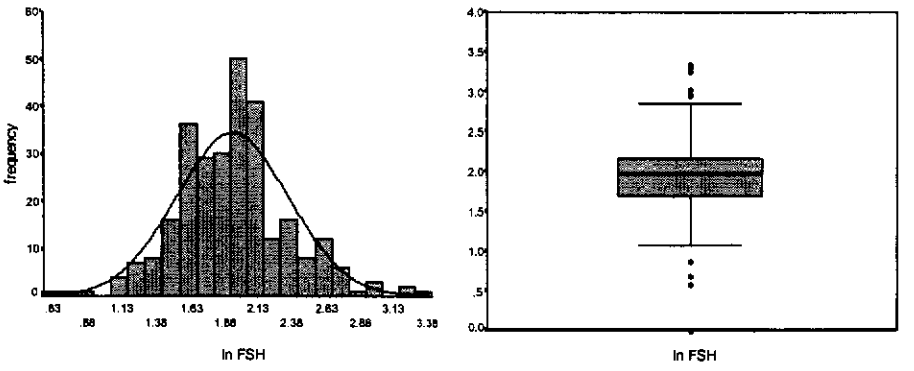


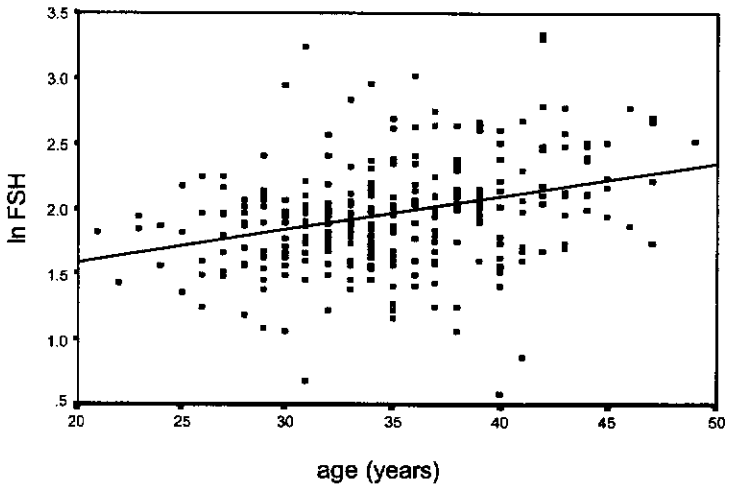
Figure 4.30: Histogram and Boxplot of ln(FSH)



4.4.1. Univariate Analysis

As shown in Figure 4.31, a relatively weak linear association was indicated between age and ln(FSH) (r=0.33, $P<0.001$).

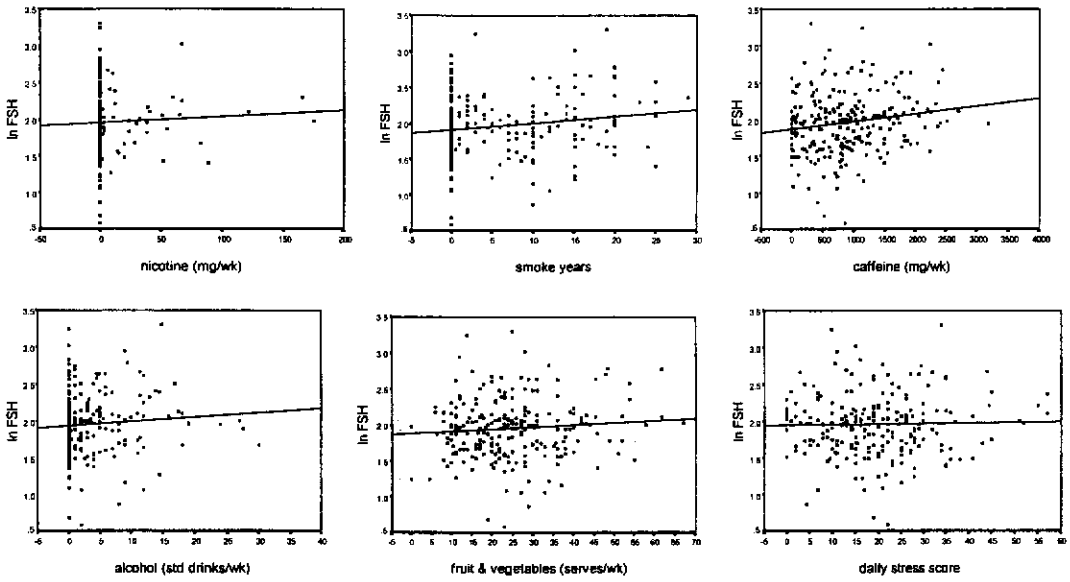
Figure 4.31: Scatterplot of Age and ln oocytes



One way ANOVA was used to compare the mean ln(FSH) of the female infertility groups. A significant ln(FSH) mean difference was found between at least two of the infertility groups ($F_{5,279}=2.673$, $P=0.022$). From the Tukey's Post Hoc test, it was established that the mean ln(FSH) of women whose infertility was of nil significance was significantly higher, on average, than that of women whose infertility was unexplained.

Against ln(FSH), each lifestyle variable is presented in a scatterplot (Figure 4.32). No single lifestyle variable was shown to have a noteworthy linear association with ln(FSH).

Figure 4.32: Scatterplots of ln(FSH) vs Each Lifestyle Variable



4.4.2 Multiple Linear Regression Analysis

The results of three multiple linear regression (MLR) model fitting strategies on ln(FSH) are presented in Table 4.15.

Table 4.15: MLR Model Fitting Strategies on ln(FSH)[a]

variable		model fitting strategy		
		full model *P* value	stepwise forward *P* value	backward elimination *P* value
age		<0.001	<0.001	<0.001
infertility aetiology (6) :	unexplained[b]	0.011	<0.001	<0.001
	endometriosis[b]	-	0.017	0.017
smoke years		-	0.045	0.045

[a] only variables with $P<0.05$ displayed
[b] as compared to infertility of nil significance

Infertility aetiology is a variable of six levels (infertility aetiology (6)), with infertility of nil significance the base category. In each model fitting strategy, infertility aetiology (6) was represented by five dummy variables. As compared to infertility of nil significance, unexplained infertility was significant in all three model fitting strategies, and endometriosis was significant in two of the three. Consequently,

infertility aetiology (6) was recoded into a new variable, infertility aetiology (3), which had three levels: unexplained, endometriosis and the base category, all other types of infertility including nil significance. The three different model fitting strategies were applied to the data containing infertility aetiology (3), which was represented by two dummy variables (Table 4.16). Age, unexplained infertility and endometriosis were significant in all three model fitting strategies, and smoke years was significant in two of the three.

Table 4.16: MLR Model Fitting Strategies on ln(FSH)[a]

variable		model fitting strategy		
		full model *P* value	stepwise forward *P* value	backward elimination *P* value
age		<0.001	<0.001	<0.001
infertility aetiology (3) :	unexplained[b]	<0.001	<0.001	<0.001
	endometriosis[b]	0.014	0.017	0.017
smoke years		-	0.045	0.045

[a] only variables with $P<0.05$ displayed
[b] as compared to all other types of infertility, including nil significance

Hierarchical regression analysis was performed to assess whether smoke years improved the fit of the model. A significant F-change of 4.504 based on 1 and 227 df ($P=0.035$) confirmed the significance of smoke years in relation to ln(FSH). Consequently, smoke years was included in the ln(FSH) model of main effects (Table 4.17).

Table 4.17: MLR ln(FSH) Model of Main Effects[a]

		coefficient[b]	std error	*P* value
constant		0.932	0.179	<0.001
age		0.026	0.005	<0.001
infertility aetiology (3):	unexplained[c]	0.221	0.059	<0.001
	endometriosis[c]	0.193	0.079	0.015
smoke years		0.007	0.003	0.035

[a] adjusted $R^2=0.163$, $F_{5, 222}=10.02$, 5&226df, $P<0.001$
[b] unstandarised
[c] as compared to all other types of infertility, including nil significance

For each dual combination of the main effect variables, an interaction term was created (Table 4.18). The main effects and all of the interaction terms were then collectively regressed on FSH. None of the interaction terms were significant. Using hierarchical regression analysis, each interaction term in turn was added to the ln(FSH) main effects model (Table 4.19). Of the interaction terms, only age*unex significantly improved the model fit, and was included in the final MLR ln(FSH) model (Table 4.20).

Table 4.18: Two-Way Interaction Terms

	age	**unexplained infertility**	**endometriosis**	**smoke years**
age	-	age*unex	age*endo	age*smyr
unexplained infertility	-	-	-	unex*smyr
endometriosis	-	-	-	endo*smyr
smoke years	-	-	-	-

Table 4.19: F-test of 2-Way Interaction Terms in the ln(FSH) Model of Main Effects

variables in the model					F change	*P* value
age	unexplained IF	endo	smoke yrs	-	-	-
age	**unexplained IF**	**endo**	**smoke yrs**	**age*unex**	**4.718**	**0.031**
age	unexplained IF	endo	smoke yrs	age*endo	0.325	0.569
age	unexplained IF	endo	smoke yrs	age*smyr	1.230	0.269
age	unexplained IF	endo	smoke yrs	unex*smyr	1.877	0.172
age	unexplained IF	endo	smoke yrs	endo*smyr	0.125	0.724

Table 4.20: MLR ln(FSH) Model[a]

		coefficient[b]	95% CI (lower, upper)	std error	*P* value
constant		1.170	0.759, 1.580	0.208	<0.001
age		0.019	0.008, 030	0.006	0.001
infertility aetiology (3):	unexplained[c]	-0.595	-1.343, 0.154	0.380	0.119
	endometriosis[c]	0.172	0.017, 0.327	0.079	0.030
smoke years		0.007	0.001, 0.014	0.003	0.035
age*unexplained infertility		0.023	0.002, 0.045	0.011	0.031

[a] adjusted R^2=0.163, $F_{5, 222}$=10.02, 5&226df, P<0.001
[b] unstandarised
[c] as compared to all other types of infertility, including nil significance

4.4.3. Model Diagnostics

4.4.3.1 Fit of the Model

The adjusted R^2 of the ln(FSH) was 0.163. This indicates that 16.3% of the variability in basal FSH levels was explained by the variation in age, smoke years and infertility aetiology, namely unexplained infertility and endometriosis. The overall F-test ($F_{5,222}$=10.02, P<0.001) shows that the regression was significant.

4.4.3.2 Non-Linear Regression Function

The standardised residuals are plotted against the standardised predicted values in Figure 4.33. The relatively random scatter of the points suggests that the function of the model is not non-linear. The standardised residuals were also plotted against age and smoke years (Figure 4.34), both of which confirms a lack of non-linearity.

Figure 4.33: Scatterplot of Standardised Residuals vs Standardised Predicted Values

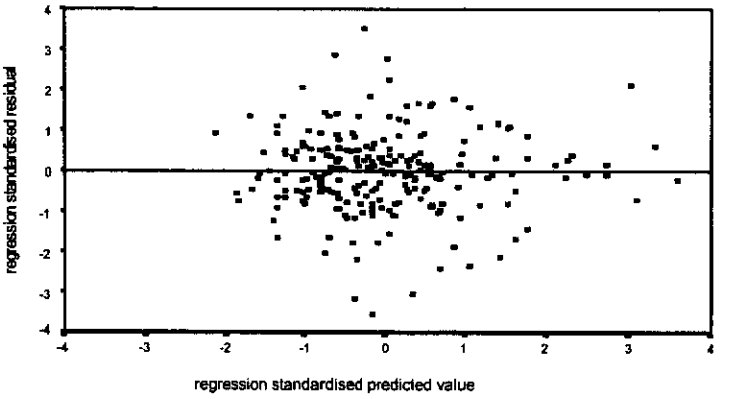
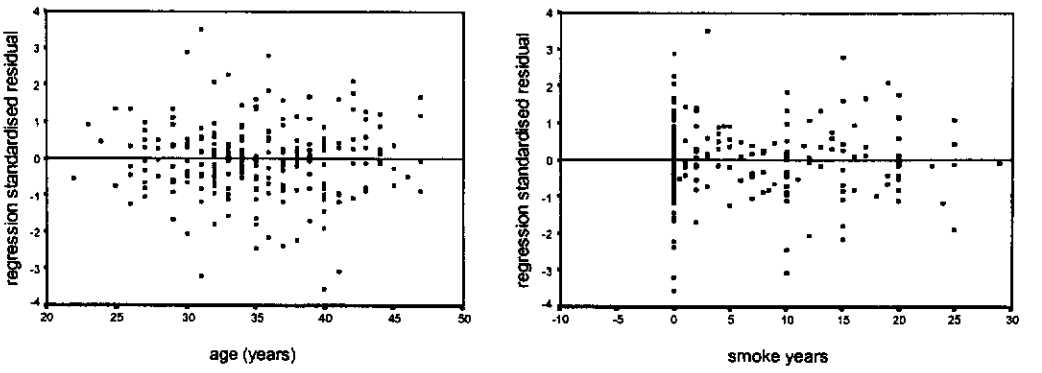


Figure 4.34: Scatterplots of Regression Standardised Residuals vs Age and Smoke Years



4.4.3.3 Heteroscedasticity

As shown in Figures 4.33 and 4.34, the variance of ln(FSH) did not show any systematic pattern over the range of values of age and smoke years.

4.4.3.4 Non-Normality

The normal P-P plot of regression standardised residuals is presented in Figure 4.35. Normality can be assumed as the plotted values do not systematically depart from the linear trend. The distribution of the regression standardised residuals in Figure 4.36 also confirms that the assumption of normality holds.

Figure 4.35: Normal P-P Plot of Regression Standardised Residuals

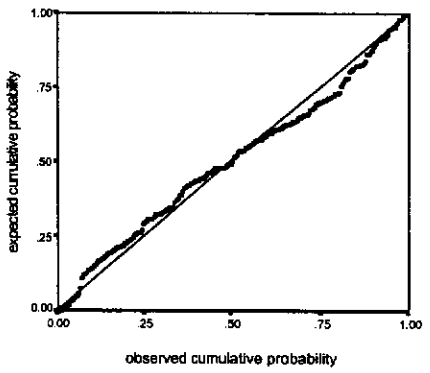
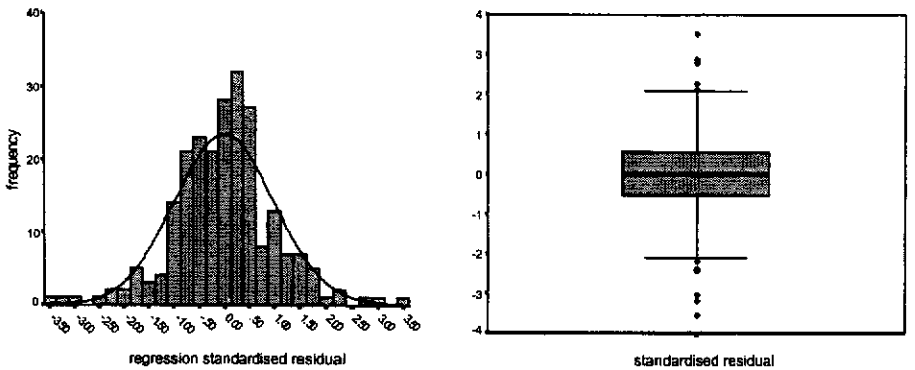


Figure 4.36: Histogram and Boxplot of Regression Standardized Residuals



4.4.3.5 Collinearity

The VIP values for age, smoke years and the diagnosis of endometriosis were 1.06, 1.07, 1.10 and 1.03, respectively. Therefore, collinearity among the independent variables was not suspected.

4.4.3.6 Outliers

The studentised deleted residual (SDR) values were plotted for each case (Figure 4.37). Four cases were identified as outliers (SDR ± 3.0): female ID no. 24, 42, 100 and 224. For each, a summary is presented in Table 4.21.

Figure 4.37: Sequence Plot of Regression Studentised Deleted Residuals

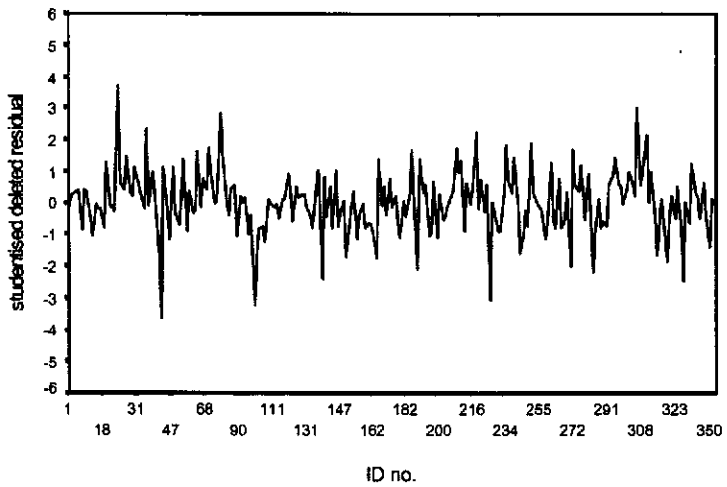


Table 4.21: Cases with an Elevated Studentised Deleted Residual

ID no.	SDR	age (years)	smoke years	unexplained infertility	endo-metriosis	FSH (IU/l)
24	3.673	31	3.0	yes	no	22.7
42	-3.663	40	0.0	no	no	6.1
100	-3.263	31	0.0	yes	no	2.0
224	-3.101	41	10.0	no	no	2.4

4.4.3.7 Leverage

Cases with an elevated centred leverage (CL) value were identified from Figure 4.38, and their summaries detailed in Table 4.22.

Figure 4.38: Sequence Plot of Leverage

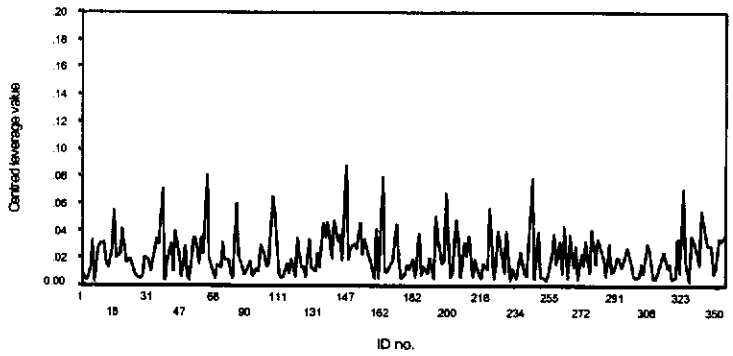


Table 4.22: Leverage Cases

ID no.	CL	age (years)	smoke years	unexplained infertility	endo-metriosis	FSH (IU/l)
37	0.070	44	0.0	yes	no	11.1
60	0.081	45	15.0	yes	no	12.3
103	0.065	25	0.0	yes	no	3.9
146	0.087	34	4.5	yes	no	7.0
162	0.079	45	3.0	yes	no	9.4
199	0.067	43	20.0	yes	no	16.2
242	0.078	47	0.0	no	yes	9.2
323	0.070	44	0.0	yes	no	12.3

4.4.3.8 Influential Cases

The Cook's distance (CD) values were plotted (Figure 4.39). Five cases were identified as having an elevated CD: female ID no. 24, 100, 218, 308 and 330. Their case summaries are presented in Table 4.23.

Figure 4.39: Sequence Plot of Cook's Distance

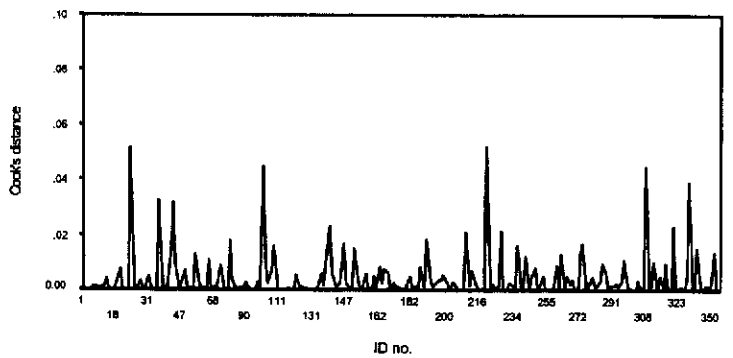
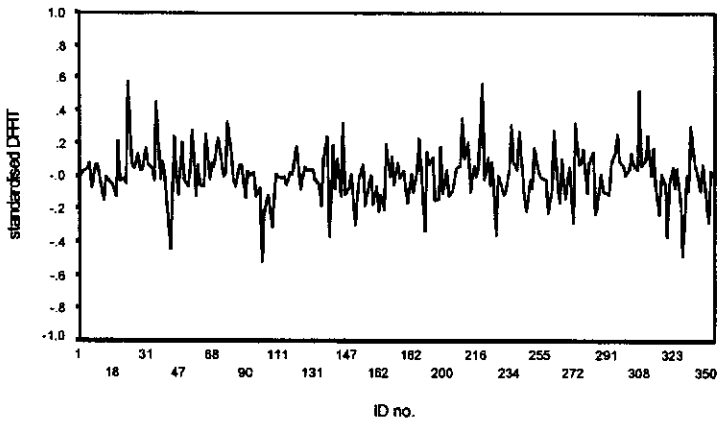


Table 4.23: Cases with an Elevated Cook's Distance Value

ID no.	CD	age (years)	smoke years	unexplained infertility	endo-metriosis	FSH (IU/l)
24	0.051	31	3.0	yes	no	22.7
100	0.044	31	0.0	yes	no	2.0
218	0.052	42	19.0	yes	no	27.3
308	0.044	30	0.0	yes	no	19.1
330	0.039	35	10.0	no	yes	3.2

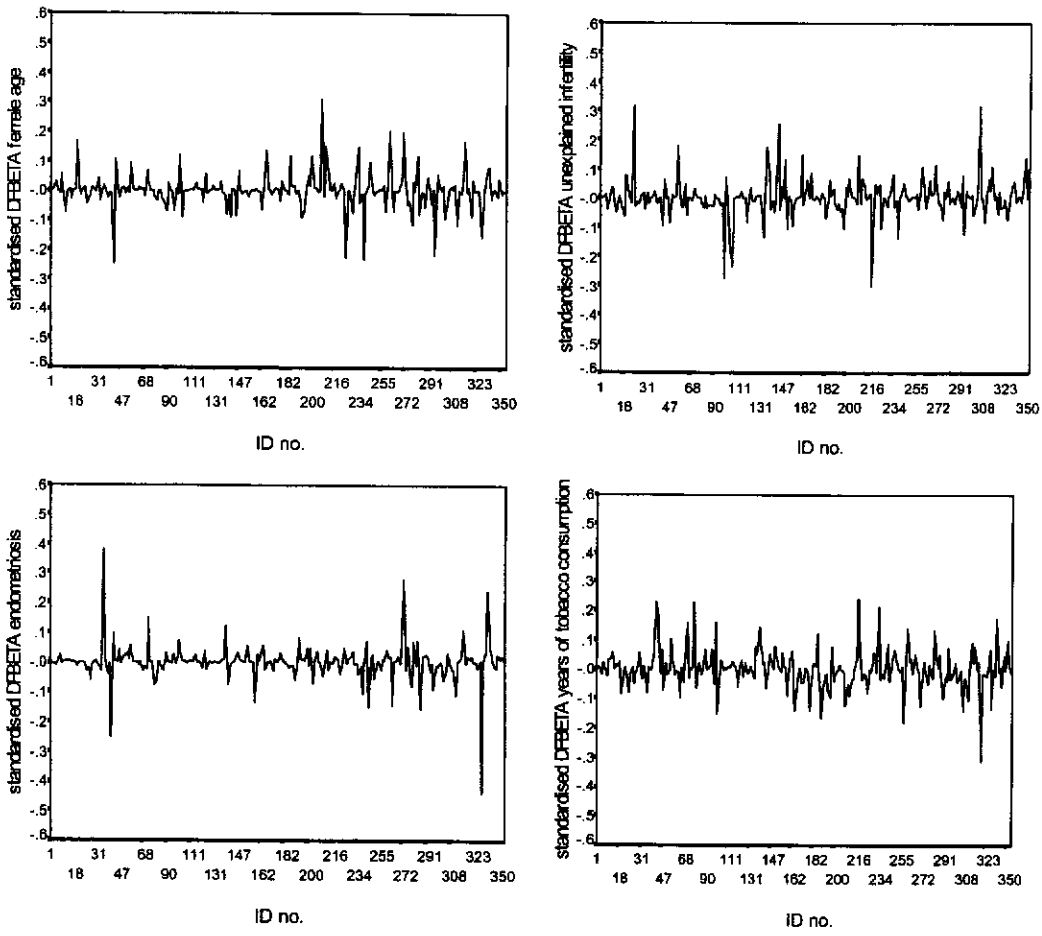
The standardised change in the fitted values (SDFFITS), which is a measure of the influence of each case on the model fit, was examined (Figure 4.40). As none of the SDFFITS values exceeded ±1.0, no potentially influential cases were noted.

Figure 4.40: Sequence Plot of Standardised Change in the Fitted Value



The standardised change in the regression coefficient (SDFBETAS) was plotted for each of the main effects: age, unexplained infertility, infertility due to endometriosis and smoke years (Figure 4.41). It would appear that female ID no. 42 was identified as potentially influential due to an extreme SDFBETA for age. Female ID no. 24, 100, 103, 146, 218 and 308, the unexplained infertility SDFBETAs was extreme, and a likely source of having been identified as potentially influential. The endometriosis SDFBETA was unusually high for female ID no. 308. For the remaining potentially influential cases (female patient ID no. 37, 60, 162, 199, 224, 242 and 323), no one SDFBETAs was remarkable.

Figure 4.41: Sequence Plots of SDFBETAS for Age, Unexplained Infertility, Endometriosis and Smoke Years



The impact of potentially influential cases on the significance of the parameters in the final model was assessed. Each potentially influential case was sequentially omitted from the ln(FSH) model. Detailed in Table 4.24 is the *P* value of each regression coefficient after the omission of the respective case. Smoke years was rendered non-significant following the omission of female ID no. 42, and the omission of female ID no. 218. Age*unexplained infertility was also non-significant upon deletion of female ID no. 218.

Table 4.24: Influence of Cases on the ln(FSH) Model

	adjusted R^2	*P* value of the coefficient					SDR	CL	CD	SDFITTS
		age	unexplained infertility	endo-metrosis	smoke years	age*unex				
	0.163	**0.001**	**0.119**	**0.030**	**0.035**	**0.031**	-	-	-	-
ID no. of deleted case										
24	0.176	0.001	0.057	0.025	0.025	0.014	3.673	-	0.051	0.570
37	0.159	0.001	0.121	0.030	0.037	0.033	-	0.070	-	-
42	0.171	<0.001	0.132	0.033	**0.053**	0.036	-3.663	-	-	-
60	0.157	0.001	0.116	0.030	0.035	0.031	-	0.081	-	-
100	0.167	0.001	0.189	0.027	0.046	0.048	-3.264	-	0.044	-0.527
103	0.158	0.001	0.170	0.030	0.038	0.049	-	0.065	-	-
146	0.167	0.001	0.079	0.030	0.036	0.020	-	0.087	-	-
162	0.164	0.001	0.095	0.030	0.039	0.024	-	0.079	-	-
199	0.0150	0.001	0.151	0.030	0.044	0.045	-	0.067	-	-
218	0.142	0.001	0.209	0.030	**0.060**	**0.071**	-	-	0.052	0.563
224	0.173	<0.001	0.139	0.030	0.026	0.038	-3.101	-	-	-
242	0.162	0.001	0.124	0.033	0.036	0.033	-	0.078	-	-
308	0.175	0.001	0.059	0.026	0.023	0.014	-	-	0.044	0.525
323	0.157	0.001	0.134	0.030	0.035	0.038	-	0.070	-	-
330	0.172	0.001	0.126	0.010	0.024	0.033	-	-	0.039	-

4.4.4 Interpretation of the Regression Model

The ln(FSH) regression equation is:

$$\ln(FSH) = 1.170 + 0.019(age) - 0.595(\text{unexplained IF}) + 0.172(\text{endometriosis}) + 0.007(\text{smoke years}) + 0.023(\text{age*unexplained infertility})$$

The natural logarithm of basal FSH levels, ln(FSH), increased with age (P=0.001). Basal FSH levels were also related to infertility aetiology, namely endometriosis and unexplained infertility. Women with endometriosis had significantly higher ln(FSH) levels than women with all other types of infertility, including 'nil significance' (P=0.030). The significant interaction term between unexplained infertility and age (age*unexplained infertility)(P=0.031) indicated that the effect of unexplained infertility on basal FSH depended upon the patient's age. This means that the effect of age on ln(FSH) is different among patients with unexplained infertility. As the coefficient for age*unexplained infertility is positive, the impact of age on basal FSH among women with unexplained infertility is greater than that of women with all other types of infertility; the slope of the regression line is steeper. It is noted, however, that one patient had an influential effect on the significance of this interaction term (Table 4.24).

Of the lifestyle factors, years of tobacco consumption (smoke years) was shown to significantly affect ln(FSH) (P=0.035). Specifically, basal FSH increased with the total number of years that the female patient had smoked, an effect irrespective of whether they were a current or ex-smoker. Though, two cases were identified as influential in regard to the significance of this effect of 'smoke years' on basal FSH levels, as is shown in Table 4.24.

4.5 Fertilisation

The investigation of factors affecting fertilisation was undertaken among the couples whose treatment involved oocyte fertilisation *in vitro* ($n_{3.2}$)(Figure 4.42); couples whose treatment involved oocyte fertilisation by ICSI were excluded. By TVOA, 332 couples had at least one oocyte retrieved (n_3). The male partner was ineligible for inclusion among eight of these 332 couples (n_3, 2.5%), seven whose treatment involved donor sperm and one couple because the male was a smoker at the time of spermatozoa cryopreservation but an ex-smoker at the time of treatment.

Two hundred and thirteen ($n_{4.2}$) of the 324 eligible couples ($n_{4.1}$, 63.6%) had treatment that involved oocyte fertilisation *in vitro*. Of these 213 couples ($n_{3.2}$), only the 154 couples who were fully compliant ($n_{4.3}$, 72.3%) were considered for data analysis. Among two of these 154 couples ($n_{4.3}$), the female experienced ovarian hyperstimulation syndrome, resulting in the retrieval of more than 60 oocytes in each instance. Consequently, these two couples were excluded from the data analysis, as their excessive production of oocytes was unusual and not representative of the majority of couples undertaking IVF treatment.

A total of 152 couples were included in the investigation of factors on fertilisation ($n_{3.4}$). Only two of these 152 couples' treatment involved a previously cryopreserved semen samples; the post-thawing progressive motility counts were 28.0 and 34.5 $\times$ 10^6/mL spermatozoa.

4.5.1. Descriptive Statistics

For the 152 couples ($n_{3.4}$), descriptive statistics on the number of oocytes that exhibited normal fertilisation (2PN) are presented in Table 4.25. Multiple logistic regression analysis involved weighting the dependent variable, the number of 2PN oocytes by the number of retrieved oocytes; therefore, descriptive statistics are also presented for the number of retrieved oocytes (Table 4.25).

Figure 4.42: Flowchart of Fertilisation Sub-Samples

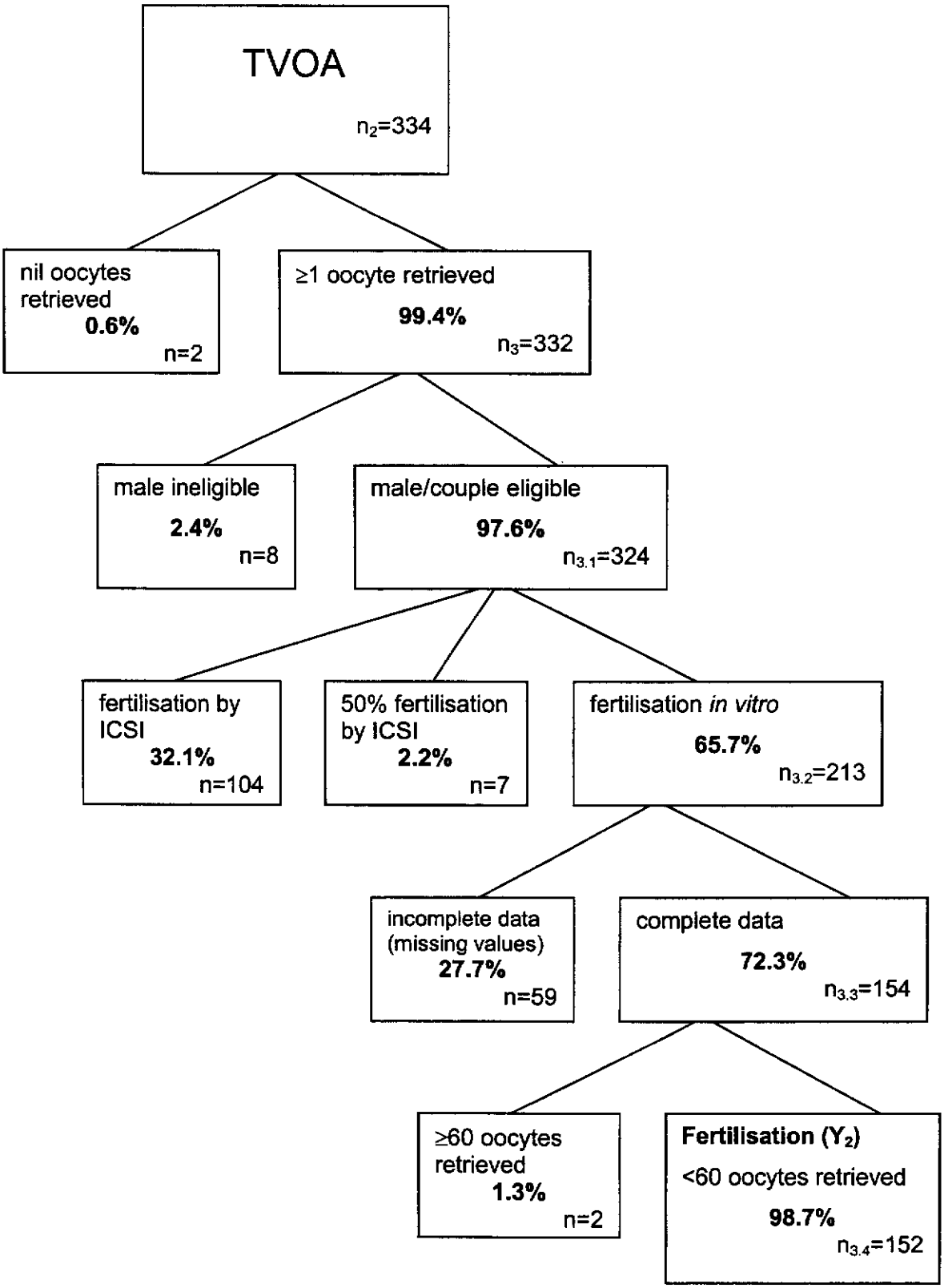


Table 4.25: Descriptive Statistics of the Number of Aspirated and Fertilised Oocytes

	n	min	max	mean	median	IQR
no. of 2PN oocytes	152	0	22	6.9	6.0	6.0
total no. of oocytes	152	1	38	10.9	10.0	10.8

Failed fertilisation, the failure of all of the retrieved oocytes to fertilise, was the outcome for four of the 152 couples ($n_{3.4}$, 2.6%). The one and only oocyte failed to fertilise among two such couples; the females were 36 and 42 years of age. For one couple, all of the four oocytes did not fertilise; the female was 42 years old. All of the 16 oocytes failed to fertilise in the remaining couple, the female who was 31 years of age.

Patient and lifestyle variables were examined among the females and males. The effect of age and gender on fertilisation was investigated, as was spermatozoa classification. Female and male lifestyle variables included nicotine consumption and years of tobacco consumption (smoke years), caffeine, alcohol and fruit and vegetable consumption, and daily and IVF stress. Among the males, dietary vitamin E supplementation was also investigated.

As demonstrated in Table 4.26, females ranged in age from 22 to 47 years, with a mean of 34.8 (SD 5.2). The age range among the males was 24 to 56 years, and the mean age was 35.8 (SD 5.6) years (Table 4.26).

Males were classified as oligospermic, asthenozoospermic and/or teratozoospermic based on their semen sample which was collected for oocyte insemination. The classification of oligozoospermia was given to 7.2%, asthenozoospermia to 21.1% and teratozoospermic to 16.4% of the males.

Presented in Table 4.26 is a description of the fertilisation sub-sample by lifestyle variables.

Table 4.26: Descriptive Statistics of Lifestyle Variables

gender	variable	min	max	median	IQR	mean	SD
female	nicotine (mg/wk)	0	175	0	0.0	6.6	-
	smoke years	0	25	1.0	10.0	5.2	-
	caffeine (mg/wk)	0	2706	846	-	902	644
	alcohol (std dks/wk)	0	27	0.8	5.0	3.0	-
	fruit & vege (serves/wk)	4.5	62	23.0	-	23.9	10.8
	daily stress	0	57	19.0	-	19.4	11.4
	IVF stress	0	45	17.0	-	17.7	11.9
male	nicotine (mg/wk)	0	188.5	0	0.0	14.1	-
	smoke years	0	29	0	12.0	6.2	-
	caffeine (mg/wk)	0	4495	1033	-	1152	845
	alcohol (std dks/wk)	0	79	7.0	11.8	9.9	-
	fruit & vege (serves/wk)	0	54	20.0	-	21.2	10.8
	daily stress	0	53	19.8	-	21.4	12.6
	IVF stress	0	63	7.0	16.0	11.5	-
	vitamin E (IU/l/wk)	0	4697	0	0.0	30.1	-

With reference to smoking status, almost half of the females had never smoked (46.7%), 36.8% were ex-smokers, 12.5% were regular smokers and 3.9% smoked occasionally. One-half of the males had also never smoked (49.3%), 25.7% were ex-smokers, 21.1% smoked on a regular basis and 3.9% occasionally smoked.

Twenty-three of the 152 females reported to have consumed nicotine (15.1%), as did 35 of the 152 males (23.0%). Of the female nicotine consumers, nicotine consumption varied between 1.6 and 175.0mg/wk, with a median value of 26.8 (IQR 57.8). Among the male nicotine consumers, nicotine consumption ranged from 1.4 to 188.5mg/wk, with a median of 40.4 (IQR 84.5).

Smoke years were examined among the ex-smokers and regular smokers. Of the 56 female ex-smokers, smoke years ranged from one week to 25 years, with a mean of 7.7 (SD 6.3). Among the 19 female regular smokers, smoke years ranged between eight and 25 years, and the median was 15.6 (SD 5.2). The 39 male ex-smokers reported to have had smoked for between one month and 27 years, with the mean of 8.8 (SD 6.0). Of the 32 male regular smokers, six months and 29 years was the range of smoke years and the mean was 16.6 (SD 6.8).

Only six females (3.9%) did not consume caffeine, neither did seven males (4.6%). Of the female caffeine consumers, caffeine consumption ranged between 4 and 2706mg/wk and the mean value was 939 (SD 630). Caffeine consumption among the male caffeine consumers ranged from 20 to 4495mg/wk, with a mean of 1207 (SD 825).

Eighty of the 152 females ($n_{3.4}$, 52.6%) and 123 of the 152 males ($n_{3.4}$, 78.8%) consumed alcohol. Among these 80 female alcohol consumers, weekly alcohol consumption ranged from 0.3 to 27.0 standard drinks, with a median of 5.0 (IQR 6.0). Among the 123 male alcohol consumers, consumption of alcohol ranged from 1.0 to 79.0 standard drinks per week, with a median value of 9.0 (IQR 10.0). Of note, one male consumed alcohol that was in excess of that of the other males, 79 standard drinks over the one-week period, an average of 11.3 standard drinks per day.

The mean weekly serves of fruit and/or vegetables among the females was 23.9 (SD 10.8), an average of 3.4 serves per day. Among the males, the mean weekly serves of fruit and/or vegetable was 21.2 (SD 10.8), which averages 3.0 serves per day.

Vitamin E supplementation was reported by almost one-fifth of the male respondents (19.7%). Of these 30 men, weekly vitamin E supplementation ranged from 10 to 4697mg, with a median of 198 (IQR 3441).

The weekly score from daily stress and from IVF stress were examined. Both stress from daily living and stress due to IVF treatment varied considerably among the females and males (Table 4.26). Of interest, thirty-one of the males (20.4%) reported zero IVF stress.

Correlations between lifestyle variables among the females, and among the males, were examined. As was the correlation of each lifestyle variable between that of the females and males. Correlations in excess of 0.4 (positive or negative) are listed in Table 4.27.

Table 4.27: Correlations Between Lifestyle Variables[a]

X_1	X_2	Pearson's r
female/female		
female nicotine	female tobacco years	0.498
female daily stress	female IVF stress	0.488
male/male		
male nicotine	male tobacco years	0.508
male daily stress	male IVF stress	0.479
female/male		
female IVF stress	male IVF stress	0.550
female fruit and vegetable	male fruit and vegetable	0.525
female daily stress	male daily stress	0.486
female caffeine	male caffeine	0.429

[a] includes only correlations with Pearson's r statistic that exceeded 0.4 (negative or positive).

4.5.2. Univariate Analysis

Simple logistic regression was undertaken between each independent variable and the number of 2PN oocytes, which was weighted by the number of retrieved oocytes. Initially, a 2PN model containing only the constant term was constructed, and the deviance was documented (Table 4.28). To this 2PN model, each independent variable was separately fitted and the change in deviance noted, as was the beta coefficient *P* value. A summary of the findings is presented in Table 4.28.

Of the patient variables, asthenozoospermia was negatively associated with fertilisation (P=0.0343). Of significance among the female lifestyle variables, fertilisation was negatively associated with smoke years (P=0.0003) and caffeine consumption (P=0.0037), but positively associated with fruit and vegetable consumption (P=0.0125). Among the male lifestyle variables, fertilisation was negatively associated with caffeine consumption (P=0.0309) and IVF stress (P=0.005), but positively associated with fruit and vegetable consumption (P<0.0001).

As female smoke years was significantly associated with fertilisation (P=0.0003)(Table 4.28), it was deemed meaningful to involve female age with

female smoke years in the 2PN model. Consequently, a new variable, the proportion of life smoked (fsmyrs/age), was created by dividing female smoke years by female age. Validation of fsmyr/age was subsequently undertaken. The change in deviance per 1df was greater for the fit of fsmyrs/age than that of female smoke years, 15.25 vs 13.21, which confirms that fsmyrs/age is a more appropriate variable than female smoke years. Therefore, fsmyrs/age was used in the multivariate analysis in preference to female smoke years.

Table 4.28: Summary of Patient and Lifestyle Variables on Fertilisation

gender	variable	deviance	df	deviance change	β[a]	*P* value
	constant only	368.70	151	-	0.52499	<0.0001
	<u>patient variables</u>					
female	age	368.60	150	0.10	-	0.7503
male	age	368.33	150	0.37	-	0.5426
	oligozoospermic[b]	368.16	150	0.54	-	0.4596
	asthenozoospermic[c]	364.26	150	4.44	-0.25287	**0.0343**
	teratozoospermic[d]	368.34	150	0.36	-	0.5447
	<u>lifestyle variables</u>					
female	nicotine	368.70	150	0.00	-	0.9445
	smoke years	355.49	150	13.21	-0.02777	**0.0003**
	caffeine	360.30	150	8.40	-0.343×10^{-4}	**0.0037**
	alcohol	368.65	150	0.05	-	0.8205
	fruit & vegetable	362.37	150	6.33	0.01224	**0.0125**
	daily stress	367.41	150	1.29	-	0.2570
	IVF stress	365.82	150	2.88	-	**0.0890**
male	nicotine	366.20	150	2.50	-	0.1186
	smoke years	368.01	150	0.69	-	0.4046
	caffeine	364.06	150	4.64	-1.403×10^{-4}	**0.0309**
	alcohol	365.11	150	3.59	-	0.0613
	fruit and vegetable	349.73	150	18.97	0.02178	**<0.0001**
	vitamin E	368.41	150	0.29	-	0.5896
	daily stress	366.18	150	2.52	-	0.1121
	IVF stress	356.51	150	12.19	-0.01514	**0.0005**

[a] β listed for variables with a *P*-value < 0.05.
[b] 2 levels, oligozoospermic (n=11) and all other (base category, n= 141)
[c] 2 levels, asthenozoospermic (n=32) and all other (base category, n=120)
[d] 2 levels, teratozoospermic (n=25) and all other (base category, n=127)

4.5.3. Multiple Logistic Regression Modelling

The first objective was to obtain a model of significant main effects, which was achieved by consensus of model fitting strategies. Using Statistical Package for Interactive Data Analysis (SPIDA) software, the best subset of ten variables (including the constant) was generated by each of four model fitting strategies: backward elimination, forward selection, stepwise selection and exhaustive search. Three further model fitting strategies were undertaken using Statistix Analytical Software: full model (forcing the fit of all of the variables into the model), backward elimination (with sequential removal of variables from the full model based on the highest *P* value) and stepwise selection (with sequential variable selection based on the lowest *P* value in the full model). In each instance, female age was adjusted into the model. The significant variables identified through each of these selection strategies are presented in Table 4.29, as are the variables found to be significant in the univariate analysis (Table 4.28).

In Table 4.29 it is shown that six variables appeared in all seven of the logistic regression models: female nicotine and proportion of lifetime smoked, and male nicotine, alcohol, fruit and vegetable consumption and IVF stress. Female daily stress was present in six of the seven models, and male caffeine consumption was present in five models.

By consensus, female nicotine and proportion of life smoked, and male nicotine, alcohol and fruit and vegetable consumption and IVF stress were included in the base model, along with female age. In the full model, all were shown to be significant ($P<0.05$). Hierarchical regression analysis was then undertaken to ascertain whether female daily stress and/or male caffeine consumption improved the model fit. The addition of female daily stress resulted in a deviance change of 5.23 on 1df ($P=0.0222$) and male caffeine consumption resulted in a 2.85 deviance change on 1df ($P=0.0914$). Consequently, female daily stress was added to the base model. With the addition of female daily stress to the base model, hierarchical regression analysis was again used to re-examine the contribution of male caffeine consumption to the fit of the model. With a resultant deviance change of 4.43 on 1df ($P=0.0353$), male caffeine consumption was also added to the base model.

Table 4.29: Model Fitting Strategies on Fertilisation, Adjusting for Female Age

gender	variable	uni-variate	SPIDA				Statistix		
			BE	FS	SS	ES	BE	SS	full
male	age								
	oligozoospermia								
	asthenozoospermia	✓							
	teratozoospermia								
female	nicotine		✓	✓	✓	✓	✓	✓	✓
	proportion life smoked	✓	✓	✓	✓	✓	✓	✓	✓
	caffeine	✓							
	alcohol								
	fruit and vegetable	✓							
	daily stress		✓	✓	✓	✓	✓		✓
	IVF stress								
male	nicotine		✓	✓	✓	✓	✓	✓	✓
	smoke years								
	caffeine	✓	✓	✓	✓	✓			
	alcohol		✓	✓	✓	✓	✓	✓	✓
	fruit and vegetable	✓	✓	✓	✓	✓	✓	✓	✓
	vit E supplementation								
	daily stress								
	IVF stress	✓	✓	✓	✓	✓	✓	✓	✓

✓ $P<0.05$

Hierarchical regression analysis was used to ascertain the significance of the remaining factors, but none appeared to improve the model fit. The final model of main effects on fertilisation is presented in Table 4.30.

An investigation of interactions between the female main effects and the male main effects on fertilisation was undertaken. Two-way interaction terms were created for each dual combination of female factors (Table 4.31), and male factors (Table 4.32). Each interaction term was separately added to the main effects model, and the change in deviance and associated *P* value were documented (Table 4.33).

Table 4.30: Multiple Logistic Regression Model of Main Effects on Fertilisation[a]

variable	β	SE	*P* value
constant	0.307	0.412	0.4561
female age[b]	-0.003	0.012	0.7870
female nicotine	0.006	0.003	0.0324
female proportion life smoked	-1.369	0.324	<0.0001
female daily stress	0.015	0.006	0.0094
male nicotine	0.004	0.002	0.0304
male caffeine	-0.147×10^{-3}	0.070×10^{-3}	0.0350
male alcohol	0.014	0.005	0.0110
male fruit and vegetable	0.022	0.005	<0.0001
male IVF stress	-0.020	0.005	<0.0001

[a] deviance=297.14, 142df
[b] included in model to adjust for 'female proportion life smoked'

Table 4.31: Female Interaction Terms

	nicotine	**proportion of life smoked**	**daily stress**
nicotine	-	fsmyrs/age *nic	fnic*stdl
proportion of life smoked	-	-	fsmyrs/age*stdl
daily stress	-	-	-

Table 4.32: Male Interaction Terms

	nicotine	**caffeine**	**alcohol**	**F & V**	**IVF stress**
nicotine	-	mnic*caff	mnic*alc	mnic*f&v	mnic*stivf
caffeine	-	-	mcaff*alc	mcaff*f&v	mcaff*stivf
alcohol	-	-	-	malc*f&v	malc*stivf
F & V	-	-	-	-	mf&v*stivf
IVF stress			-	-	-

Of the interaction terms, mcaff*alc and malc*f&v independently improved the fit of the main effects model. The contribution of fsmyrs/age*nic to the model fit was questionable (P=0.0543). Using hierarchical regression analysis, mcaff*alc, malc*f&v and fsmyrs/age*nic each resulted in a significant deviance change; therefore, each was added to the model of main effects. The inclusion of these three interaction terms rendered male nicotine consumption non-significant (P=0.1113), and as it did not feature in any of the significant interaction terms, it was removed from the model. The final multiple logistic regression model of fertilisation is presented in Table 4.34.

Table 4.33: Assessment of Interaction Terms on Fertilisation

variables in model	deviance	df	change in deviance (1df)	P value
main effects model (MEM)[a]	297.14	142	-	-
MEM + fsmyrs/age*nic	293.42	141	3.72	0.0543
MEM + fnic*stdl	296.77	141	0.37	0.5429
MEM + fsmyrs/age*stdl	296.59	141	0.55	0.4622
MEM + mnic*caff	297.12	141	0.02	0.8992
MEM + mnic*alc	295.52	141	1.62	0.1939
MEM + mnic*f&v	296.99	141	0.15	0.7001
MEM + mnic*stivf	296.66	141	0.48	0.4911
MEM + mcaff*alc	**287.95**	**141**	**9.19**	**0.0026**
MEM + mcaff*f&v	295.95	141	1.19	0.2767
MEM + mcaff*stivf	294.24	141	2.90	0.0899
MEM + malc*f&v	**289.48**	**141**	**7.66**	**0.0054**
MEM + malc*stivf	296.94	141	0.20	0.6539
MEM + mf&v*stivf	295.70	141	1.44	0.2324

[a] see Table 4.30

Table 4.34: Final Logistic Regression Model of Factors on Fertilisation[a]

factor	β	SE	OR (95% CI)	*P* value
constant	-0.016	0.441	-	0.9718
female age	-0.007	0.012	0.99 (0.97, 1.02)	0.5470
female nicotine	-0.025	0.013	0.98 (0.95, 1.01)	0.0559
fsmyrs/age	-1.650	0.330	0.19 (0.10, 0.28)	<0.0001
fsmyrs/age*nicotine	0.059	0.025	1.06 (1.01, 1.11)	0.0187
female daily stress	0.016	0.006	1.02 (1.01, 1.03)	0.0059
male caffeine	0.120×10^{-3}	0.101×10^{-3}	1.00 (0.99, 1.01)	0.2350
male alcohol	0.073	0.014	1.08 (1.05, 1.11)	<0.0001
male fruit & vege	0.032	0.007	1.03 (1.02, 1.05)	<0.0001
male IVF stress	-0.020	0.005	0.98 (0.97, 0.99)	<0.0001
mcaff*alc	-0.026×10^{-3}	0.008×10^{-3}	0.97 (0.96, 0.99)	0.0007
malc*f&v	-0.001	0.516×10^{-3}	<1.00 (<1.00, <1.00)	0.0144

[a] deviance=279.06, 140df

4.5.4 Model Diagnostics

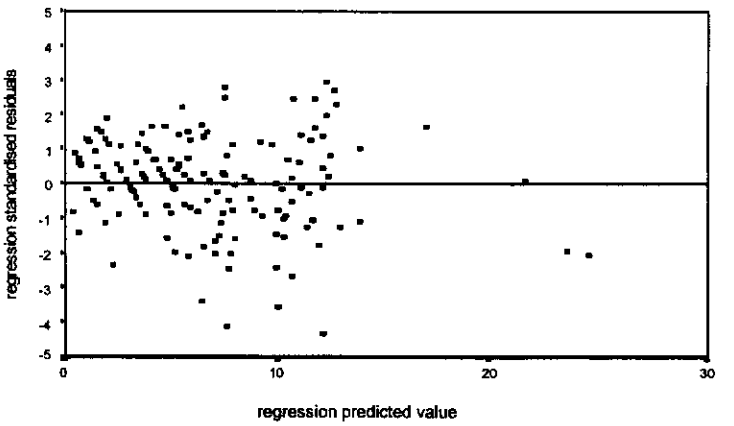
4.5.4.1 Fit of the Model

The pseudo R^2 for the final model was 0.242. A conservative estimate is that almost one-quarter of the variation in fertilisation was explained by the factors in the final model.

4.5.4.2 Non-Linear Regression Function

As the standardised residuals (SDR) plotted against the predicted values are randomly scattered (Figure 4.43), there is no evidence that the function of the model is non-linear.

Figure 4.43: Scatterplot of Standardised Residuals vs Predicted Values



4.5.4.3 Outliers

Four cases were identified as outliers (SDR ± 3.0): couple ID no. 19, 36, 321 and 322. A case summary of these couples is presented in Table 4.35. Of note, a relatively poor rate of fertilisation was evident among all of these couples: 0%, 22%, 31% and 7%, respectively. In particular, none of the 16 oocytes from couple ID no. 19 fertilised, despite all indications to the contrary. The female partner of couple ID no. 321 was 40 years of age and had smoked tobacco for 63% of her life (25 years). Potentially of significance was the high male caffeine consumption of couple ID no. 322, which was found to be within the 90th percentile. Of the factors listed in Table 4.35, none were remarkable with regard to the low fertilisation rate experienced by couple ID no. 36.

Table 4.35: Summary of Cases with an Extreme Standardised Residual Value

ID	SR	female age	female nic	fsmyrs/ age	female daily stress	male caff	male alc	male F&V	male IVF stress	PN 2	total oocytes
19	-4.109	31	0	0.06	2.0	137	1	5	0	0	16
36	-3.351	33	0	0	16.0	939	0	33	12	2	9
321	-3.519	40	88.5	0.63	23	284	0	23	17	5	16
322	-4.284	31	20.1	0.32	29.0	2367	15	17	32	2	30

nic = nic
alc = alcohol
caff = caffeine

4.5.4.4 Leverage

Outlying cases were also identified through the centred leverage (CL) value (Figure 4.44). Seven cases, in particular, had an elevated CL value: couple ID no. 34, 96, 197, 291, 320, 321 and 355. A case summary of these couples is displayed in Table 4.36.

A high fertilisation rate of 80% was experienced by couple ID no. 96, despite the 34 year old female smoker having smoked for 47% of her life (16 years) and the male's caffeine consumption being within the 90th percentile. Couple ID no. 197 also experienced a high fertilisation rate of 82%, which coincided with high levels of male caffeine consumption and alcohol consumption, both of which were in the 90th percentile. With a 40% fertilisation rate, couple ID no. 291 had relatively extreme values for most factors. This female patient was 32 years of age, had smoked for 59% of her life (19 years) and had levels of daily stress within the 75th percentile. The male partner of female patient ID no. 291 had caffeine consumption within the 75th percentile, alcohol consumption within the 90th percentile, zero consumption of fruit and vegetables and no IVF stress. Only 50% of the oocytes fertilised for couple ID no. 320, which was in spite of good female prognostic factors; however, the male partner had a markedly high level of IVF stress (95th percentile), with concomitant low fruit and vegetable consumption (10 percentile) and alcohol consumption within the 75th percentile.

Figure 4.44: Sequence Plot of Centred Leverage

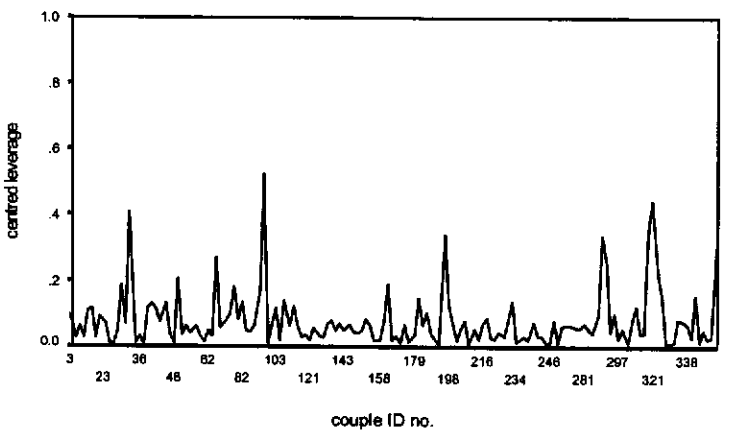


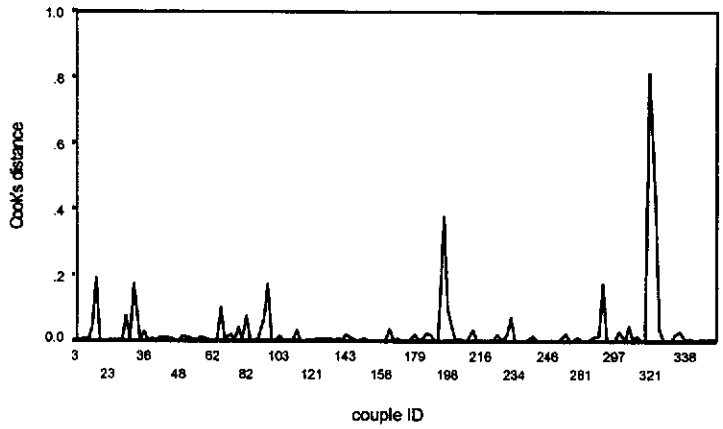
Table 4.36: Summary of Cases with an Extreme Centred Leverage Value

id	centred leverage	female age	female nicotine	fsmyrs /age	female daily stress	male caffeine	male alcohol	male F&V	male IVF stress	pn2	total oocytes
34	0.406	28	55.3	0.29	22.0	0	7	14	11	9	14
96	0.521	34	175.0	0.47	3.0	2780	4	28	0	8	10
197	0.340	34	0	0	32.0	2226	31	32	26	18	22
291	0.335	32	28.4	0.59	7.0	2184	30	0	0	4	10
320	0.346	34	0	0	15.0	1062	24	8	56	11	22
321	0.439	40	88.5	0.63	23.0	284	0	23	17	5	16
322	0.335	31	20.1	0.32	29.0	2367	15	17	32	2	30

4.5.4.5 Influential Observations

The Cook's distance (CD) value was used to identify potentially influential cases (Figure 4.45). Three cases were shown to have an elevated CD value: couple ID no. 197, 321 and 322 (the largest peak in Figure 4.45 represents both couple ID no. 321 and 322), all of which had been identified previously as outliers, and discussed accordingly.

Figure 4.45: Sequence Plot of Cook's Distance



4.5.4.6 Model Sensitivity

The impact of the potentially influential couples on the significance of the parameters in the final model was examined. Listed in Table 4.37 are the couples who had previously been identified as an outlier and/or as potentially influential. Each couple, in isolation, was omitted from the data set, and the final model was re-fitted. For

each parameter in the final model, the subsequent change in the beta coefficient and it's corresponding *P* value was noted (Table 4.37). Of specific interest was whether removal of a given couple changed the apparent significance of the regression parameter.

The data from three couples were influential in regard to female nicotine consumption (Table 4.37). Whilst non-significant in the final model, following the independent omission of couple ID no. 34, 96 and 321, female nicotine consumption was shown to be significant, specifically from P=0.0559 to P=0.0102, from P=0.0559 to P=0.0201 and from P=0.0559 to P=0.0013, respectively. The interaction term between female proportion of years smoked and female nicotine consumption was rendered non-significant by the independent omission of couples ID no. 292 and 322, from P=0.0187 to P=0.1030 and from P=0.0187 to P=0.2083, respectively. The independent exclusion of couple ID no. 19 and 67 rendered the interaction term between male alcohol consumption and male fruit and vegetable consumption (malc*f&v) non-significant, from P=0.0144 to P=0.0609, and from P=0.0144 to P=0.1361, respectively.

Table 4.37: Sensitivity of the Final Logistic Regression Model on Fertilisation for Selected Couples of Interest

Couple ID no.	deviance	df	coefficient *P* value										
			female age	female nicotine	fsy_ag_p	fsmyrs_age *nic	female daily stress	male alcohol	male f&v	male IVF stress	male caffeine	male caff*alc	male alc*f&v
	279.06	**140**	**0.5470**	**0.0559**	**<0.0001**	**0.0187**	**0.0059**	**<0.0001**	**<0.0001**	**<0.0001**	**0.2350**	**0.0007**	**0.0144**
ID no. of deleted couple													
19	256.03	139	0.4732	0.0616	<0.0001	0.0199	0.0401	<0.0001	0.0003	<0.0001	0.5874	0.0039	**0.0609**
34	276.09	139	0.5271	**0.0102**	<0.0001	0.0037	0.0096	<0.0001	<0.0001	0.0001	0.1163	0.0003	0.0122
36	269.23	139	0.4894	0.0520	<0.0001	0.0168	0.0080	<0.0001	<0.0001	<0.0001	0.2178	0.0006	0.0066
50	278.35	139	0.5854	0.0594	<0.0001	0.0208	0.0059	<0.0001	<0.0001	<0.0001	0.2507	0.0031	0.0114
67	275.88	139	0.5688	0.0519	<0.0001	0.0170	0.0088	<0.0001	<0.0001	0.0001	0.3060	0.0023	**0.1361**
96	277.08	139	0.5748	**0.0201**	<0.0001	0.0078	0.0028	<0.0001	<0.0001	<0.0001	0.3568	0.0007	0.0154
197	269.65	139	0.5777	0.0578	<0.0001	0.0208	0.0123	<0.0001	<0.0001	<0.0001	0.0885	<0.0001	0.0016
291	278.66	139	0.5317	0.0505	<0.0001	0.0166	0.0067	<0.0001	<0.0001	<0.0001	0.2937	0.0033	0.0124
292	272.17	139	0.5906	0.1595	<0.0001	**0.1030**	0.0022	<0.0001	<0.0001	<0.0001	0.1265	0.0005	0.0102
320	278.99	139	0.5535	0.0539	<0.0001	0.0180	0.0092	<0.0001	<0.0001	0.0005	0.2308	0.0006	0.0143
321	266.45	139	0.6225	**0.0013**	<0.0001	0.0002	0.0038	<0.0001	<0.0001	0.0002	0.6879	0.0036	0.0198
322[a]	256.95	139	0.3408	0.4157	<0.0001	**0.2083**	0.0029	<0.0001	<0.0001	0.0012	0.1773	0.0078	0.0116
355	279.05	139	0.5470	0.0722	<0.0001	0.0312	0.0087	<0.0001	<0.0001	<0.0001	0.2557	0.0008	0.0143

[a] previously cryopreserved semen sample used for *in vitro* fertilisation.

4.5.5 Interpretation of the Regression Model

The fertilisation regression equation is:

$$\ln [\ \hat{p}_i / (\ 1 - \hat{p}_i\ )\ ] = u_i$$

where $\hat{p}_i$ is the estimated probability of fertilisation *in vitro* for the *i*th patient ($i = 1, \ldots, n$) is pregnant and u_i is the linear predictor:

$$\begin{aligned} u_i = {} & -0.016 - 0.007(\text{female age}) - 0.025(\text{female nicotine}) - \\ & 1.650(\text{fsmyrs/age}) + 0.059(\text{fsmyrs/age*female nicotine}) + \\ & 0.016(\text{female daily stress}) + 0.120\times10^{-3}(\text{male caffeine}) + 0.073\ (\text{male} \\ & \text{alcohol}) + 0.032(\text{male fruit \& vege}) - 0.020(\text{male IVF stress}) - \\ & 0.026\times10^{-3}(\text{male caffeine*alcohol}) - 0.001(\text{male alcohol*fruit \& vege}). \end{aligned}$$

Fertilisation *in vitro* was not affected by any of the patient factors examined, including female age, male age, oligozoospermia, asthenozoospermia and teratozoospermia. However, numerous lifestyle factors were identified as having a significant effect on fertilisation, those pertaining to both the male and female. Based on the pseudo R^2, these significant lifestyle factors collectively accounted for a conservative 24.2% of the variability in the rates of fertilisation *in vitro* between the couples.

Of the female lifestyle factors, cigarette smoking and daily stress had a significant effect on the rate of fertilisation *in vitro*. Contrary to expected, fertilisation increased with daily stress levels of the female patient. In regard to smoking, fertilisation decreased with years of tobacco consumption ($P<0.0001$), measured as the proportion of a woman's life that she has/had smoked adjusted for age (fsmyrs/age). The effect of female nicotine consumption on fertilisation was tenuous. Although of borderline significance ($P=0.0559$) in the final model, the effect became significant following the simultaneous and independent removal of three couples from the model

fit. Nonetheless, the significant interaction term between female nicotine consumption and proportion of years smoked (fsmyrs/age*nicotine)(P=0.087) suggests that female nicotine consumption exacerbates the detrimental effect of fsmyrs/age on the rate of fertilisation (P=0.0144). It is noted, however, that the independent omission of two couples from the model fit changed the significance of this interaction term from $P<0.05$ to $P\geq0.05$.

In relation to male lifestyle factors, alcohol consumption and fruit and vegetable consumption were beneficial in respect to fertilisation *in vitro*. Specifically, the rate of fertilisation increased with male alcohol consumption ($P<0.0001$), and with male fruit and vegetable consumption ($P<0.0001$). A significant interaction term between male alcohol consumption and male fruit and vegetable consumption (malc*f&v) was also found (P=0.0144). The negative coefficient of malc*f&v (β=-0.001) suggests that this relationship is asymptotic, that being, a threshold of benefit that can be derived from the combined effect of male alcohol and fruit and vegetable consumption. Of note, however, this interaction term was rendered non-significant following the simultaneous and independent omission of two couples.

By contrast, male IVF-related stress and caffeine consumption had a detrimental effect on fertilisation. Increasing levels of stress due to IVF treatment significantly decreased fertilisation *in vitro* ($P<0.0001$). Although male caffeine consumption was not significant as a main effect (P=0.2350), it did exert an effect, as is demonstrated by the significant interaction term between male caffeine and alcohol consumption (mcaff*alc). The negative coefficient of mcaff*alc ($\beta=-0.026\times10^{-3}$) implies that male caffeine consumption negates, to some extent, the beneficial effect of male alcohol consumption on fertilisation.

4.6 Pregnancy

Factors affecting pregnancy following IVF treatment were investigated. By definition, pregnancy is a positive β-hCG pregnancy test outcome at 16 days post-embryo transfer. The treatment of 296 couples (n_4) proceeded to the stage of embryo transfer (Figure 4.46). Eight of these 296 couples were ineligible for inclusion. Consequently, 288 couples were included in the analysis of factors affecting pregnancy outcome ($n_{4.1}$), of which a pregnancy ensued among 90 of these 288 couples ($n_{4.1}$), generating a pregnancy rate of 31.3%.

4.6.1 Descriptive Statistics

Females ranged in age from 21 to 47 years, with a mean of 34.5 (SD 5.3). The age range of the males was 21 to 57 years, and the mean was 36.3 (SD 5.8).

The distribution of females by infertility aetiology is displayed in Table 4.38, as is the method of fertilisation. One in every three couples' treatment involved ICSI (34.0%).

Despite having more than two embryos available, 25.3% of the couples had the transfer of only two embryos (2-ET), which was at the recommendation of the Embryologist and/or the Clinician. As such couples were considered to have a better than average prognosis of pregnancy, the transfer of only two embryos reduced the likelihood of a multiple pregnancy. As the number of embryos transferred impacts on the value of the modified Cumulative Embryo Score (mCES), 2-ET was treated as an additional factor in the analysis on pregnancy outcome.

Embryo quality was also included in the investigation on pregnancy outcome. The mCES was used as a measured collective quality of the embryos transferred into the uterine. Values of the mCES ranged from 1 to 44, with a mean of 34.5 (SD 5.3)(Figure 4.47).

Figure 4.46: Flowchart of Pregnancy and Pregnancy Loss Sub-Samples

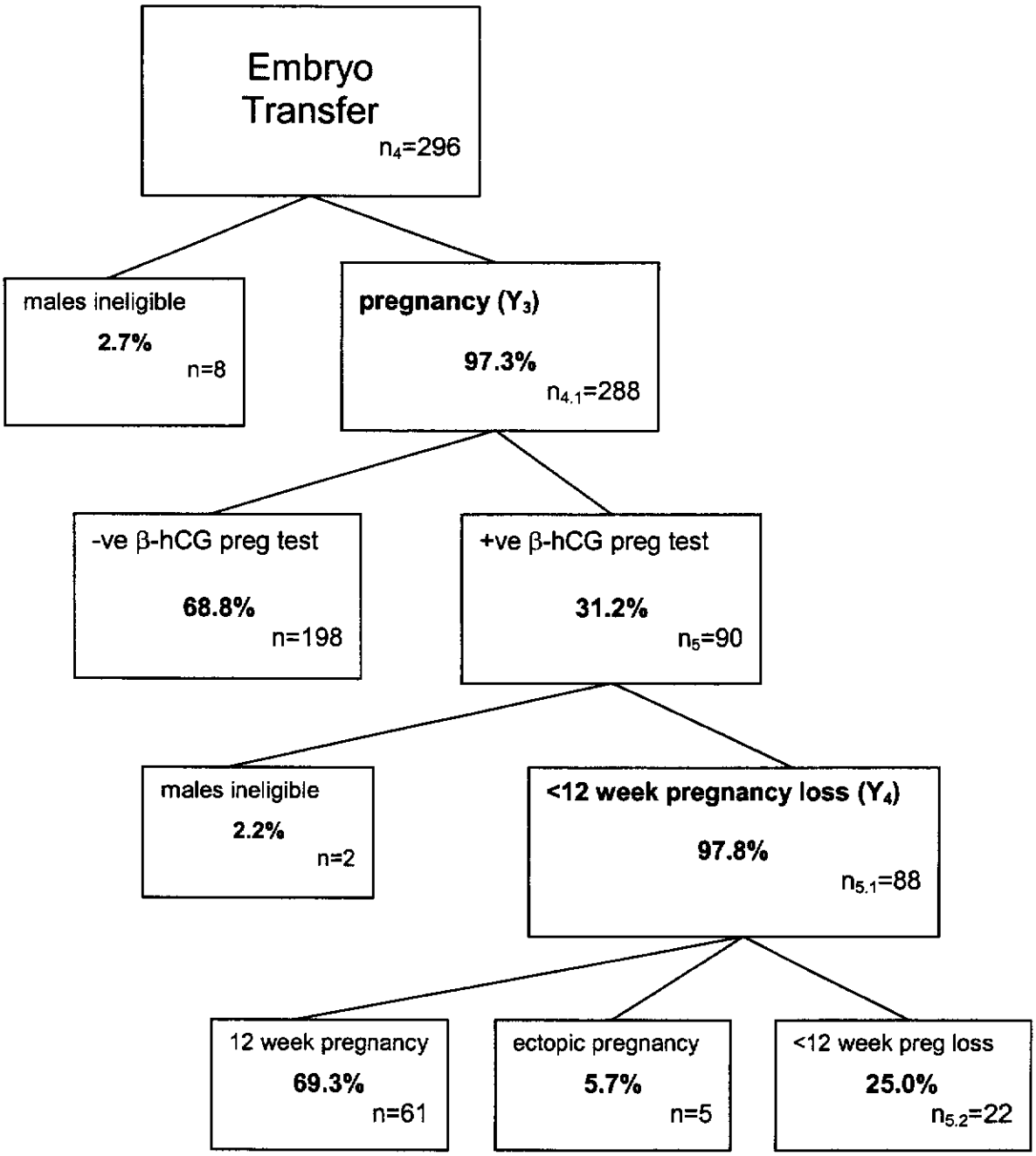
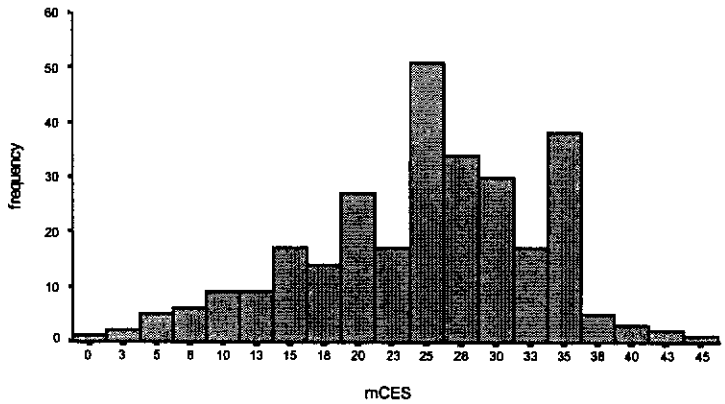


Table 4.38: Distribution of Patient and Treatment Factors

variable	categories	frequency (n)	percent (%)
infertility aetiology:	nil significance	71	24.7
	unexplained	69	24.0
	tubal factor only	83	28.8
	endometriosis only	40	13.9
	uterine only	10	3.5
	multiple factors	15	5.2
fertilisation method	ICSI	98	34.0
	in vitro	190	66.0
2-ET	yes	73	25.3
	no	215	74.7

Figure 4.47: Histogram of mCES



Descriptive statistics for each lifestyle factor investigated in relation to pregnancy outcome are presented in Table 4.39.

Of the 288 couples, smoking status was self-reported by 258 (89.6%) of the females, and by 218 (75.5%) of the males. One in five of the women was a regular smoker (20.2%), 4.3% were occasional smokers, almost one-third were ex-smokers (31.4%) and 44.2% had never smoked. Among the males, 21.1% were regular smokers, 3.7% occasional smokers, 27.5% were ex-smokers and nearly half of the men (47.7%) had never smoked.

Table 4.39: Descriptive Statistics of Lifestyle Factors

gender	factor	n[a]	min	max	median	IQR	mean	SD
female	nicotine (mg/wk)	239	0	175	0	0.0	5.9	-
	smoke years	239	0	29	1.0	10.0	5.6	-
	caffeine (mg/wk)	241	0	2706	780	-	851	631
	alcohol (std dks/wk)	241	0	30	0.2	4.5	3.2	-
	fruit & veg (serves/wk)	240	0	67	23.5	-	24.9	12.1
	daily stress	238	0	57.0	19.0	-	19.1	11.5
	IVF stress	238	0	65.0	17.0	-	17.6	12.2
male	nicotine (mg/wk)	219	0	245	0	0.0	15.5	-
	smoke years	218	0	30	1.0	12.2	6.7	-
	caffeine (mg/wk)	217	0	5376	1062	-	1198	876
	alcohol (std dks/wk)	217	0	79	6.0	13.0	9.8	-
	fruit & veg(serves/wk)	211	4	54	20.0	-	21.0	10.6
	daily stress	209	0	53	19.0	-	21.0	12.2
	IVF stress	209	0	62	7.0	16.0	11.0	-

[a] n varied due to response rate; patients' completion and return of LQ/D.

Over the period covered by the diary, seven of the 11 female occasional smokers consumed nicotine. Weekly nicotine consumption ranged from 0.6 to 3.6mg. Only three of the male occasional smokers consumed nicotine during the week in question; their weekly nicotine consumption was 0.7, 3.6 and 4.1mg. Nicotine consumption among the 34 female regular smokers ranged from 0.8 to 175.0mg/wk, with a median value of 31.4mg (IQR 48.9). Among the 46 male regular smokers, weekly nicotine consumption ranged from 2.9 to 245.0mg, and the median was 60.0mg (IQR 78.1).

Among the 81 female ex-smokers, smoke years ranged from one week to 25 years, but the median was 7.0 (IQR 10.0). Of the 34 female regular smokers, smoke years ranged from 6 to 25, with a mean of 14.9 (SD 5.4). Of the 60 male ex-smokers, smoke years ranged from one month to 30 years, and the median was 16.5 (IQR 10.8). Smoke years ranged from six months to 30 years among the 46 male regular smokers, with a mean of 17.0 (SD 6.7).

Thirteen of 241 female respondents (5.4%) did not consume caffeine, neither did 11 of the 217 male respondents (5.1%). While one-half of the females did not consume any alcohol (49.8%) over this same period of time, only 22.6% of the males were non-alcohol consumers. Of the 121 female respondents who did consume alcohol, consumption ranged from 0.3 to 30 standard drinks per week, with a median value of 4.5 (IQR 7.0). Among the 168 male alcohol consumers, consumption ranged from 1 to 79 standard drinks per week, with a median of 8.5 (IQR 11.0). Two males, in particular, had extreme levels of alcohol consumption: 59 and 79 standard drinks per week, an average of 8.4 and 11.3 standard drinks per day, respectively.

As shown in Table 4.39, weekly fruit and vegetable consumption varied considerably among both the males and females. Consumption among the females ranged from 0 to 67 serves per week, with a mean of 24.9 (SD 12.1). For males, weekly consumption of fruit and vegetables ranged from 4 to 54 serves, with a mean of 21.0 (SD 12.2).

Stress levels among the males and females also showed considerable variability (Table 4.39). Among the females, the weekly daily stress scores ranged from 0 to 57, with a mean of 19.1 (SD 11.5), and the weekly IVF stress scores ranged from 0 to 65, with a mean of 17.6 (SD 12.2). The weekly daily stress scores among the males varied between 0 and 53, with a mean of 21.0 (SD 12.2). The distribution of male weekly IVF stress scores was right-skewed, with values ranging from 0 to 62, and a median of 7.0 (IQR 16.0).

4.6.2 Univariate Analysis

A comparative analysis between the pregnant and non-pregnant women, and between the male partners' of these two groups of women is presented in Table 4.40.

Embryo quality, on average, was significantly higher among the pregnant couples than the non-pregnant couples (P=0.001). Specifically, the mean mCES of the pregnant and

non-pregnant couples was 26.9 (SD 6.6) and 23.7 (SD 9.0), respectively (Table 4.40), a significant mean difference of -3.2 (95% CI -5.0 to -1.3).

Table 4.40: Summary of Univariate Comparative Analysis Between Pregnant[a] and Non-Pregnant Females and Between Their Male Partners

			univariate analysis				
gender	variable	n	test	statistic	value	df	*P* value
	mCES	288	t test	t	-3.39	230.9	**0.001**
	2-ET[b]	288	chi-square	χ^2	10.69	1	**0.001**
female	age	288	t-test	t	2.31	286	**0.022**
	infertility aetiology[c]	288	chi-square	χ^2	10.86	5	0.054
male	age	288	t test	t	1.62	286	0.106
	ICSI[d]	288	chi-square	χ^2	0.82	1	0.365
	lifestyle factors						
female	nicotine	239	MW[e]	U	5649		**0.044**
	yrs of tobacco	239	MW	U	5896	-	0.372
	caffeine	241	t-test	t	-0.49	239	0.623
	alcohol	241	MW	U	6437	-	0.995
	fruit & vegetables	240	t-test	t	1.75	238	0.081
	daily stress	238	t-test	t	-2.27	236	**0.024**
	IVF stress	238	t-test	t	-1.59	236	0.113
male	nicotine	219	MW	U	4994	-	0.251
	yrs of tobacco	218	MW	U	4856	-	0.257
	caffeine	217	t-test	t	-0.63	109.0	0.532
	alcohol	217	MW	U	5130	-	0.772
	fruit & vegetables	211	t-test	t	0.92	209	0.360
	daily stress	209	t-test	t	0.21	207	0.836
	IVF stress	209	MW	U	4510	-	0.434

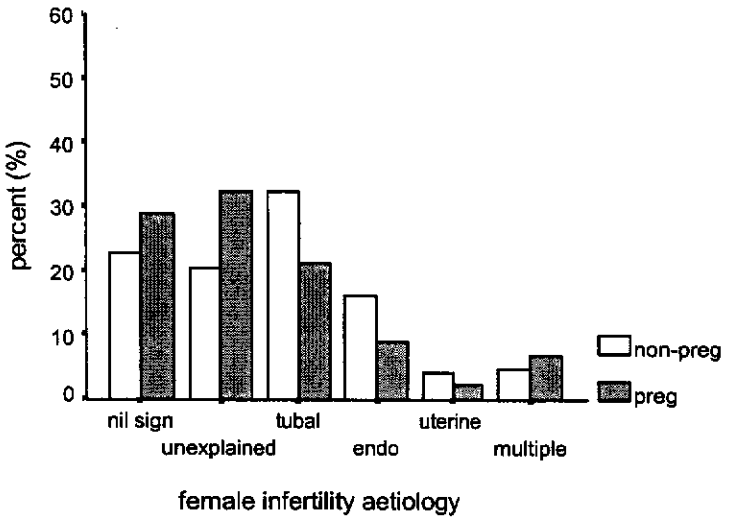
[a] pregnancy test 16 days post-ET
[b] 2 levels, non-2-ET (base category, n=215) and 2-ET (n=73)
[c] 6 levels, nil significance (base category, n=71), unexplained (n=69), tubal (n=83), endometriosis (n=40), uterine (n=10) and multiple (n=15)
[d] 2 levels, fertilisation in vitro (base category, n=190) and fertilisation by ICSI (n=98)
[e] Mann-Whitney non-parametric test of two independent samples

Of the 73 couples who had a 2-ET transfer, 46.6% had a positive pregnancy outcome as compared to 26.0% of the 215 non-2-ET couples. This higher rate of pregnancy among the 2-ET couples was of statistical significance (P=0.001)(Table 4.40).

Women who had a positive pregnancy outcome were significantly younger on average than the non-pregnant women (P=0.022)(Table 4.40). Specifically, the mean age of pregnant and non-pregnant women was 33.4 (SD 4.7) and 35.0 (5.5) years, respectively; a significant mean age difference of 1.5 years (95% CI 0.23 to 2.8). No difference of significance was evident between the mean age of male partners' of the pregnant and non-pregnant women (Table 4.40).

The relationship between pregnancy outcome and female infertility aetiology was examined (Figure 4.48). A pregnancy resulted among 36.6% of the women with infertility of nil significance (26/71), 42.0% with unexplained infertility (29/69), 22.9% with tubal factor (19/83), 20% with endometriosis only (8/32), 20% with uterine factor and 40.0% with multiple infertility factors (6/15). This association between female infertility and pregnancy outcome was of borderline significance (P=0.054)(Table 4.40).

Figure 4.48: Histogram of Pregnancy Outcome by Female Infertility Aetiology



nil sign = infertility of nil significance
endo = endometriosis

Of the 98 couples whose treatment involved ICSI for oocyte fertilisation, 34.7% had a positive pregnancy outcome, which compared with 29.5% of the 190 non-ICSI couples (P=0.365)(Table 4.40).

Of the female lifestyle factors, significant differences in nicotine consumption and daily stress were found between the pregnant and non-pregnant women (Table 4.40). In relation to nicotine consumption, the mean rank was significantly higher among the pregnant women than that of the non-pregnant women, 128.5 vs 115.8 (P=0.044), indicating women who achieved a pregnancy had significantly higher levels of nicotine consumption. Significantly higher levels of daily stress were reported among female patients who became pregnant than those who did not (P=0.024), with mean weekly daily stress scores of 21.5 (SD 12.8) and 17.9 (SD 10.6), respectively.

Of the lifestyle factors investigated, no differences were evident between the male partners' of the pregnant and non-pregnant women (Table 4.40).

4.6.3 Multiple Logistic Regression Modelling

Multiple logistic regression analysis was used to assess the effect of patient, treatment and lifestyle factors on pregnancy outcome. All of the variables listed in Table 4.40 were examined in the modelling procedure.

The first objective was to acquire a model of main effects. The results of three different modelling strategies were compared: stepwise forward, backward elimination and the full model, which included all of the variables. The stepwise forward method involved stepwise selection, with entry based on the significance of the score statistic, and removal based on the probability of a likelihood ratio (LR) statistic. With the backward elimination method, removal was based on the probability of the LR statistic.

Embryo quality (mCES) was significant in all three models (Table 4.41). Tubal infertility and female daily stress were significant in two of the three models. One of the three models featured female unexplained infertility, as did 2-ET.

Table 4.41: Multiple Logistic Regression Model Fitting Strategies

variable	model fitting strategies		
	full model *P* value[a]	stepwise forward *P* value	backward elimination *P* value
mCES	0.0079	0.0108	0.0090
2-ET by choice	-	0.0395	-
unexplained female infertility[b]	-	0.0247	-
tubal factor infertility[b]	0.0370	-	0.0303
female daily stress	-	0.0402	0.0484

[a] only variables with $P<0.05$ are listed
[b] female infertility of nil significance as base category

Hierarchical regression analysis was subsequently undertaken to further investigate the significance of the variables identified through the three different model fitting strategies: mCES, 2-ET, female infertility aetiology and female daily stress. To a base model comprised of mCES, 2-ET was added. As 2-ET significantly improved the model fit (χ^2=13.877, 1df, P=0.0002), it was included in the base model.

Next, the significance of female infertility, specifically tubal factor and unexplained, was examined. As female infertility was a variable with six levels, it was re-coded into a 3-level variable (unexplained infertility, tubal factor and all other types of infertility) and added to the base model comprised of mCES and 2-ET. Female unexplained infertility was shown to be of questionable significance (P=0.0534) and tubal factor was non-significant (P=0.2837). Female infertility was re-coded further into a 2-level variable (unexplained infertility and all other), and re-examined in the base model. As its inclusion improved the model fit, it remained in the base model along with mCES and 2-ET.

Female daily stress was added to the base model of mCES, 2-ET and unexplained infertility. With a resultant χ^2 statistic of 5.446 on 1df, female daily stress was shown to improve the fit of the model (P=0.0196), and was added to the base model.

Following a further investigation, none of the remaining variables improved the model fit. Therefore, the multiple logistic regression model of main effects on pregnancy included mCES, 2-ET, unexplained infertility and female daily stress (Table 4.42).

Table 4.42: Multiple Logistic Regression Model of Main Effects on Pregnancy[a,b]

factor	β	SE	Wald	*P* value
constant	-3.329	0.669	24.744	<0.0001
mCES	0.060	0.199	9.282	0.0023
2-ET	1.107	0.331	11.168	0.0008
unexplained infertility	0.787	0.329	5.727	0.0167
female daily stress	0.030	0.013	5.353	0.0207

[a] +ve β-hCG pregnancy test 16 days post-ET
[b] goodness-of-fit χ^2=28.863, 4df, P<0.0001

Interrelationships between the main effects on pregnancy outcome were subsequently examined. An interaction term was created for each dual combination of the main effects (Table 4.43).

Table 4.43: Interaction Terms

	mCES	**2-ET**	**unexplained infertility**	**female daily stress**
mCES	-	mCES*ET2	mCES*unex	mCES*fstdl
2-ET	-	-	ET2*unex	ET2*fstdl
unexplained infertility	-	-	-	unex*fstdl
female daily stress	-	-	-	-

Hierarchical regression analysis was then used to assess whether any of the interaction terms improved the fit of the main effects model. As shown in Table 4.44, none of the interaction terms significantly improved the model fit. Therefore, the final multiple logistic regression model of pregnancy is that displayed in Table 4.42.

As relative chance (RC) is a more appropriate term for the odds ratio when the outcome is a positive event, the associated RC for each main effect is presented in Table 4.45.

Table 4.44: Examination of Interaction Terms on Pregnancy[a]

variables in the model					β *P*-value	χ^2 (1df)	goodness of fit
mCES	**2-ET**	**unex IF**	**f dl stress**	-	-	-	235.853
mCES	2-ET	unex IF	f dl stress	mCES*2-ET	0.3209	1.013	235.850
mCES	2-ET	unex IF	f dl stress	mCES*unex	0.1601	2.093	240.711
mCES	2-ET	unex IF	f dl stress	mCES*fdlst	0.4307	0.642	235.679
mCES	2-ET	unex IF	f dl stress	2-ET*unex	0.3506	0.864	236.103
mCES	2-ET	unex IF	f dl stress	2-ET*fdlst	0.1213	2.400	238.333
mCES	2-ET	unex IF	f dl stress	unex*fdlst	0.2469	1.336	236.067

[a] +ve β-hCG pregnancy test 16 days post-ET

Table 4.45: Relative Chance of Main Effects on Pregnancy[a]

main effect	relative chance (RC)	95% CI for RC low, upper
mCES	1.062	1.022, 1.105
2-ET	3.025	1.581, 5.791
unexplained female infertility	2.196	1.153, 4.182
female daily stress	1.030	1.005, 1.057

[a] +ve β-hCG pregnancy test 16 days post-ET

4.6.4 Model Diagnostics

4.6.4.1 Outliers

As no one couple had a standardised residual nor a studentised residual of ±3.0, no outliers were identified (Figures 4.49 and 4.50).

Figure 4.49: Sequence Plot of Standardised Residuals

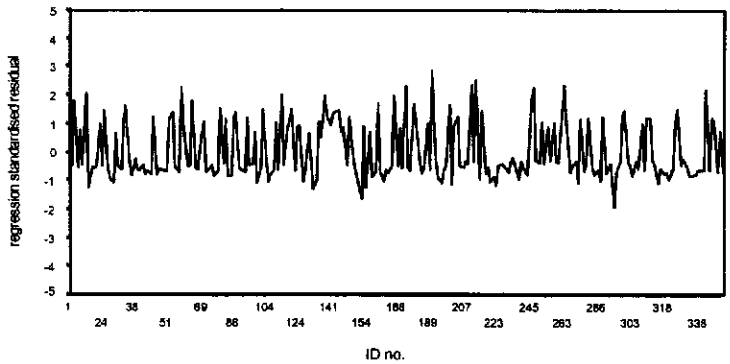
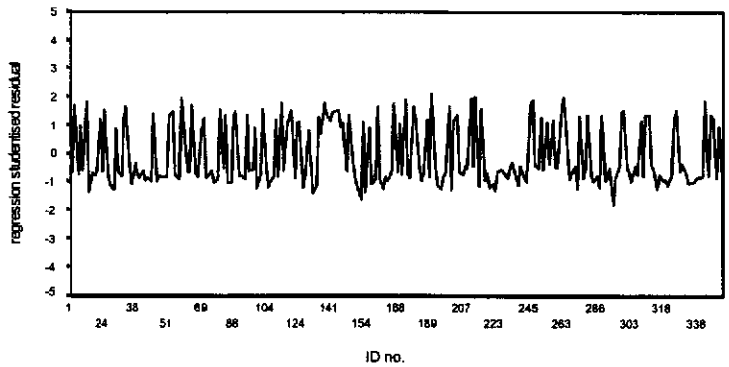


Figure 4.50: Sequence Plot of Studentised Residuals



4.6.4.2 Leverage

A plot of the centred leverage (CL) values is presented in Figure 4.51. Six couples had a CL value that exceeded 0.050. A summary of these six couples is displayed in Table 4.46.

Figure 4.51: Sequence Plot of Centred Leverage

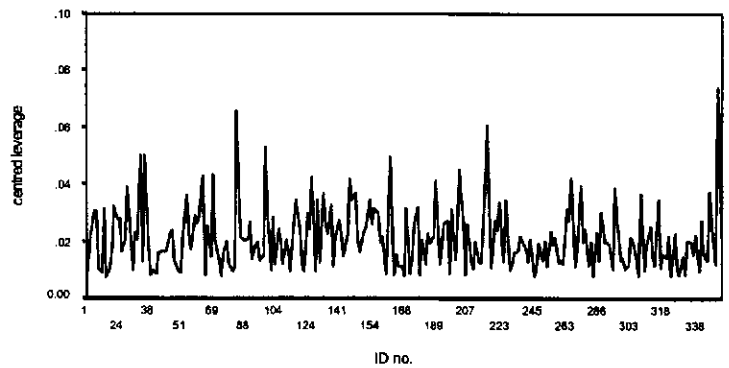


Table 4.46: Summary of Cases with Extreme Centred Leverage Values

ID no.	CL	mCES	2-ET	female unexplained infertility	female daily stress	pregnancy outcome
35	0.050	14.0	no	no	51.0	+ve
37	0.050	15.0	no	yes	45.0	-ve
84	0.065	22.0	no	no	57.0	+ve
99	0.053	30.0	no	yes	52.0	+ve
217	0.060	16.0	no	no	55.0	+ve
355	0.074	38.0	no	no	57.0	+ve

4.6.4.3 Influential Observations

The value of the Cook's distance (CD) was also examined (Figure 4.52). Six cases had CD values that were elevated relative to all other cases and were, therefore, considered to be potentially influential. A summary of these six couples is presented in Table 4.47.

Figure 4.52: Sequence Plot of Cook's Distance

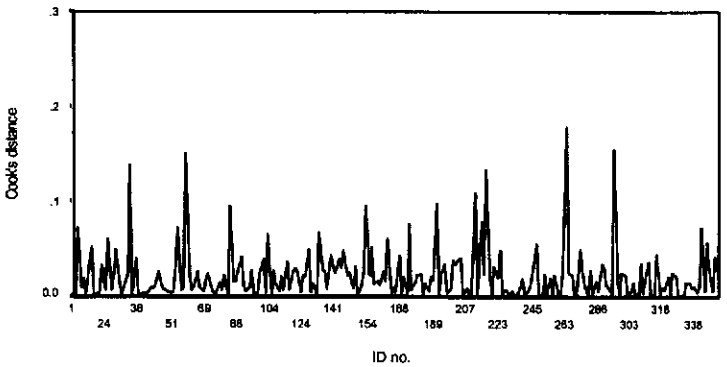
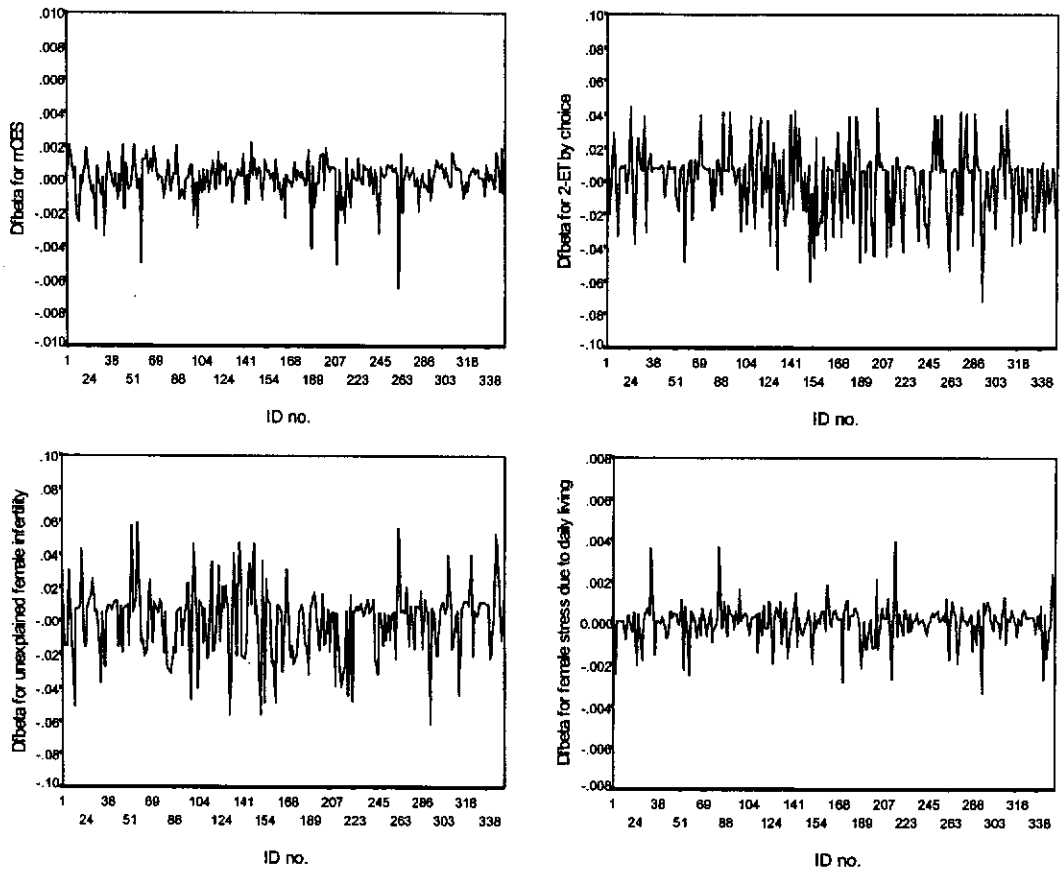


Table 4.47: Summary of Cases with Extreme Cook's Distance Values

ID no.	CD	mCES	2-ET	unexplained female infertility	female daily stress	pregnancy test outcome
35	0.138	14.0	no	no	51.0	+ve
58	0.150	12.0	no	yes	7.0	+ve
211	0.108	12.0	no	no	31.0	+ve
217	0.132	16.0	no	no	55.0	+ve
263	0.177	7.5	no	yes	13.0	+ve
294	0.153	24.0	yes	yes	44.5	-ve

A DFBETA sequence plot for each main effect in the final model is plotted (Figure 4.53). The DFBETA value represents the change in logistic coefficients when the respective case is deleted from the model. Based on these DFBETA plots, it would appear that female daily stress was the principle reason for the identification of couple ID no. 35 and 84 as potentially influential. Couple ID no. 58 was identified due to mCES, 2-ET and unexplained female infertility, as was couple ID no. 263. It would appear that couple ID no. 211 was identified based on all of the factors in the final model. The predominate reason for the identification of couple ID no. 217 was unexplained female infertility. 2-ET, unexplained female infertility and female daily stress were the reason that couple ID no. 294 was identified as potentially influential. No one factor was shown to account for the identification of couples ID no. 37, 99 and 355.

Figure 4.53: Sequence Plots of DFBETA for mCES, 2-ET, Female Unexplained Infertility and Female Daily Stress



All of the couples who had been identified as potentially influential are listed in Table 4.48. Each couple was independently omitted from the data set and the final model re-fitted; the resultant coefficient *P* values are also displayed in Table 4.48. For the purpose of comparison, the coefficient *P* values for each factor in the final model are also presented. As shown, no one case influenced the model to the extent that the coefficient *P* value change from significant (<0.05) to non-significant (≥0.05).

4.6.5 Interpretation of the Regression Model

The pregnancy regression equation is:

$$\ln [\ \hat{p}_i / (1 - \hat{p}_i)\ ] = u_i$$

where $\hat{p}_i$ is the estimated probability of pregnancy for the ith patient ($i = 1, \ldots, n$) and u_i is the linear predictor:

$$u_i = -3.329 + 0.060(\text{mCES}) + 1.107(\text{2-ET}) + 0.787(\text{unexplained female infertility}) + 0.030(\text{female daily stress score})$$

The relative chance (RC) of a pregnancy increased with embryo quality. Specifically, the RC increased by a factor of 1.06 (95% CI 1.02 to 1.10) for every one unit increase in the mCES (P=0.0023). Female patients who had two embryos transferred by recommendation (2-ET) were significantly more likely to achieve a pregnancy than non-2-ET women: RC of 3.0 (95% CI 1.6 to 5.8). The chance of pregnancy was also elevated among female patients with unexplained infertility aetiology: RC of 2.2 (95% CI 1.2 to 4.2).

Daily stress was the one and only lifestyle factor related to pregnancy outcome. Contrary to the expected, the RC of pregnancy increased with increasing levels of daily stress. For every one unit increase in the weekly stress score, the RC increased by 3.0%. None of the lifestyle factors of the male partners were shown to affect pregnancy outcome.

Although univariate analysis indicated that female age was significant in relation to pregnancy, no such association was evident following multivariate analysis. On further examination, 2-ET women were shown to be significantly younger than non-2-ET women, 30.2 (SD 3.8) vs 36.0 (SD 4.9) years (t=10.40, 159.9df, $P<0.001$). This significant age difference between 2-ET and non-2-ET women appeared to over-shadow the effect of female age on pregnancy.

Table 4.48: Sensitivity of the Final Logistic Regression Model on Pregnancy[a] for Selected Couples of Interest

ID	goodness of fit	p-value of coefficient in the final model				std residual	CL	CD
		mCES	ET2	unexplained infertility	female daily stress			
model	**235.853**	**0.0023**	**0.0008**	**0.0167**	**0.0207**	-	-	-
35	234.751	0.0016	0.0006	0.0127	0.0452	-	0.050	0.138
37	234.920	0.0030	0.0010	0.0136	0.0159	-	0.050	-
58	234.641	0.0013	0.0005	0.0286	0.0132	-	-	0.150
84	234.661	0.0021	0.0007	0.0135	0.0480	-	0.065	-
99	234.596	0.0026	0.0008	0.0206	0.0313	-	0.053	-
211	233.961	0.0013	0.0006	0.0126	0.0273	-	-	0.108
217	234.768	0.0017	0.0007	0.0129	0.0489	-	0.060	0.132
263	234.185	0.0011	0.0005	0.0280	0.0152	-	-	0.177
294	235.133	0.0020	0.0004	0.0106	0.0112	-	-	0.153
355	234.471	0.0032	0.0008	0.0151	0.0381	-	0.074	-

[a] +ve β-hCG pregnancy test 16 days post-ET

4.7 Pregnancy Loss

Factors affecting a pregnancy loss were investigated among the 90 couples who had a positive β-hCG pregnancy test outcome 16 days post-ET (n_5)(Figure 4.46). The couples who experienced a pregnancy loss before the 12th week of gestation (<12 week pregnancy loss) were compared to the couples whose pregnancy was ongoing at 12 weeks gestation. Paradoxically, two of the 90 couples experienced both outcomes of interest; each had an ongoing singleton pregnancy at 12 weeks, but also had evidence of a loss of another conceptus at an earlier stage of pregnancy. Consequently, these two couples were excluded from the data analysis. Of the 88 eligible couples ($n_{5.1}$)(Figure 4.46), 27 experienced a pregnancy loss prior to the 12th week of gestation: a <12 week pregnancy loss rate of 30.7%.

4.7.1 Descriptive Statistics

Patient, treatment and lifestyle variables examined in relation to pregnancy loss are described in Table 4.49. As there were too few cases in each category, female infertility aetiology was not examined in the regression analysis on pregnancy loss. For interest, however, 26 of the women had infertility of nil significance (29.5%), 28 had unexplained infertility (31.8%), 18 had tubal infertility (20.5%), eight had endometriosis (9.1%), two had infertility associated with uterine factors (2.3%) and six women were infertile due to multiple factors.

In relation to treatment factors, 34 of the 88 (38.6%) couples had a two embryo transfer by recommendation (2-ET). The method of oocyte fertilisation was by ICSI for 34 of the 88 couples (38.6%).

Eighteen of the 77 females had smoked at least one cigarette during the week of the diary (30.5%), of which nicotine consumption ranged from 1.6 to 107.1mg/week, with a median value of 34.8 (IQR 34.9). During the week in question, 19 of the 72 male respondents had smoked a tobacco product (26.4%), with nicotine consumption ranging from 4.1 to 245.0mg/week, with a median value of 61.2 (IQR 76.4).

Table 4.49: Descriptive Statistics of Treatment, Patient and Lifestyle Factors

	factor	n[a]	min	max	Median	IQR	mean	SD
	mCES	88	7.5	42.0	28.0	-	26.8	6.5
female:	age	88	23	45	33.0	-	33.4	4.6
male:	age	88	26	57	34.5	-	35.4	6.0
female:	nicotine (mg/wk)	77	0	107.1	0	0.0	7.9	-
	smoke years	77	0	25	3.0	10.0	5.6	-
	caffeine (mg/wk)	78	0	2706	821	837	867	-
	alcohol (std dks/wk)	78	0	24	0.8	4.0	2.7	-
	fruit & veg (serves/wk)	78	0	52	22.2	-	23.2	10.7
	daily stress	78	0	57	21.0	-	20.9	12.3
	IVF stress	78	0	65	18.8	-	19.2	12.5
male:	nicotine (mg/wk)	72	0	245.0	0	4.7	18.6	-
	smoke years	72	0	30	3.0	11.8	7.1	-
	caffeine (mg/wk)	71	0	5376	1045	1184	1220	-
	alcohol (std dks/wk)	71	0	37	6.0	11.5	9.0	-
	fruit & veg (serves/wk)	69	4	54	20.0	-	20.4	10.7
	daily stress	67	0	48	19.5	-	20.7	1.6
		67	0	62	8.0	16.0	12.0	-

[a] n varied due to response rate; patients' completion and return of LQ/D.

Of the 77 females, 45 reported to have smoked either in the past or the present (58.4%), of which smoke years ranged from 1 week to 25 years, with a mean of 9.6 (SD 6.2). Forty-two of the 72 males had also consumed tobacco (58.3%) with duration ranging from 6 months to 30 years and a median of 10.0 (IQR 12.5).

Caffeine was consumed among the majority of females (72/78, 92.3%) and males (66/71, 93.0%). Among the 72 female caffeine consumers, caffeine consumption varied between 19 and 2706mg/week, with a median value of 917 (IQR 768). Of the 66 male caffeine consumers, consumption ranged from 72 to 5376mg of caffeine per/week, with a median value of 1105 (IQR 1135).

Alcohol was consumed by approximately half of the females (52.6%). Of these 41 alcohol drinkers, consumption ranged from 0.5 to 24 standard drinks during the week

of the diary; mean and median values were 5.1 (SD 4.9) and 3.5 (IQR 4.5), respectively. The majority of male respondents consumed alcohol (59/71, 83.1%). Weekly consumption among these 59 males varied between one and 37 standard alcoholic drinks, although the median was only 8.0 std dk/wk (IQR 10).

4.7.2 Univariate Analysis

A comparative analysis was undertaken between the women who experienced a <12 week pregnancy loss and those whose pregnancy was ongoing at 12 weeks, and between the male partners' of these two groups of women (Table 4.50).

Both female age and 2-ET were significant in relation to a pregnancy loss (Table 4.50). Specifically, the women who had a <12 week pregnancy loss were significantly older, on average, than the women whose pregnancy was ongoing at 12 weeks, 36.0 (SD 4.4) vs 32.2 (SD 4.3) years ($P<0.001$). The pregnancy loss rate was significantly higher among the non-2-ET couples than the 2-ET couples, 40.7% vs 14.7% ($P=0.010$).

No one female lifestyle variable was associated with pregnancy loss (Table 4.50). Among the male lifestyle variables, however, years of tobacco consumption was significantly higher among those males whose female partner experienced a pregnancy loss than those whose female partner did not (mean rank 45.2 vs 32.4, $P=0.012$).

There were too few cases in each of the infertility categories for meaningful statistical inferences to be drawn from the data. Nevertheless, a pregnancy loss was experienced by 23.1% of the women with infertility of nil significance (6/26), 25.0% with unexplained infertility (7/21), 38.9% with tubal factor (7/11), 37.5% with endometriosis only (3/8), and by four of the six women with multiple factor infertility. Neither of the two women with uterine factor infertility experienced a pregnancy loss.

Table 4.50: Comparative Analysis Between <12 Week Pregnancy Loss and Non-Pregnancy Loss Females and Between Their Male Partners.

			Univariate analysis				
gender	variable	n	test	statistic	value	df	*P* value
	mCES	88	t test	t	0.82	86	0.415
	2-ET[a]	88	chi-square	χ^2	6.65	1	**0.010**
female	age	88	t-test	t	-3.90	86	**<0.001**
male	age	88	t test	t	-1.29	86	0.201
	ICSI[b]	88	chi-square	χ^2	0.04	1	0.838
	lifestyle factors						
female	nicotine	77	MW[c]	U	617	-	0.504
	yrs of tobacco	77	MW	U	622	-	0.642
	caffeine	78	MW	U	660	-	0.865
	alcohol	78	MW	U	546	-	0.145
	fruit & vegetables	78	t-test	t	-0.32	76	0.753
	daily stress	78	t-test	t	0.39	74	0.699
	IVF stress[d]	76	t-test	t	-1.43	74	0.157
male	nicotine	72	MW	U	444	-	0.063
	yrs of tobacco	72	MW	U	363	-	**0.012**
	caffeine	71	MW	U	409	-	0.079
	alcohol	71	MW	U	536	-	0.839
	fruit & vegetables	69	t-test	t	0.43	67	0.669
	daily stress	67	t-test	t	-0.46	65	0.645
	IVF stress[d]	67	MW	U	372	-	0.099

[a] 2 levels, non-2-ET (base category, n=54) and 2-ET (n=34)
[b] 2 levels, fertilisation in vitro (base category, n=54) and fertilisation by ICSI (n=34)
[c] Mann-Whitney non-parametric test of two independent samples
[d] logarithm transformation

4.7.3 Multiple Logistic Regression Modelling

All of the variables that were examined in the multiple logistic regression modelling procedures are listed in Table 4.50. The results of the three different model fitting strategies are presented in Table 4.51. As shown, female age was significant ($P<0.05$) in all of the three models and male nicotine consumption was significant in the stepwise forward model.

By hierarchical regression analysis, to the base model comprised of female age, male nicotine consumption was added. Male nicotine consumption significantly improved the fit of the model (χ^2=5.163, 1df, P=0.0231), and was added to the base model. Consequently, the main effects pregnancy loss model comprised female age and male nicotine consumption.

Table 4.51: Multiple Logistic Regression Selection Strategies on Pregnancy Loss[a,b]

variable	selection strategy		
	full model P value	stepwise forward P value	backward elimination P value
female age	0.0051	0.0019	0.0008
male nicotine consumption	-	0.0458	-

[a] <12 week pregnancy loss; n=68
[b] only significant variables listed (P<0.05)

To investigate whether the effect of female age and male nicotine consumption on pregnancy loss was interactive, an interaction term as such was created (fage*mnic). To the main effects model, fage*mnic was added, but it did not improve the model fit. Therefore, the final pregnancy loss model is presented in Table 4.52, along with the relevant relative risks.

Table 4.52: Final Pregnancy Loss Model[a,b,c,d]

variable	β	SE	Wald	P value	OR	95% OR CI
constant	-9.564	2.704	12.507	0.0004	-	-
female age	0.249	0.076	10.631	0.0011	1.283	1.104, 1.490
male nicotine	0.017	0.008	4.755	0.0292	1.018	1.002, 1.034

[a] <12 weeks
[b] n=72, data incomplete due to missing values of lifestyle variables
[c] goodness-of-fit χ^2=18.392, 2df, P=0.0001

Five of the pregnancy loss cases were the result of an ectopic pregnancy. The aetiology of an ectopic pregnancy is predominantly female-related, and as such, it was hypothesised that male nicotine consumption is unrelated to a ectopic pregnancy loss. In order to test this hypothesis, the five ectopic pregnancy loss cases were

excluded from the data, and the model fitting strategies repeated (Table 4.53). Evidence that the relationship between male nicotine consumption and pregnancy loss was strengthened following the exclusion of ectopic pregnancies would lend support to the proposed hypothesis. Therefore, the model was refitted for the remaining 83 couples ($n_{4.2.2}$), of which 22 experienced a non-ectopic pregnancy loss before the 12th week of gestation.

Female age was significant in two of the three models (full and backward elimination models)(Table 4.53). Male nicotine consumption was significant in the stepwise forward model. 2-ET, mCES and male alcohol consumption were all significant in the backward elimination model.

Table 4.53: Modelling Selection Strategies on Pregnancy Loss[a,b,c,d]

variables	regression model		
	full model *P* value[b]	stepwise forward *P* value	backward elimination *P* value
female age	0.0464	0.0009	-
2-ET	-	-	0.0404
mCES	-	-	0.0292
male nicotine consumption	-	0.0109	-
male alcohol consumption	-	-	0.0257

[a] <12 weeks
[b] excludes ectopic cases
[c] n=68, data incomplete due to missing values of lifestyle variables
[d] only significant variables listed ($P<0.05$)

To the base model comprised of female age, 2-ET was added. 2-ET did not improve the fit of the model. Subsequent analysis revealed that neither mCES nor male alcohol consumption improved the model fit. Male nicotine consumption, however, did improve the fit of the model ($\chi^2=7.708$, 1df, $P=0.0055$), and was added to the base model. Once again, the interaction between female age and male nicotine consumption was investigated, but was not found to be of significance. The final non-ectopic pregnancy loss model is presented in Table 4.54, as are the associated odds ratio (OR).

A comparative analysis of the final pregnancy loss model (Table 4.52) and the final non-ectopic pregnancy loss model (Table 4.54) shows that the relationship between male nicotine consumption and pregnancy loss is strengthened following the exclusion of the ectopic pregnancies. The hypothesis that male nicotine consumption is not related to ectopic pregnancies is supported, and that the exclusion of the ectopic pregnancy cases was an appropriate action. Moreover, this finding supports the specificity of the relationship between male nicotine consumption and pregnancy loss.

Table 4.54: Final Non-Ectopic Pregnancy Loss Model[a,b,c]

variable	β	SE	Wald	*P* value	OR	95% OR CI
constant	-12.116	3.330	13.241	0.0003	-	-
female age	0.311	0.092	11.342	0.0008	1.365	(1.139, 1.636)
male nicotine	0.024	0.009	6.942	0.0084	1.024	(1.006, 1.042)

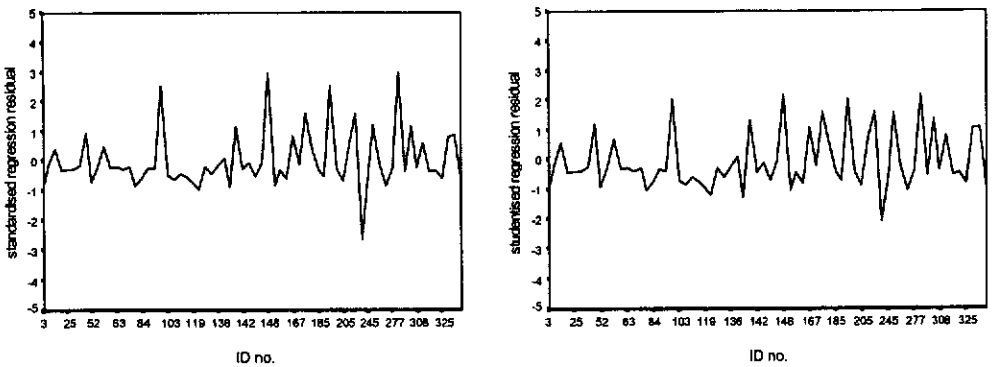
[a] <12 weeks
[b] n=68; data incomplete due to missing values of lifestyle variables
[c] goodness-of-fit χ^2=22.776, 2df, *P*<0.0001

4.7.4 Model Diagnostics

4.7.4.1 Outliers

Following examination of the regression residuals, none of the couples were identified as an outlier. Specifically, no one couple had a standardised or studentised residual of ±3.0 (Figure 4.54).

Figure 4.54: Sequence Plots of Standardised Residuals and Studentised Residuals



4.7.4.2 Leverage

Centred leverage (CL) values were presented in a sequence plot (Figure 4.55). Three cases were identified as having an elevated CL value relative to that of the other cases: couple ID no. 103, 138 and 248. Summaries of these three couples are presented Table 4.55.

Figure 4.55: Sequence Plot of Centred Leverage

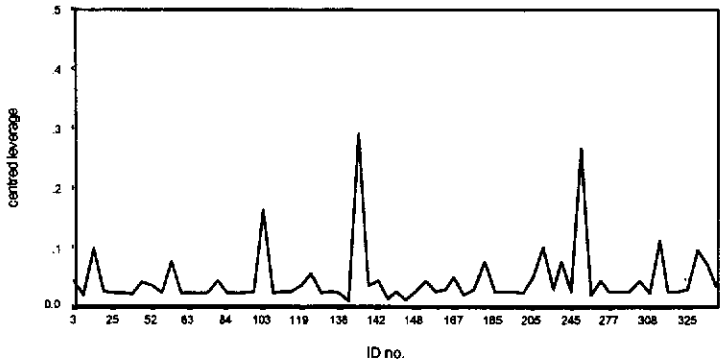


Table 4.55: Summary of Cases with Extreme Centred Leverage Values

ID no.	CL value	female age	male nicotine consumption	pregnancy loss
103	0.163	25	126.1	no
138	0.289	27	150.0	no
248	0.265	27	144.3	yes

4.7.4.3 Influential Observations

The Cook's distance (CD) values were plotted (Figure 4.56). Five cases were identified as having an elevated CD: couple ID no. 138, 148, 217, 248 and 288. A summary is presented for each case in Table 4.56.

Figure 4.56: Sequence Plot of Cook's Distance

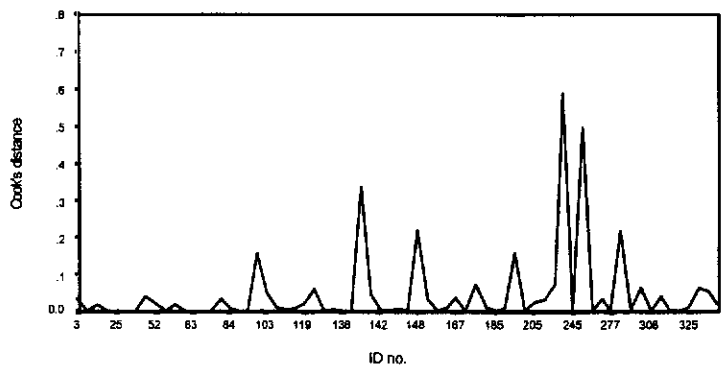
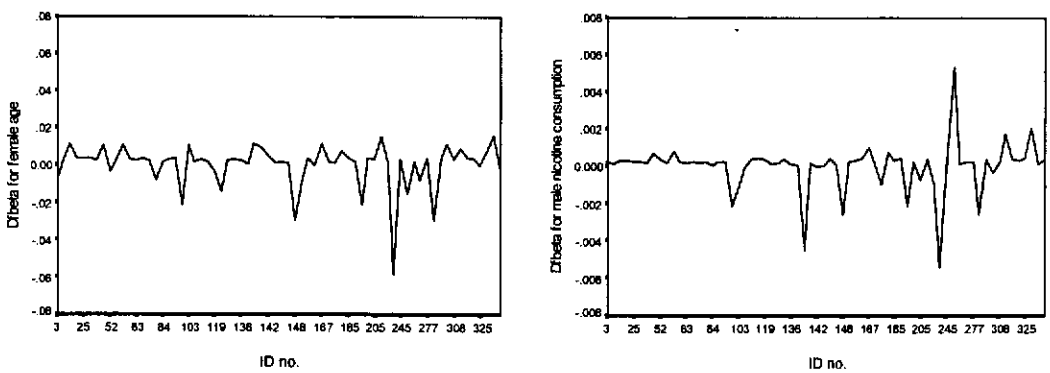


Table 4.56: Summary of Cases with Extreme Cook's Distance Values

ID no.	CD value	female age	male nicotine consumption	pregnancy loss
138	0.336	27	150.0	no
148	0.217	32	0	yes
217	0.588	40	70.0	no
248	0.500	27	144.3	yes
288	0.217	32	0	yes

The DFBETA sequence plot, which represents the change in logistic coefficients when a case is deleted from the model, for female age is presented in Figure 4.57, as is that for male nicotine consumption. Couple ID no. 248 was identified as potentially influential due to the relatively young age of the female and to the relatively high consumption of nicotine by the male. The male member of couple ID no. 138 and couple ID no. 217 had relatively high nicotine consumption, and was the likely explanation for these two couples having been identified as potentially influential. No specific factor was noteworthy for couples ID no. 103, 148 and 288, although a combination of both female age and male nicotine consumption is indicated.

Figure 4.57: Sequence Plots of DFBETA for Female Age and Male Nicotine Consumption



Each potentially influential couple was examined further. To determine the relative influence of each of these couples on the significance of the main effects in the final non-ectopic pregnancy loss model, each couple was independently omitted from the data, and this final model refitted. Following the exclusion of each potentially influential couple from the analysis, the resultant coefficient *P* values for female age and male nicotine consumption are presented in Table 4.57, as are the coefficient *P* values from the final non-ectopic pregnancy loss model (Table 4.54). As shown, the omission of couple ID no. 248 from the pregnancy loss model rendered the variable, male nicotine consumption, non-significant (*P*=0.0723).

Table 4.57: Sensitivity of the Final Non-Ectopic <12 Week Pregnancy Loss Model on Selected Couples of Interest

couple ID	deviance	parameters in model		CL	CD
		female age	male nicotine		
	62.634	**0.0008**	**0.0084**	-	-
ID no. of deleted case					
103	61.778	0.0012	0.0070	0.163	-
138	63.366	0.0011	0.0053	0.289	0.336
148	60.974	0.0007	0.0054	-	0.217
217	60.791	0.0005	0.0030	-	0.588
248	60.067	0.0006	**0.0723**	0.265	0.500
288	60.974	0.0007	0.0054	-	0.217

4.7.5 Interpretation of the Regression Model

The pregnancy loss regression equation is:

$$\ln [\ \hat{p}_i / (\ 1 - \hat{p}_i\ )\ ] = u_i$$

where $\hat{p}_i$ is the estimated probability of <12 week non-ectopic pregnancy loss for the ith patient ($i = 1, \ldots, n$) and u_i is the linear predictor:

$$u_i = -12.116 + 0.311(\text{female age}) + 0.024(\text{male nicotine consumption})$$

As expected, pregnancy loss increased with female age. Each additional year presented a 36% increase in risk (that being relative to the former year). More specifically, a 40 year old patient had a risk of pregnancy loss that was 3.6 times that of a 30 year old.

The risk of the female patient experiencing a pregnancy loss was also elevated if her male partner was a smoker. Specifically, the risk increased by 2.4% for every 1mg increase in male nicotine consumption per week. It is noted, however, that one couple was especially influential in this regard (couple ID no. 248)(Table 4.57).

Univariate data analysis suggested that the risk of pregnancy loss was lower among couples who had a two-embryo uterine transfer by recommendation (2-ET). However, 2-ET was not identified as a significant factor in regard to pregnancy loss following multivariate data analysis. Further analysis revealed that 2-ET women were significantly younger than non-2-ET women, 30.4 (SD 3.4) vs 35.1 (SD 4.4) years (t=5.04, 81df, P<0.001). It is likely that this significant difference in age of almost five years between 2-ET and non-2-ET women accounted for the univariate association evident between 2-ET and pregnancy loss.

For interest, a summary of the couples by female age, male nicotine consumption (mg/wk) and pregnancy loss status is detailed in Table 4.58. With the exception of the five couples whose treatment ended in an ectopic pregnancy, these 83 couples

include all couples whereby the female had a positive β-hCG pregnancy test outcome 16 days post-ET. Couples are displayed by female age <35 and ≥35 years, and all <12 week non-ectopic pregnancy cases are highlighted.

Table 4.58: Summary by Female Age, Male Nicotine Consumption and Pregnancy Loss

ID	female age	male nic[a] mg/wk[b]	<12 week preg loss	ID	female age	male nic mg/wk	<12 week preg loss
female <35 years (n=48)							
9	27	0	no	311	33	0	no
23	32	0	no	324	32	0	no
25	31	0	no	346	31	-	no
30	31	0	no	**349**	**34**	**88**	**yes**
34	28	0	no				
53	29	0	no	female ≥ 35 years (n=35)			
59	30	0	no	3	38	0	no
63	29	0	no	**11**	**45**	**0**	**yes**
69	31	0	no	**35**	**37**	**29**	**yes**
71	29	0	no	46	40	-	no
84	34	24	no	52	37	0	no
88	30	0	no	**58**	**40**	**51**	**yes**
89	31	0	no	82	38	0	no
92	25	-	no	97	37	-	no
95	**33**	**-**	**yes**	116	35	-	no
99	**33**	**0**	**yes**	117	35	0	no
103	25	126	no	119	37	0	no
111	34	30	no	121	39	0	no
114	34	0	no	**124**	**40**	**-**	**yes**
125	29	0	no	**137**	**41**	**245**	**yes**
130	34	0	no	**141**	**37**	**15**	**yes**
136	27	18	no	145	35	0	no
138	27	150	no	154	38	0	no
142	27	61	no	160	36	0	no
144	24	0	no	**167**	**37**	**40**	**yes**
146	23	0	no	**171**	**36**	**0**	**yes**
148	**32**	**0**	**yes**	**176**	**40**	**75**	**yes**
152	31	-	no	190	35	0	no
157	32	0	no	**211**	**43**	**0**	**yes**
169	27	0	no	**215**	**36**	**0**	**yes**
185	32	0	no	217	40	70	no
188	28	-	no	**225**	**38**	**-**	**yes**
200	**33**	**0**	**yes**	262	38	0	no
204	31	0	no	263	36	-	no
205	32	62	no	269	39	-	no
235	27	-	no	274	35	-	no
245	34	0	no	**298**	**38**	**0**	**yes**
248	**27**	**144**	**yes**	**310**	**35**	**100**	**yes**
255	27	0	no	325	36	0	no
257	29	-	no	**351**	**40**	**0**	**yes**
277	31	4.9	no	352	37	0	no
288	**32**	**0**	**yes**				
297	33	0	no				
308	30	4.1	no				

[a] nicotine

[b] - indicates missing data; this couple was not included in the final regression model

Chapter 5
Discussion

The major shortcoming of most former studies that investigated the effect of female cigarette smoking on the clinical outcomes of IVF treatment was the limited application of univariate statistical methods of data analysis. Multiple regression analysis was used in this study to examine not only the effect of smoking but that of other lifestyle factors, including caffeine, alcohol and fruit and vegetable consumption, and stress. Moreover, a detailed record of lifestyle factors was ascertained by means of a 7-day lifestyle diary. Determination of weekly tobacco consumption took into account the nicotine levels in the brand of cigarette smoked. In accord with the recommendations by Schreiber et al. (1988), the quantification of caffeine intake accounted for tea and coffee beverages (caffeinated and decaffeinated), brewing method, proportion of beverage consumed, iced coffee beverages, kola soft drinks and caffeine-containing medications. To date, it is the most detailed examination of lifestyle factors and clinical outcomes among IVF couples.

5.1 Smoking

5.1.1 Female Smoking

The most widely investigated lifestyle factor is cigarette smoking. In this study, firstly, its effect on oocyte production was examined. Regression analysis on oocyte production revealed age, eFSH, basal FSH levels and daily stress (to be discussed in Section 5.2.1) were significant. Taking into account these factors, neither cigarette smoking at the time of treatment (current smoking) nor the total number of years of smoking exposure (smoke years) was statistically significant in terms of the number of oocytes retrieved (ln(oocytes)) by TVOA.

The conviction that cigarette smoking accelerates the age-related depletion of ovarian primordial oocytes is well supported (Joesbury et al. 1998; Sharara et al. 1994; Van Voorhis et al. 1996; Westhoff, Murphy & Heller 2000). Evidence includes an earlier onset of menopause among smokers (Jick, Porter & Morrison 1977; Midgette & Baron 1990). This study showed that as levels of basal FSH increased, oocyte

production decreased (P<0.001). Basal FSH level is an indirect measure of ovarian reserve and elevated levels are indicative of primordial follicle depletion. After controlling for the significant effect of female age and infertility aetiology, a significant positive linear relationship between ln(basal FSH) and smoke years was shown here (P=0.035). This dose-dependent effect is further evidence that smoking accelerates depletion of the ovarian reserve. In this study, smoke years was a measure of the total number of years of active cigarette smoking among smokers and ex-smokers. This negative effect of smoking even among ex-smokers implies that its damaging affect on ovarian reserve is irreversible. This was also the conclusion reached by Westhoff, Murphy and Heller (2000).

Whilst smoke years has an adverse affect on ovarian reserve, which in turn reduces oocyte production, cigarette smoking at the time of IVF treatment was not shown in this study to affect the number of oocytes retrieved. This finding is consistent with that of Zenzes, Reed and Casper (1997), where the number of oocytes retrieved did not correlate with smoking exposure (log follicular fluid cotinine values), even after adjusting for age. It may well be that the administration of eFSH and the dose in accord with ovarian response may compensate for an inhibitory effect of smoking on oocyte production. It was demonstrated by this study that the administration of high quantities of eFSH is associated with the retrieval of fewer oocytes; an effect exacerbated by age (P=0.025).

Female smoking also had a detrimental effect on rates of fertilisation *in vitro*. This effect was independent of male factors, including smoking by the male partner. After taking into account female age, fertilisation rates decreased with female smoke years (P<0.0001). Although questionable as a main effect (P=0.0559), cigarette smoking at the time of treatment intensified the negative effect of smoke years on fertilisation (P=0.0187). This adverse effect of female smoking on rates of fertilisation *in vitro* is in keeping with the negative effect on ovarian reserve. Smoking has a detrimental and irreversible effect on oocyte quality that is evident through reduced fertilisability.

Based on the literature, the smoking-related impairment to fertilisation *in vi*tro observed here seems to be a result of oocyte immaturity or cytotoxin-induced

dysfunction of cytoplasmic organelles. Purported to be affected by cigarette smoking are gap junctions (Paksy et al. 1997), meiotic spindles (Zenzes 2000), and mechanisms involved in the release of internal stores of calcium (Canipari 2000). Whereas oocyte immaturity appears to be related to altered follicular steroidogenesis, the evidence points to morphological changes being the direct result of the cytotoxic constituents in cigarette smoke or indirectly through oxidative stress caused by a smoking-induced increase in ROS generation. The latter may also be accompanied by smoking-related compromise in antioxidants that protect against oxidative stress.

In this study, cigarette smoking by the female patient at the time of treatment affected neither the relative chance of β-hCG pregnancy nor risk of <12 week pregnancy loss. Two meta-analyses on female smoking and pregnancy outcome independently reported that the relative chance of pregnancy among smokers was reduced to almost half that of non-smokers (Feichtinger et al. 1997; Hughes et al. 1994). However, the causality of this relationship is questionable, as the findings are based on crude pregnancy rates that are subject to bias due to confounding effects. To determine the effect of smoking on pregnancy, multiple logistic regression analysis was used in the present study to statistically control for the potential confounding effect of patient, treatment and other lifestyle factors. In light of this statistical rigour, no adverse effect of female smoking on either a β-hCG pregnancy outcome 16-days post-ET or <12 week pregnancy loss was demonstrated.

It is well established that the smoking habit of female patients is associated with that of the male partner. In the present study, smoking by the male partner significantly increased the risk of his female partner experiencing a <12 week pregnancy loss. In a previous study by the Investigator and colleagues, which was a prelude to the current study and involved data from 498 different IVF treatment cycles, male smoking was shown to significantly reduce the likelihood of a couple achieving a 12 week pregnancy (Joesbury et al. 1998). This triad relationship between male and female smoking and pregnancy outcome renders male smoking a potential confounder in the relationship between female smoking and pregnancy. Therefore, paternal smoking cannot be ruled out as accounting for the differences between female smokers and

non-smokers reported by former studies that simply compared crude rates of pregnancy and pregnancy loss.

5.1.2 Male Smoking

In this study, the male patients included in the data analysis on fertilisation *in vitro* represent a relatively homogenous group, as all had sperm quality adequate for the technique of fertilisation *in vitro*. ICSI is the treatment of choice for couples when the spermatozoa are of poor quality or severely defective. Couples whose treatment involved ICSI were not included in this data analysis on fertilisation *in vitro*. Laboratory techniques used in the preparation of sperm for insemination result in the selection of spermatozoa of the highest quality, best in terms of motility and morphology. Furthermore, a uniform number of 50 000 to 100000 spermatozoa per oocyte are routinely used for insemination. In essence, the technique of fertilisation *in vitro* exerts a reasonable degree of control over individual variability in sperm count, morphology and motility; thereby, facilitating an examination of the less understood events of fertilisation: capacitation, hyperactivation and sperm-oolemma fusion.

The current study is the first to simultaneously examine both male and female lifestyle factors on rates of fertilisation *in vitro*. Neither cigarette smoking by males at the time of treatment nor past exposure among current and ex-smokers (smoke years) was shown to have a negative effect on rates of fertilisation *in vitro*. However, this does not rule out any adverse effect of smoking on sperm quality. Almost two-thirds of the 152 males included in the regression analysis on fertilisation *in vitro* were classified as normozoospermic (65.8%). The prevalence of oligo-, astheno- and teratozoospermia was 7.2%, 21.1% and 16.4%, respectively. Each was included as separate independent variables in the regression analysis. Yet, no one form of sperm abnormality was shown to significantly affect rates of fertilisation *in vitro*.

Preliminary univariate data analysis undertaken in this study showed that rates of fertilisation were significantly reduced among asthenozoospermic men (P=0.0343). Consequently, the association between spermatozoa classification and smoking status was examined in a subsequent analysis of the data (see Appendix F: Supplementary Data Analysis). As shown in Table F (Appendix F), male smoking was not associated with asthenozoospermia; the prevalence of asthenospermia in smokers and non-smokers was 20.0% and 21.4%, respectively (P>0.05). Nor was male smoking status associated with oligozoospermia or teratozoospermia in these 152 males whose treatment involved fertilisation *in vitro* and whom were included in the analysis on fertilisation.

In spite of the abundance of anecdotal evidence, this is the first study to show unequivocally that cigarette smoking by the male partner increases the risk of his partner experiencing a <12 week pregnancy loss. Specifically, the risk of pregnancy loss increased 2.4% for every 1mg incremental increase in nicotine consumption (P=0.0084). The most plausible explanation, and one in keeping with the anecdotal evidence to date, is that this adverse effect of paternal smoking stems from preconception DNA damage of the fertilising spermatozoa; subsequent embryonic/foetal development is impaired, and the result is an early pregnancy loss. This confirms Wyrobek's statement in that '...the impact of male reproduction goes well beyond fertilisation' (Wyrobek 1993, p. 6).

This adverse effect of male smoking on pregnancy was evident even in this relatively small sample of 68 pregnancies, in which the <12 week pregnancy loss rate was 27.9%. By the application of multiple logistic regression analysis, the present study showed that cigarette smoking by the male, but not the female, increased the risk of pregnancy loss. However, two former studies reported that rates of pregnancy loss were higher among female smokers than non-smokers (Harrison, Breen & Hennessey 1990; Maximovich & Beyler 1995). Both studies used crude pregnancy loss rates to compare female smokers and non-smokers. Based on the findings of this study and the previous one (Joesbury et al. 1998), it possible that significantly higher rates of pregnancy loss among female smokers resulted from the confounding effect of male smoking.

Consistent with previous studies, female age was the only other significant factor in regard to pregnancy loss, with rates increasing with age (P=0.0008). It is not clearly understood why the risk of pregnancy loss increases with age, although an age-related decline in oocyte quality is well established. Recently, it was suggested that poorer quality oocytes, such as those from older women, have impaired DNA repair mechanisms. In effect, the ability of aged oocytes to repair DNA damage introduced by the fertilising spermatozoa may be compromised (Ollero et al. 2001). The load of genetically abnormal spermatozoa has been shown to increase with age (Morris et al. 2002; Spanò et al. 1998). This age-related increase in spermatozoa DNA damage in combination with the purported female age-associated compromise in DNA repair mechanisms may well explain the significant relationship of increasing rates of pregnancy loss with female age.

This study's finding that paternal smoking increases the risk of an early pregnancy loss is well supported. Cigarette smoking increases the risk of DNA damage 1) in male germ cells because spermatogenesis is continuous from puberty to advanced age, and 2) in spermatozoa due to the high number of mitotic and meiotic cell divisions from spermatogenesis to zygote (Zenzes, Bielecki & Reed 1999). Carp et al. (2001) showed only 29% of abortuses had detectable chromosomal abnormalities, mostly aneuploidy, which tend to be maternal in origin (Little & Vainio 1994). Therefore, the cause of the majority of pregnancy losses is not known (Carp et al. 2001). Point mutations, which are not detectable through karyotyping of abortuses, are more frequent in male than female gametes (Little & Vainio 1994). Further evidence of the adverse effect of paternal smoking is supported by studies in which male smoking has been linked with increased morbidity in offspring: birth defects (Zhang et al. 1992) and childhood cancers (Ji et al. 1997; Sorahan et al. 1997a, 1997b).

Convincing evidence in further support of this male-mediated, cigarette smoke-related increased risk of pregnancy loss is derived from studies that compared smokers and non-smokers in terms of DNA damage in spermatozoa. It has been consistently shown among males attending infertility clinics that levels of DNA

damage are higher in spermatozoa of smokers (Fraga et al. 1996; Potts et al. 1999; Shen et al. 1997; Sun, Jurisicova & Casper 1997; Zenzes, Bielecki & Reed 1999). More importantly, the evidence suggests that spermatozoa carrying damaged DNA are capable of fertilisation *in vitro* (Peluso, Luciano & Nulsen 1992) and pronucleus formation following intracytoplasmic injection (Twigg, Irvine & Aitken 1998).

Yet, the most telling of evidence of an adverse paternal smoking effect is that from a study by Zenzes, Bielecki and Reed (1999) in which the frequency of benzo(a)pyrene-induced guanosine adducts (BPDE-I-dG-DNA) was investigated in 4-8 cell embryos. Benzo(a)pyrene is a constituent of cigarette smoke and is highly mutagenic (Zenzes, Bielecki & Reed 1999). The frequency of BPDE-I-dG-DNA adducts was 3.7 times higher in embryos from couples in which the male smoked, irrespective of the smoking status of the female. Zenzes, Bielecki and Reed (1999) concluded that BPDE-I-dG-DNA adducts in embryos were mainly paternal in origin.

Underpinning all of this evidence is the fact that spermatozoal DNA is especially vulnerable to oxidative stress. This is primarily due to 1) limited intracellular antioxidative defense mechanisms due to the virtual non-existence of cytoplasm, and 2) high levels of polyunsaturated fatty acids (PUFA) in the phospholipids of the plasma membrane renders spermatozoa especially vulnerable to the destructive actions of ROS (Aitken 1999). In confirmation, semen samples with high levels of ROS produce spermatozoa that exhibit the most extensive DNA damage (Ollero et al. 2001). It has been observed that spermatozoa retrieved from the epididymides exhibit more DNA damage than that from the testes (O'Connell, McClure & Lewis 2002). This suggests that spermatozoa are susceptible to oxidative stress during transit through the epididymides.

The highly compacted chromatin of spermatozoa is believed to protect the DNA from damage. Therefore, abnormal chromatin packaging due to incomplete or impaired condensation will predispose spermatozoa to damage of the DNA (Zini et al. 2001). As protamines play a major role in sperm chromatin condensation (Spanò et al. 1998), any modification, deficiency or complete absence of these protamines is likely to lead to anomalies in the tight packaging of sperm chromatin (Bianchi et al. 1996).

In fact, access by fluorescent dyes to DNA occurred mostly in spermatozoa with decondensed or damaged chromatin (Bianchi et al. 1996). Sakkas et al. (1996) showed that spermatozoa from patients with poorly packaged chromatin have high levels of endogenous DNA nicks. Similarly, abnormal chromatin packaging is correlated with the presence of DNA strand breaks (Manicardi et al. 1995 and Sailer et al. 1995 cited in Sakkas et al. 1996). The molecular basis for sperm chromatin anomalies is poorly understood (Sakkas et al. 1996). Whilst Sakkas et al. (1996) suggested abnormal chromatin package may have a genetic basis, the fact that there is a wide spectrum of DNA damage both within and between men, forming a spectrum and not discrete sub-populations (Morris et al. 2002), does not support this hypothesis. It is plausible, however, that nutritive factors play a role, as protamines have high levels of the amino acids, arginine and cysteine (Seligman et al. 1994).

IVF clinics routinely use sperm preparation techniques to isolate 'functionally normal' spermatozoa: 1) swim-up or sperm migration, which separates the spermatozoa into motile and non-motile fractions, and 2) density gradient centrifugation (e.g. Percoll® and PureSperm®), which separates spermatozoa according to density and selects in favour of motile and normal morphological spermatozoa (Mortimer 1999 cited in Sakkas et al. 2000). [Sakkas et al. (2000) made note of the fact that the manufacturers have now withdrawn Percoll® from use in sperm preparation techniques.] Although there is not total agreement between studies, the evidence to date as a whole suggests that the percentage of spermatozoa with DNA anomalies is lower in the spermatozoa selected for insemination than that of the ejaculate (Angelopoulos et al. 1998; Gandini et al. 2000; Ollero et al. 2001; Sakkas et al. 2000; Spanò et al. 1999). Sakkas et al. (2000) compared swim-up and density gradient centrifugation, and found the latter to be superior in efficiency to isolate a population of spermatozoa with low percentage of DNA anomalies. In the majority of patients with high levels of DNA damage, Sakkas et al. (2000) reported that both Percoll® and PureSperm® significantly reduced the percentage of spermatozoa with DNA damage.

5.2 Stress

5.2.1 Female Stress

Apart from cigarette smoking, stress was the only other female lifestyle factor shown to have an impact on the clinical outcomes of IVF treatment. More specifically, the effect was related to stress from daily living in general (daily stress) and not stress from IVF treatment. By inference, daily stress is more akin to chronic stress than the acute nature of stress from IVF treatment. Oocyte production was adversely affected by daily stress; the number of oocytes retrieved (ln(oocytes)) decreased with increasing levels of daily stress (P=0.039). Only two other studies have examined stress and oocyte production, and both used univariate methods of data analysis (Boivin & Takefman 1995; Demyttenaere et al. 1998). Neither showed stress to be statistically significant. It has been suggested that eFSH administered for ovarian follicle stimulation treatment compensates for infertility caused by psychological factors (Harlow et al. 1996). This is not supported by the findings of the current study: daily stress had a significant adverse effect on oocyte production even after controlling for eFSH administration, age and basal FSH levels.

Whilst shown to impair oocyte production, female daily stress was positively associated with fertilisation *in vitro* (P=0.0059) and β-hCG pregnancy (P=0.0207) in this study. Although measures of stress differed, the results of this study are in accord with that of Sanders and Bruce (1999). They showed rates of pregnancy were highest among female patients who 1) scored toward the depression pole of the POMS elated-depressed scale, or 2) with tendencies toward low or high levels of anxiety in stressful circumstances. If it can be assumed that female IVF patients who engage in paid work experience higher levels of daily stress, that being due to juggling the demands of treatment and work commitments, then the finding by Facchinetti et al. (1997) is in discord with this study. Facchinetti et al. (1997) showed employment in a job outside of the home was associated with a poor pregnancy outcome, whereas patients with high daily stress levels in the current study had the highest rates of pregnancy. Given this, the assumption that daily stress is correlated with paid employment may be an erroneous one. Regardless of its source, female daily stress was shown here to impair oocyte production, but not fertilisation or pregnancy.

Hypothalamic amenorrhea is associated with periods of chronic depression (Thiering et al. 1993), stressful life events (Gallinelli et al. 2000) and stressful lifestyle and occupations (Negro-Vilar 1993). In terms of infertility aetiology, stress-related hypothalamic amenorrhea presents as an ovulatory disorder. This type of infertility is readily remedied with eFSH (Vermeulen 1993), as is administered for the purpose of stimulating multiple ovarian follicles in IVF treatment. As supported by the findings of this study, a compromise in the quality oocytes, resultant embryos retrieved or implantation is unlikely, as the dysfunction is at the level of the hypothalamus and not the ovary or uterus. Hence, without any further impediment to fertility other than a reduction in the number of oocytes retrieved, the female IVF patients in this study with high levels of daily stress had higher than average rates of fertilisation *in vitro* and pregnancy.

At first glance, this positive effect of daily stress on fertilisation and pregnancy is contrary to the expected. As alluded to, it is unlikely to be a result of daily stress *per se*, but rather, that associated with ovulatory infertility aetiology. This finding is consistent with that shown by Sanders and Bruce (1999); women with tendencies toward depression or high anxiety had elevated rates of pregnancy, as did women with low levels of anxiety. In fact these authors commented that it was difficult to explain the increased chance of pregnancy among women with high anxiety scores. Unlike the current study, oocyte production and fertilisation were not examined (or at least not reported), which precluded an understanding of the 'bigger clinical picture'.

5.2.2 Male Stress

Whilst implicated in the literature, it was quite unexpected to have shown that male stress from IVF treatment had an adverse effect on rates of fertilisation *in vitro*. Derived from multivariate regression analysis with a resultant $P<0.0001$, it is unlikely to be a chance finding. Most former studies used psychometric instruments to assess the effect of stress on sperm quality. Stress was measured in this study using two general appraisal questions: stress due to IVF treatment (IVF stress) and stress due to daily living in general (daily stress). Of note, stress levels were ascertained for the duration of one whole week, that being the week ending within

days of producing the ejaculate for insemination. The negative effect of stress is that associated with IVF treatment and not daily stress in general, suggesting that spermatozoa are susceptible to acute stress. This is in keeping with the temporal relationship between stress exposure (just prior to the production of the semen sample for insemination) and the observed adverse effect on rates of fertilisation *in vitro*.

As noted by others, an adverse effect of stress on male factor infertility is difficult to explain. The exception here being that the effect is unlikely to be due to motility, morphology or count, as none of these factors were significant in the fertilisation model. Clarke et al. (1999) suggested that a stress-induced alteration of the hypothalamic-pituitary-adrenal axis might suppress secretion of LH, and in turn, lower testosterone production. Androgens have a role in the epididymides, as different regions have different androgen requirements (Hinton et al. 1995). According to Donnelly, McClure and Lewis 2000, androgens modulate the production of hypotaurine. This non-enzymatic and sulphur-based antioxidant is found in high concentrations within the fluids of the male reproductive tract, and plays a vital role in protection against oxidative stress by scavenging the highly reactive and lethal hydroxyl radical (OH·)(Halliwell 1994). Therefore, a potential stress-induced suppression of hypotaurine may compromise hypotaurine production, and in turn, the overall antioxidative defenses of the male reproductive tract.

Although based on animal models, high levels of corticosterone have been shown to influence epididymal lipid composition (Balasubramanian, Aruldhas & Govindarajulu 1987). In a study of experimental design, rats were given high doses of corticosterone daily, administered intra-muscularly for 20 days. Corticosterone treatment significantly depressed serum levels of testosterone and prolactin. Lipid analysis included spermatozoa, luminal fluid and epididymal tissue. Total phospholipid and phospholipid fractions were significantly decreased in the cauda region, but there was no accompanying change in total lipid or cholesterol content. In the caput region, a significant reduction in total lipids, cholesterol and phospholipids, including the phosphatidylinositol, phosphatidylcholine and phosphatidyethanolamine fractions was demonstrated. When the epididymal tissue

was washed free of spermatozoa and luminal fluid, corticosterone-treated rats had significantly increased total lipid, glyceride, and the mono-, di-, and triglyercides fractions, but total phospholipids and its fractions remained unaltered. Moreover, all lipid classes returned to baseline levels after 20 days withdrawal of corticosterone treatment. Balasubramanian, Aruldhas and Govindarajulu (1987) concluded that high levels of corticosterone alter epididymal lipids, which may reflect infertility disorders in patients with glucocorticoid excess. The applicability of this animal model to that of human male infertility is not without question. Nonetheless, it is plausible that the significant relationship between male IVF stress and decreased rates of fertilisation *in vitro* observed in this study is due to reduced fluidity of the spermatozoa plasma membrane. In light of the fact that alternative explanations are lacking, it is a hypothesis worthy of further investigation.

5.3 Nutritive Factors

5.3.1 Female Nutritive Factors

There is no evidence from this study that female caffeine, alcohol and fruit and vegetable consumption affects either oocyte production, fertilisation, pregnancy or pregnancy loss. This is consistent with the findings of others. In addition to smoking, Hughes et al. (1994) included female caffeine and alcohol consumption as covariates in the regression analysis on clinical pregnancy. None of these lifestyle factors were statistically significant. Similarly, Klonoff-Cohen, Bleha and Lam-Kruglick (2002) showed neither female caffeine nor alcohol consumption had an affect on oocyte production, fertilisation, pregnancy or pregnancy loss. Like that of Klonoff-Cohen, Bleha and Lam-Kruglick (2002), the current study involved a detailed quantitative assessment of caffeine consumption. Despite this, no effect of female caffeine consumption on IVF treatment was shown by this study nor by Klonoff-Cohen, Bleha and Lam-Kruglick (2002). In conclusion, there exists no evidence to date that female alcohol or other dietary factors affect the clinical outcomes of IVF treatment.

Of relevance, however, female patients undergoing IVF treatment tend to modify their health behaviours so as to enhance even the smallest chance of a successful clinical outcome. For example, almost half of the women in this study did not consume alcohol during the follicular phase of treatment, and the average consumption among those that did was, on average, less than one standard alcoholic drink per day. Caffeine intake was relatively low, with consumption averaging the daily equivalent of 1.5 cups of instant coffee. In a like manner, Klonoff-Cohen, Bleha and Lam-Kruglick (2002) reported that only 37% of female IVF patients consumed caffeine during treatment, yet 83% were regular caffeine consumers at the initial clinic visit. In regard to diet, female patients tend to adapt good health behaviours during IVF treatment, and this in part may account for this lack of effect of nutritive factors on the clinical outcomes of IVF treatment. An alternative explanation is the effectiveness of eFSH for oocyte production, which may overcome any nutritive deficiency or factor that is an impediment to conceiving naturally.

5.3.2 Male Nutritive Factors

Dietary deficiencies may be associated with compromised antioxidative defense mechanisms against oxidative stress (Aitken 1999). Wong et al. (2000) stated this may account for as many as one-third of cases of male sub-fertility. Male IVF patients are less inclined to modify health behaviours to facilitate treatment outcome, in part, because there is little reason to suspect that nutritive factors have an effect on male fertility. Not because there is evidence of no effect, but rather that nutrition and male fertility has received little attention to date (Wong et al. 2000). The majority of existing studies have focused on sperm quality. This is the first study to investigate, in detail, male lifestyle factors on fertilisation *in vitro*.

Among males, a number of nutritive factors were shown by this study to have a significant impact on rates of fertilisation *in vitro*: alcohol, caffeine, and fruit and vegetable consumption. Rates of fertilisation *in vitro* increased with male fruit and vegetable consumption ($P<0.0001$). The effect of male alcohol consumption was not as anticipated; rates of fertilisation increased with increasing levels of alcohol consumption ($P<0.0001$); an effect highly unlikely to be due to statistical chance. It was stated by Wong et al. (2000) that nutritional deficiency may be an important neglected cause of reproductive impairment in man. The significance of nutritional factors on fertilisation demonstrated by this study supports this contention.

In addition, a significant interaction between male fruit and vegetable consumption and male alcohol consumption on fertilisation was demonstrated ($P=0.0144$). The negative sign of this interaction term indicates that the relationship is asymptotic; there is a threshold of benefit that can be attained from the combined effect of these two factors. Moreover, it suggests that the factor exerting a positive effect on fertilisation *in vitro* is common to both the consumption of fruit and vegetables and alcohol.

Before speculating as to the biological mechanisms of these observed effects, pertinent and related aspects of fertilisation discussed previously will be recapitulated. Once released from the protection of the Sertoli cells within the seminiferous tubules, spermatozoa are especially susceptible to the destructive

activity of ROS due to 1) lack of cytoplasm and associated intra-cellular antioxidant defense mechanisms, and 2) high levels of PUFA in the plasma membrane; intracellular enzymes cannot protect the outer plasma membranes from ROS (Hinton et al. 1995). Consequently, spermatozoa are highly reliant on extracellular antioxidant defense systems (Aitken 1999). In IVF treatment, seminal plasma is of little relevance as an extracellular defense system, as spermatozoa are washed free from this protective medium during sperm preparation for insemination. It is estimated that spermatozoa spend 70% of their lifespan within the epididymides and, therefore, the site whereby spermatozoa are most affected by ROS.

Only recently has the significance of the epididymides role in protection been recognised (Hinton et al. 1995). During transit through the epididymides, the plasma membrane of spermatozoa is remodelled; the total composition of PUFA increases with migration along the epididymides. In effect, membrane fluidity is enhanced in preparation for motility and membrane fusion (Ollero et al. 2001). Reduced glutathione (GSH) is believed to be the predominant, non-enzymatic antioxidant within the epididymides (Hinton et al. 1995). GSH acts as an antioxidant by serving as a substrate for glutathione peroxidase (Guérin, El Mouatassim & Ménézo 2001). Other thiol-compounds, hypotaurine (2-aminoethane sulfinic acid) and taurine (2-aminoethane sulphonic acid), are also actively engaged in antioxidant roles (Hinton et al. 1995). They are also thought to be essential for sperm capacitation, motility, fertilisation and early embryonic development (Donnelly, McClure & Lewis 2000).

5.3.2.1 Folate and Vitamin B_6

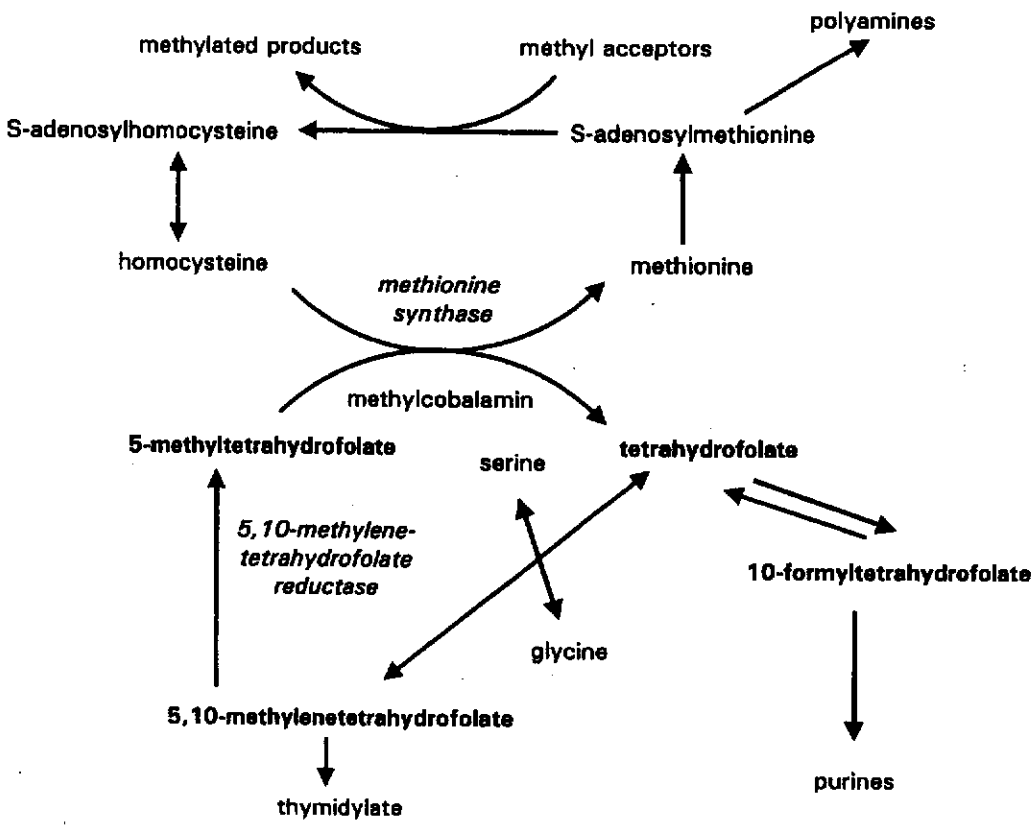
In addition to the independent significant effect of male fruit and vegetable consumption ($P<0.001$) and male alcohol consumption ($P<0.001$), there was also a significant interaction between these two factors on fertilisation ($P=0.0144$). The negative sign of this interactive term implies that at least one source of benefit is of the same origin. Folate and vitamin B_6 (pyridoxine) are potential candidates, as these vitamin B groups originate in beer from the malted barley (Mayer, Šimon & Rosolová 2001). Mayer, Šimon and Rosolová (2001) showed that beer intake was positively associated with blood folate levels and negatively associated with homocysteine concentrations. Regular consumption of beer is believed to be an

important source of folate and vitamin B_6 in diets inadequate in fresh fruit, vegetables and legumes (Mayer, Šimon & Rosolová 2001).

Vitamins of group B, mainly folate, vitamin B_6 (pyridoxine) and B_{12} are important coenzymes involved in the homocysteine-methionine metabolic pathway (Mayer, Šimon & Rosolová 2001). Homocysteine is a sulphur-containing amino acid that results from demethylation of dietary methionine (Mayer, Šimon & Rosolová 2001). As shown in Figure 5.1 (Wallock et al. 2001), 5-methyltetrahydrofolate (5-methyl-THF) is a co-factor in the remethylation pathway, and vitamin B12 is a co-factor of methionine synthase (Mayer, Šimon & Rosolová 2001). Figure 5.2 (Steegers-Theunissen et al. 1993) shows the transsulfuration pathway in which pyridoxine is a co-factor of cystathion beta synthase and that which leads to the production of cysteine (Mayer, Šimon & Rosolová 2001). Cysteine is the precursor compound of hypotaurine and taurine (Figure 5.3)(Guérin, El Mouatassim & Ménézo 2001), and in turn, glutathione, as shown in the oocyte model in Figure 5.4 (Guérin, El Mouatassim & Ménézo 2001).

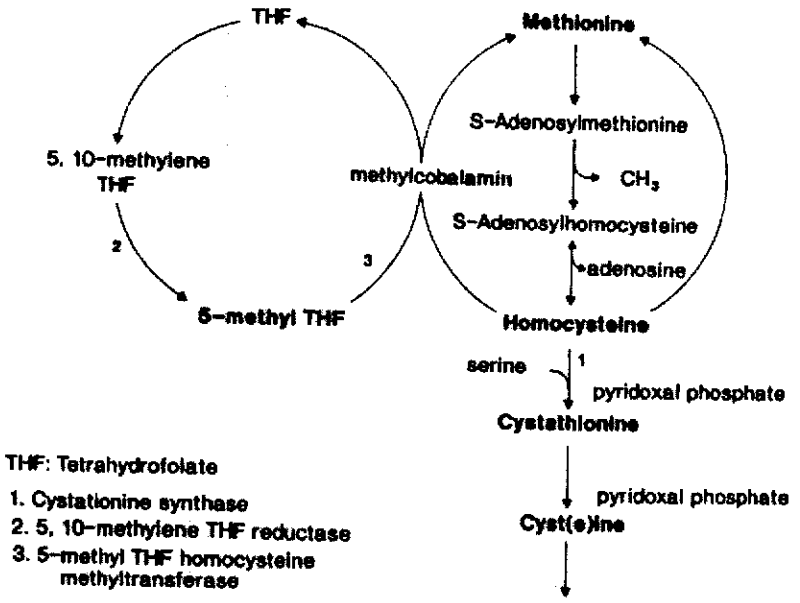
The role of folate in male fertility has only recently been investigated (Wallock et al. 2001). Levels of folate were shown to be 1.5 times higher in seminal plasma than in blood plasma, and strongly correlated. Non-methyltetrahydrofolate (non-methyl-THF) accounted for 26% of total folate in seminal plasma (Wallock et al. 2001). Various co-enzyme forms of folate participate in metabolic pathways leading to the production of polyamines. It was noted by Wallock et al. (2001) that the concentration of polyamines are higher in seminal fluid than in any other body fluid or organ. The dietary protein, methionine, is demethylated to homocysteine (a sulfur, amino acid), of which 5-methyl-THF is the substrate for the methionine synthase reaction to remethylate homocysteine to form methionine. Of significance, non-methyl-THF is involved in the synthesis of cysteine, from which cysteamine, hypotaurine and taurine originate, as is illustrated in Figures 5.1 (Wallock et al. 2001), Figure 5.2 (Steegers-Theunissen et al. 1993) and Figure 5.3 (Guérin, El Mouatassim & Ménézo 2001). Cysteine is also the precursor molecule in the production of glutathione (Figure 5.4)(Guérin, El Mouatassim & Ménézo 2001).

Figure 5.1: The Role of Folate and Vitamin B_{12} (Cobalamin) in the Homocysteine-Methionine Metabolic Pathway



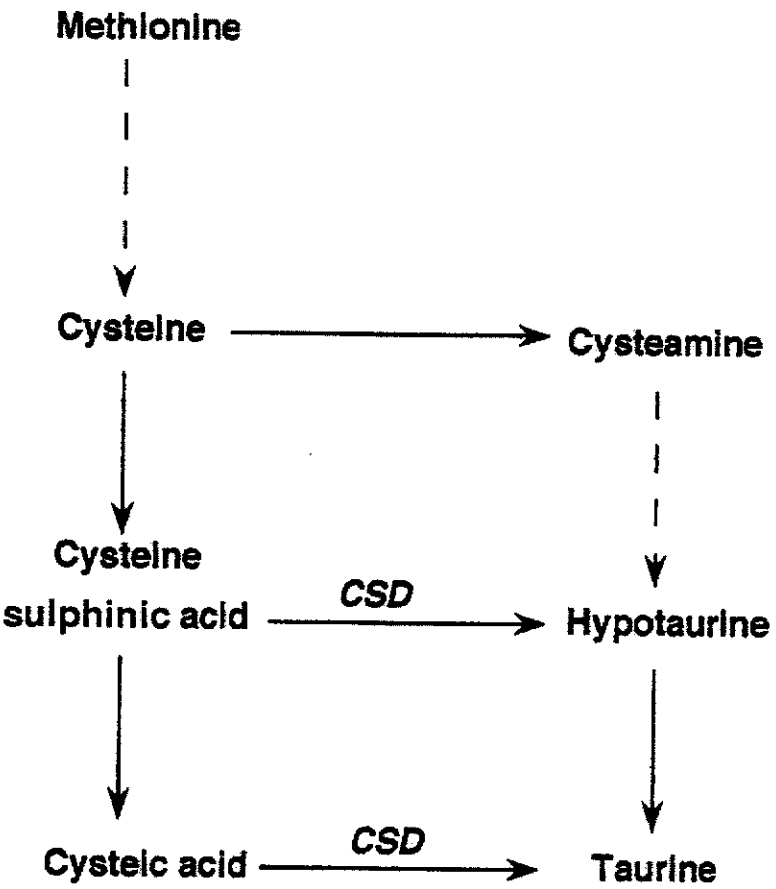
Wallock et al. (2001)

Figure 5.2: The Role of Vitamin B_6 in Cysteine Production



Steegers-Theunissen et al. (1993)

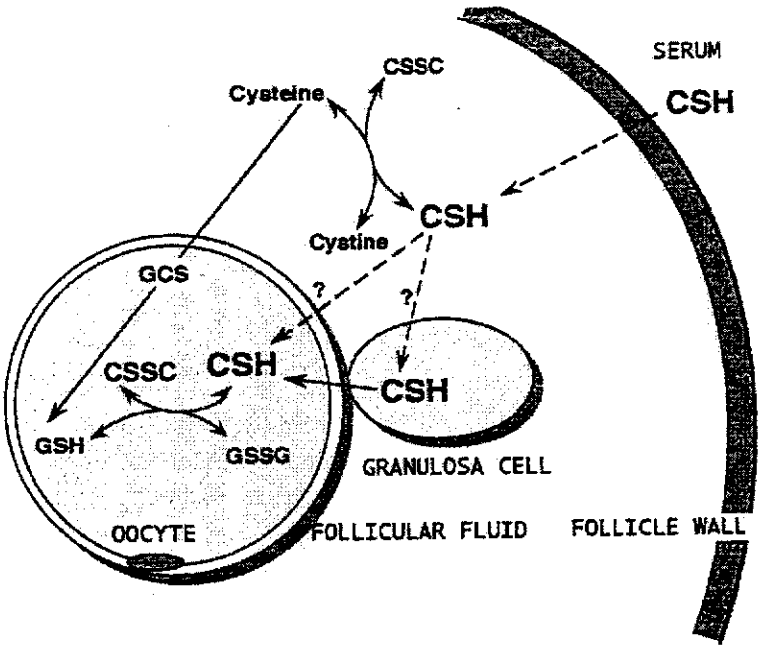
Figure 5.3: The Methione-Hypotaurine-Taurine Metabolic Pathway



CSD = cysteine sulphinate decarboxylase

Guérin, El Mouatassim & Ménézo 2001

Figure 5.4: Cysteine-Glutathione Metabolic Pathway



CSH: cysteamine
CSSC: cystamine
GCS: glutamylcysteine synthetase

Guérin, El Mouatassim & Ménézo 2001

Folate is essential for DNA synthesis and repair (Wallock et al. 2001). In women, supplementation of folate pre-conception and during early pregnancy has been shown to prevent 70% of neural tube defects (NTD) in offspring (Greene & Copp 1997). (There is evidence that myo-inositol may be effective in prevention of folate-resistant NTD (Greene & Copp 1997)). Inadequate 5-methyl-THF causes massive mis-incorporation of uracil in human DNA. This predisposes the chromosomes to breakage and DNA damage (Blount et al. 1997 cited in Wallock et al. 2001).

5.3.2.2 Myo-inositol

There is increasing evidence that myo-inositol (MI), an isomer of the C6 sugar alcohol of the vitamin B complex group, plays a crucial role in gamete development, including oocyte maturation, fertilisation and embryonic development (Chiu et al. 2002). MI is the precursor molecule for the synthesis of phosphoinositides. These molecules, in turn, are involved in the phosphatidylinositol (PtdIns) signal induction system, which is responsible for the generation of intracellular signals (Chiu et al. 2002). Activated in response to hormonal or other stimuli, the PtdIns cycle is a transducer of information across plasma membrane. It involves a receptor-dependent hydrolysis of an inositol lipid precursor to generate the inositol 1,4,5-triphosphate [Ins(1,4,5) P_3], which is a second messenger that regulates many cellular processes by modulating the release of intracellular calcium (Berridge 1993). Recently, phosphatidylinositol 3-kinase (PI3K) was demonstrated in mouse spermatozoa (Feng et al. 1998 cited in Luconi et al. 2001). It was suggested that PI3K is involved in the induction of the acrosome reaction (Luconi et al. 2001).

5.3.2.3 Caffeine Consumption

Caffeine consumption was yet another male lifestyle factor found to be of significance in regard to fertilisation *in vitro*. Only one study to date has examined male caffeine consumption and fertilisation. In this study by Klonoff-Cohen, Bleha and Lam-Kruglick (2002), the dependent variable was simply the number of fertilised oocytes, which did not take account of the total number of oocytes inseminated. Furthermore, this study used only univariate methods of data analysis. No effect of male caffeine consumption was evident (Klonoff-Cohen, Bleha & Lam-Kruglick 2002).

In the present study, caffeine consumption was not significant as a main effect in this study, but as a significant interaction term involving male alcohol consumption. This implies that the effect of male caffeine consumption is indirect, in that it modified the beneficial effect of alcohol consumption; the positive effect of alcohol consumption is negated by caffeine intake. In regard to fertilisation *in vitro*, regression analysis revealed that male caffeine consumption did not interact with male fruit and vegetable consumption as it did with alcohol consumption. This is particularly telling; it indicates that at least one of the beneficial effects of alcohol consumption is not one shared by the beneficial effect of fruit and vegetable consumption. Moreover, it provides a clue as to what component of alcohol is exerting this positive effect on fertilisation.

5.3.2.4 Alcohol Consumption

It is hypothesised that one of beneficial effects elicited by alcohol consumption is that derived from vitamin B_{12} (cyanocobalamin); the one which is not shared with the beneficial effect of fruit and vegetable consumption and the effect negated by caffeine consumption. Of all the vitamins, B_{12} has the most complex structure, and one of few biological molecules known to possess cobalt as part of its structure (Thomas & Gillham 1989). As plants do not manufacture or require vitamin B_{12}, animals and humans rely on microorganisms to synthesize their vitamin B_{12} requirements (Thomas & Gillham 1989). Vitamin B_{12} is found in beer and originates from the brewing yeasts (Mayer, Šimon & Rosolová 2001). Levels of vitamin B_{12} in beer vary according to the brewing process, with the highest levels found in beers where yeasts are added at the end of the brewing process (Mayer, Šimon & Rosolová 2001). In this study, however, only total weekly consumption of alcohol (by standard drinks 10gm of ethanol) was measured, which did not discriminate by type of alcoholic beverage consumed. Nonetheless, it has been shown that wine drinkers are more likely to be women, spirit drinkers more likely to be men, heavy drinkers and less educated, and beer drinkers are more likely to be young males who fall between these two groups for most traits (Denke 2000). It is likely that the men who participated in this study are more representative of the latter.

As discussed previously, vitamin B_{12} is a co-factor of methionine synthase in the remethylation of homocysteine (Figure 5.1)(Wallock et al. 2001). In this pathway, vitamin B_{12} acts as the co-enzyme, methylcobalamin, and receives a methyl group from 5-methyltetrafolate. The methyl group is transferred from methycobalamin to homocysteine to generate methionine. In effect, tetrahydrofolate is returned to the metabolic pool (Figure 5.1)(Wallock et al. 2001).

Deficiencies in vitamin B_{12} tend to arise from impaired absorption (Thomas & Gillham 1989). The most common cause is the failure of the gut to produce sufficient quantities of an 'intrinsic factor', which is a glycoprotein or possibly a group of closely related glycoproteins (Thomas & Gillham 1989). This intrinsic factor releases vitamin B_{12} from the ingested protein molecule (Thomas & Gillham 1989). The reason for inadequate production of intrinsic factor is not known, although vitamin B_{12} deficiency is associated with gastritis (Thomas & Gillham 1989). Among the males in this study, caffeine consumption was shown to negate the positive effect of alcohol consumption on rates of fertilisation *in vitro*. This vitamin B_{12} hypothesis would, in part, be confirmed if it was demonstrated that caffeine consumption in some way compromised levels of vitamin B_{12}.

It is interesting to note that Fenech (1998) questioned the sufficiency of standard recommended daily intake for vitamin B_{12}. Fenech (1998) examined the relationship between *in vivo* vitamin levels and chromosomal damage in lymphocytes. A consistent and significant negative correlation between vitamin B_{12} and chromosomal damage was observed in both young and older men. In the latter case, none of the men were in the deficient range for vitamin B_{12}. The current non-deficient range is that defined by the absence of clinical parameters for anaemia. Given this, Fenech (1998) stated that dietary intake of vitamin B_{12} for minimising chromosomal damage does not correspond with the current standard of sufficiency. Moreover, chromosomal damage was positively and significantly correlated with plasma homocysteine status in men who were neither folate nor vitamin B_{12} deficient (Fenech 1998).

Undoubtedly, this scenario also applies to the sufficiency of vitamin B_{12} required for optimal production of the polyamines resulting from the homocysteine-methionine metabolic pathway (Figure 5.1)(Wallock et al. 2001). In the process termed transsulphuration, the sulphur atom of S-adenosylmethionine (SAM) is converted via a series of enzymatic steps to cysteine. Cysteine is a precursor of hypotaurine, taurine (Figure 5.3)(Guérin, El Mouatassim & Ménézo 2001), and in turn, glutathione (Figure 5.4, Guérin, El Mouatassim & Ménézo 2001). In human liver cells, approximately half of the intracellular pool of glutathione is derived from the homocysteine-dependent transsulphuration pathway, and this pathway can be viewed as an autocorrective response that leads to increased glutathione synthesis in cells changed by oxidative stress (Lu 2000). Lu (2000) stated that given the importance of SAM, it is not surprising that this molecule is currently being investigated as a possible therapeutic agent for the treatment of various clinical disorders.

Alcohol, or ethanol, is a highly lipid-soluble compound (Adinoff, Bone & Linnoila 1988). Excessive, chronic alcohol consumption intake is generally associated with vitamin deficiency, especially folate, vitamin B_1 (thiamine) and vitamin B_6, due to malnutrition, malabsorption and ethanol toxicity (van den Berg, van der Gaag & Hendriks 2002). Yet, levels of alcohol consumption by the males in this study were low to moderate, and similar to that reported by other studies (Dunphy, Barratt & Cooke 1990; Gerhard et al. 1992; Marshburn, Sloan & Hammond 1989). The majority of men in this study (80%) consumed alcohol during the week prior to collection of the semen sample for insemination, and intake averaged (median) only 9 standard drinks per week. The consumption of alcohol among males in this study is consistent with levels in the range of low to moderate.

Although initially met with skepticism, the putative protective effect of low to moderate, regular alcohol consumption on cardiovascular disease is now widely accepted (Denke 2000; Gaziano et al. 1993). The majority of the more recent large population-based studies showed that moderate drinking in the range of 1-3 standard alcohol beverages per day is associated with a reduction in rates of coronary disease, being 30 to 40% lower than that of non-drinkers (Denke 2000). The consistency of the data in favour of a beneficial effect of regular, low-moderate alcohol

consumption on cardiovascular disease is persuasive. Denke (2002, p. 321) stated that "[T]he proposal that alcohol has a protective effect on the risk of ischaemic heart disease no longer has the charm of novelty." This study is the first to show that male alcohol consumption increases rates of fertilisation *in vitro*, within the range consumed by the males in this study. Undoubtedly, this study will be received with a similar degree of skepticism as that encountered by the first studies to report that low to moderate alcohol consumption was associated with reduced rates of cardiovascular disease.

The protective effect of alcohol against cardiovascular disease does not appear to be specific to alcoholic drink type. Although initial reports suggested that wine was superior to beer, it is now believed that, for the most part, the cardio-protective effect is caused by the alcohol content of the beverage and not inherent differences in other components found in the respective beverages (Denke 2000; Hein, Suadicani & Gyntelberg 1996; Rimm et al. 1996). Several mechanisms of benefit have been forwarded: 1) increased levels of high-density lipoprotein (HDL) cholesterol, 2) anti-thrombotic properties, 3) antioxidant properties of the beverages, and 4) enhanced resistance to insulin (Denke 2000). It is the former, however, that has received the most attention. Low-moderate, regular alcohol intake has been repeatedly shown to increase serum concentration of HDL-cholesterol levels (Rimm et al. 1996).

Rates of fertilisation *in vitro* increased with alcohol consumption by male patients in this study. One study is of particular relevance in accounting for the potential biological mechanisms of this unexpected finding: Simonetti et al. (1995) investigated the effect of moderate alcohol consumption on lipids and select vitamins of red blood cells (RBCs) and plasma among 58 healthy Italian males aged 20-75 years. Twenty-six non-alcoholic drinkers were compared with 32 average drinkers of mean daily alcohol intake 46.5$\pm$ 4.4g/day and a range of 30.2-63.4 g/day. A detailed analysis of the following phospholipid classes was undertaken: phosphatidylethanolamine (PE) and phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and sphingomyelin (SM). In a further analysis, the fatty acid composition of PE and PC was measured.

Analysis of covariance was used to statistically control for differences in age, smoking and BMI.

Like that of the phospholipid bilayer of the sperm plasma membrane, the dominant phospholipids in the RBC bilayer membrane are PE and PC (Lenzi et al. 1996; Simonetti et al. 1995; Storey 1997). Drinkers as compared to non-drinkers had a significant decrease in SM, increase in PC and decrease in PE, with a resultant overall lower SM/PC ratio. The SM/PC ratio is an index of membrane viscosity; low values are indicative of a highly viscous (or fluid) medium (Simonetti et al. 1995). It was highlighted by Simonetti et al. (1995) that the polar moiety of PC is an ester of phosphorus with ethanolamine, which is capable of binding to the contiguous phosphate group and, therefore, like SM, enhances membrane viscosity. The authors concluded that the data supported the hypothesis that alcohol intake increases RBC membrane fluidity.

The importance of the homocysteine-methionine metabolic pathway is once again underscored in regard to the synthesis of the phospholipids. As early as 1983, Sastry and Janson (1983) showed that human spermatozoa contain two phospholipid N-methyltransferases, which convert the plasma membrane phospholipid, PE to PC by stepwise methylation. Phosphatidycholine (PC) is the phospholipid associated with increased membrane fluidity. Of significance, however, is that these reactions require the substrate, S-adenosylmethionine (SAM), which acts as a methyl donor (Figure 5.1)(Wallock et al. 2001). SAM is produced by SAM synthease from adenosine-triphosphate and methionine. Conversely, S-adenosylhomocysteine (SAH) is a potent inhibitor of SAM-mediated methylation (Sastry & Janson 1983). In turn, Sastry and Janson (1983) showed SAH depressed sperm motility. More recently, Rambaldi and Gluud (2002) stated that SAM plays a fundamental role in the synthesis of membrane phospholipids, especially PC and, therefore, mandatory for the maintenance of membrane fluidity. In addition to transmethylation and transulphuration, SAM is also important for nucleic acid and protein synthesis (Rambaldi & Gluud 2002). Lu (2000) stated that interference of these reactions can affect a wide spectrum of processes ranging from gene expression to membrane fluidity.

Differences in the effect of alcohol on the PUFA composition of the two major classes of phospholipids, PE and PC, has been demonstrated by Simonetti et al. (1995). There are three families of PUFA and classification is based on the distance between the first double bond and the methyl terminal: *n-3*, *n-6* and *n-9* (Lenzi et al. 1996). α-Linolenic acid (C18:1 *n-3*), linoleic acid (C18:2 *n-6*) and oleic acid (C18:1 *n-9*) are the respective parent fatty acids (Lenzi et al. 1996). Alcohol drinkers had significantly higher RBC PUFA concentrations, of which were attributed to a 13% increase in arachidonic acid (20:4 *n-6*) and 22% increase in docosahexaenoic acid (22:6 *n-3*). Remarkably, these two PUFA were stated by Lenzi et al. (1996) to be of notably high levels in the membrane of spermatozoa.

In regard to PE, alcohol consumers had significantly increased 20:4 *n-6* and a partial increase in 22:4 *n-6*, with a concomitant decrease in the relative content of the precursor 18:2 *n-6*, 18:3 *n-6* and 20:3 *n-6*. In light of this, Simonetti et al. (1995) suggested that alcohol activates desaturase. This family of enzymes are involved in the conversion of dietary di-saturated fatty acids, α-Linolenic acid (C18:1 *n-3*) and linoleic acid (C18:2 *n-6*) into the long-chain PUFA through a series of elongation and desaturation reactions. It is believed that these reactions, which occur mainly in the liver, involve vitamin E and selenium as co-factors (Lenzi et al. 1996).

The viscosity or fluidity of biological membranes is determined by their lipid composition, and membrane integrity is maintained by antioxidant compounds, namely α-tocopherol and β-carotene (Simonetti et al. 1995). Simonetti et al. (1995) also compared plasma and RBC retinol, α-tocopherol (vitamin E) and β-carotene in drinkers and non-drinkers. After adjustment for age, smoking and BMI, drinkers had significantly higher levels of retinol and α-tocopherol in plasma. It was suggested that the elevated plasma concentrations of retinol observed among the alcohol consumers may be attributed to the effect of ethanol on retinol mobilisation from the liver, which had been implicated in previous studies (Leo, Lowe & Lieber 1987, Sato & Lieber 1982 and Lieber & Savolainen 1984 cited in Simonetti et al. 1995). Vitamin A is believed to play a fundamental role in regulating cell division, cell

differentiation and the synthesis of membrane glycoproteins, which are involved in cell recognition (Thomas & Gillham 1989).

Compelling evidence that ethanol alters cell membranes was recently demonstrated in the amphibian model. An investigation of the lipid composition of *Xenopus* embryos exposed to varying concentrations of ethanol (1-250mM) *in vitro* during their first six days embryonic development was conducted by Lindi et al. (2001). Phospholipid content increased in a dose-dependent manner from 150 to 250mM ethanol concentrations, with no concomitant change in cholesterol and protein values, which supports the specificity of the ethanol-induced effect. Further analysis of individual phospholipid classes showed that fatty acid composition was significantly affected from ethanol concentrations of 150 to 250mM. The elevated PUFA/saturated fatty acid ratio resulted from a concomitant decrease in palmitic acid (C16:0) and an increase in arachidonic (C20:4) and eicosapentaenoic (C20:5) acid.

Xenopus was noted by Lindi et al. (2001) to be an advantageous system for the studying of lipid modifications. The embryo is a closed system during early development and utilises stored material of maternal original until day 4 or 5 of development. Although exposed to varying concentrations of ethanol, the *Xenopus* embryos were not fed; therefore, differences in dietary factors across embryos were eliminated. Lindi et al. (2001) concluded that the observed changes to the phospholipid fatty acid composition were caused by ethanol *per se* (Lindi et al. 2001).

Some of the beneficial effects of alcohol are likely to be attributed to the non-alcohol components: protein, vitamin B and polyphenols (formerly termed tannins)(Fenech 1998). Polyphenols are present in beer and wine, though beer contains more proteins and group B vitamins. It has been shown that the nutritional value of beer can contribute substantially to the diet (Denke 2000), which is consistent with that surmised by Mayer, Šimon and Rosolová (2000) in regard to adequate folate and vitamin B intake. Polyphenols prevent low-density lipoprotein (LDL) oxidation *in vitro*, and in a dose-dependent fashion (Denke 2000). Beer has a total polyphenol content approximately equivalent to that of red wine, and has a greater capacity to

prevent LDL oxidation than white wine (Denke 2000). Given the significance of lipid oxidation in spermatozoan function, it is highly plausible that these positive effects of alcohol consumption could translate to benefits in male fertility.

5.3.2.5 Healthy Diet in General

A further explanation for the positive effect of fruit and vegetable consumption on fertilisation is that fruit and vegetable consumption *per se* is an indicator of overall quality or healthiness of diet in general. A well-balanced diet is more likely to provide the full repertoire of essential nutrients. This is a tenable theory as the metabolic pathways resulting in the production of antioxidants are complex and involve many co-factors, including zinc and selenium.

Zinc is 100 times more concentrated in seminal plasma than blood serum and testament to its physiological role in male fertility (Eggert-Kruse et al. 2002). In spermatozoa, SOD is an important intracellular, enzymatic antioxidant, of which the Cu/Zn isozyme is the most representative (Storey 1997). In this form, zinc plays a vital role in plasma membrane stability by the prevention of lipoperoxidation (Eggert-Kruse et al. 2002; Storey 1997; Wong et al. 2000). A recent study suggested that the removal of zinc from the plasma membrane destabilises it in preparation for capacitation and the acrosome reaction (Eggert-Kruse et al. 2002). Zinc deficiency adversely affects the absorption and metabolism of folic acid (Wong et al. 2000). It is necessary for the conversion of polyglutamylfolates to the monoglutamate; this latter form of folate is that absorbed in the intestine (Wong et al. 2000).

There is little doubt that selenium plays a vital role in male fertility, and one in which is traced to the enzyme glutathione peroxidase (GPX)(Thomas & Gillham 1989). In addition to SOD, GPX is the other vital intracellular, antioxidant enzyme within spermatozoa. Of significance, selenium forms part of the active centre of this enzyme (Storey 1997; Thomas & Gillham 1989). In its protective role, GPX reduces lipid hydroperoxides to relatively inert hydroxyl fatty acids. As GPX acts on the products of oxidation, it is complementary to the role of SOD, which acts to limit initiation of the oxidative reactions (Storey 1997). Therefore, the selenium-isoform,

GPX protects spermatozoa against lipoperoxidation (Storey 1997; Thomas & Gillham 1989).

A healthy diet, rich in fruits, nuts, grains and vegetables seems to offer the best protection against oxidative stress in general, due to the presence of antioxidants, to the many other vital nutrients or both (Halliwell 1994). Fenech (1998) concluded that based on knowledge to date, modulation of oxidative stress by diet may best be by way of the optimisation of vitamin B status.

5.4 Conclusions

Specific lifestyle factors were shown by this study to impact on the clinical outcomes of IVF treatment. Daily stress and cigarette smoking were of clinical significance among female patients. This study adds to the growing body of evidence that cigarette smoking has an adverse and irreversible effect on ovarian reserve. It was also shown that the fertilisability of oocytes was impaired among female patients with a history of smoking, which supports a smoking-related compromise in oocyte quality. Of note, female smoking did not affect rates of pregnancy or pregnancy loss. Nor did female caffeine and alcohol intake affect any of the clinical outcomes of IVF treatment.

This study makes an original contribution to understanding the effect of stress on patients undergoing IVF treatment. Acute stress from IVF treatment was shown among males to negatively effect rates of fertilisation *in vitro*. Among females, daily stress, purportedly chronic in effect, compromised oocyte production. Based on the evidence from this study, it would appear that females with high levels of daily stress present with infertility associated with an anovulatory factor. As the dysfunction of stress-related ovulatory infertility is at the level of the hypothalamus, anovulation is readily remedied with eFSH, with no subsequent impediment to fertilisation, embryo quality or implantation. The evidence from this study suggests that female patients with stress-related ovulatory factor infertility experience successful outcomes following IVF treatment due to higher than average rates of fertilisation and pregnancy.

All of the male lifestyle factors measured in this study, including stress, impacted on at least one of the clinical outcomes investigated. The most notable was male cigarette smoking. The risk of a male's partner experiencing a <12 week pregnancy loss increased with smoking exposure. As indicated by the literature, this is likely due to pre-conceptual DNA damage that fatally impairs embryonic development subsequent to implantation. This is the first study to show empirically and unequivocally that male smoking is linked with early pregnancy loss among IVF couples.

Two former studies had reported an increase in crude rates of pregnancy loss among female smokers following IVF treatment. The significant association between male smoking and early pregnancy loss shown by this study renders the validity of the female smoking-related increase in pregnancy loss highly questionable. It is well established that the smoking habit of the female is associated with that of the male partner. Therefore, smoking by the male partner cannot be ruled out as accounting for the previously reported elevated rate of pregnancy loss experienced by female smokers as compared to non-smokers. It was demonstrated by this study that the smoking habit of the male, but not the female, had an adverse effect on pregnancy outcome.

Most profound was the impact of male lifestyle factors, especially nutritive factors, on rates of fertilisation *in vitro*. Fertilisation increased with male consumption of fruit and vegetables and alcohol. However, this beneficial effect of male alcohol consumption was negated by male caffeine intake. Interactions between the effects of fruit and vegetable, alcohol and caffeine consumption on fertilisation provided valuable clues as to the biological mechanism of these relationships. Based on the literature, the beneficial effects of these nutritive factors on fertilisation *in vitro* appear to stem from vitamins of group B, namely folate, vitamin B_6 and B_{12}, and PUFAs. In general, the B group vitamins are important cofactors and substrates in the homocysteine-methionine metabolic pathway. This pathway is responsible for the production of polyamines and purines that protect against the damaging effects of ROS. Specific sub-fractions of PUFAs enhance fluidity of the sperm plasma membrane, which is vital for spermatozoan motility and oolemma fusion.

5.5 Implications

Cigarette smoking has an adverse effect on both male and female fertility. Therefore, acquiring a history of cigarette smoking for patients attending infertility clinics may assist in understanding their cause of infertility. It is with this knowledge that both male and female patients undertaking infertility treatment should be encouraged to cease, or at least reduce, their smoking habit.

Cigarette smoking is a highly addictive habit and one that is exacerbated by stress, making cessation difficult to achieve during IVF treatment. In light of this, IVF treatment needs to focus on limiting the damage already caused by cigarette smoking exposure. Recently, sperm preparation techniques have been investigated for their efficiency at isolating spermatozoa with normal nuclear DNA. There is some indication that the density gradient centrifugation technique is superior to the swim-up technique. In which case, it may prove advantageous to apply the density gradient centrifugation technique (or superior techniques that may become available in the future) to all male patients who currently smoke and those with a prolonged or recent history of smoking. This may reduce the likelihood of their female partners experiencing a male-mediated pregnancy loss or lessening the chance of inferior DNA being transmitted to offspring.

The adverse effect of cigarette smoking is irreversible. Female smoking depletes the ovarian reserve, and male smoking is believed to cause an accumulation of DNA damage in the germ cells. Therefore, the only manner by which to prevent the damaging effect of smoking on male and female reproduction is to abolish the behaviour completely. Information gained from this study may well serve health promotion programmes in combating the commencement of cigarette smoking among young adults. Issues of fertility are more relevant to the young adult population than that of the smoking-related diseases such as lung cancer and cardiovascular disease, which tend to be associated with older age.

Stress experienced by both female and male patients impacted on the clinical outcomes of IVF treatment. Whilst it was stress from daily living that adversely affected female patients, the effect of stress was that from IVF treatment among male

patients. It is recommended that female patients with high daily stress levels be identified at the initial infertility consultation and offered 1) less invasive forms of infertility treatment, namely ovulation induction, 2) stress management during treatment, or 3) psychological counselling prior to the commencement of treatment. Less is known about the factors contributing to stress among male IVF patients, and even less in regard to strategies of management and prevention. Clearly, further research in this area is needed.

The significance of male nutritive factors on rates of fertilisation *in vitro* has been highlighted by this study. Clinicians should draw to the attention of male patients the importance of diet. Based on the findings of this study, males should be encouraged to adopt a diet rich in fruit and vegetables. Furthermore, consumption of high levels of caffeine should be curtailed. Low to moderate alcohol consumption may be beneficial, although it is premature to propose alcohol consumption as a therapeutic agent until the findings can be replicated by controlled clinical trials. It is clear from this study, however, that abstinence from alcohol consumption among male patients as a means of improving clinical outcomes is not supported. The importance of sperm plasma membrane fluidity has been reiterated by the findings of this study. In light of this, the potential benefits of dietary supplements containing the parent fatty acids, α-Linolenic acid (C18:1 *n-3*), linoleic acid (C18:2 *n-6*) and oleic acid (C18:1 *n-9*), is worthy of further consideration.

In meeting with its principal objective, this study has demonstrated that specific lifestyle factors impact on the clinical outcomes of IVF treatment. Many of the relationships between lifestyle factors and clinical outcomes shown by this study were new and unexpected. Further research is warranted into identifying specific mechanisms of effect, including those suggested. Moreover, this study has paved the way for future research into improving IVF treatment outcomes through lifestyle modification.

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Appendix A

Smoking and Clinical Outcomes of IVF: Systematic Review

Trapp, Kemeter and Feichtinger (1986) were the first to have published a study on smoking and the clinical outcomes of IVF treatment. This study involved 114 female patients who received IVF treatment during 1984 and 1985 at a medical centre in Vienna, Austria. All were younger than 40 years of age. Clomiphene citrate (CC) was used for pituitary suppression and human menopausal gonadotrophin (hMG) for ovarian follicle stimulation, with the daily dose being modified in accordance with the individual patient's ovarian response (ovarian response-dependent).

In the Trapp, Kemeter and Feichtinger (1986) study, smoking status was self-reported by questionnaire. Female patients were classified as either a smoker or non-smoker, although neither criterion was clearly defined. One in every three patients was a smoker (33.3%; 38/114). Female smokers were marginally younger than the non-smokers, with mean ages of 33.8 (standard error (SE) 0.5, standard deviation (SD 4.4)) and 34.2 (SE 1.4, SD 8.6) years, respectively. Compared to their non-smoking counterparts, tubal malformation occurred more frequently among the female smokers (86.8% versus (vs) 79.0%), as did having a male partner who had an abnormal sperm diagnosis (25.7% vs 14.7%). The statistical significance of these differences between the female smokers and non-smokers was not reported.

Trapp, Kemeter and Feichtinger (1986) also measured levels of rhodanide in serum and follicular fluid. Rhodanide (SCN) is a metabolite of cyanide, a chemical component of cigarette smoke. As compared to female non-smokers, smokers had significantly higher mean serum SCN levels, 114.9 (SD 69.7) vs 80.8 (SD 81.9) μmol/l ($P<0.05$). Likewise, mean SCN levels were significantly higher in the follicular fluid of female smokers than non-smokers, 124.1 (SD 49.3) vs 98.9 (SD 66.3) μmol/l ($P<0.05$). The considerable overlap in both serum and follicular fluid SCN levels between the female smokers and

non-smokers renders the validity of SCN as a biochemical measure of smoking exposure questionable.

The clinical outcomes of IVF treatment investigated by Trapp, Kemeter and Feichtinger (1986) included oocyte production, fertilisation and pregnancy. Pregnancy was that confirmed by beta-human chorionic gonadotrophin (β-hCG) levels on days 15 and 20 post-embryo transfer (β-hCG pregnancy). As it was not specifically stated, it is inferred that the measure of fertilisation presented is the proportion of oocytes (including immature) that fertilised per couple presented as a group average (mean fertilisation rate). Although not significant, fewer oocytes were retrieved from smokers than non-smokers (mean 3.6 (SD 2.4) vs 4.3 (SD 3.2); $P>0.05$), and in turn the number of resultant fertilised oocytes was less (mean 1.8 (SD 1.8) vs 2.2 (SD 2.4); $P>0.05$). Female smokers had a lower mean fertilisation rate than the non-smokers, 45.1% (SD 39.6) vs 49.9% (SD 38.2; $P>0.05$). Three of the 38 smokers (7.9%) and 12 of the 76 non-smokers (15.8%) had a β-hCG pregnancy ($P>0.05$).

Trapp, Kemeter and Feichtinger (1986) examined the possibility of a dose-dependent effect of smoking on IVF outcomes. Female smokers were divided into light smokers (a few cigarettes per day (cig/day); n=19) and heavy smokers (>1 packet of cig/day; n=19). However, the authors reported that no differences in the results of IVF treatment were evident between the non-smokers, light smokers and heavy smokers.

The next study published on smoking and IVF outcomes was by Harrison, Breen and Hennessey (1990). It involved 650 female patients who had either IVF or gamete intra-fallopian transfer (GIFT) treatment between January and June 1988 at the Queensland Fertility Group, Brisbane. All were younger than 40 years of age. Clomiphene citrate was used for pituitary suppression and hMG for follicle stimulation; the quantities administered was ovarian response-dependent.

Harrison, Breen and Hennessey (1990) ascertained smoking status upon admission to hospital, presumably before oocyte retrieval. The inclusion criterion for smokers was a stable habit throughout the stimulation phase and the preceding month. Smokers accounted for 16.6% (108/650) of the patients, of which, 72 (66.7%) smoked 1-10 cig/day, 31 (28.7%) smoked 11-20 cig/day and 5 (4.6%) smoked 21-30 cig/day.

Harrison, Breen and Hennessey (1990) did not report the mean age of the smokers and non-smokers. They did, however, report that of the smokers and non-smokers, 49% and 44% were diagnosed with tubal infertility, 15% and 19% with endometriosis, and 14% and 13% had a partner with male factor infertility, respectively. The authors concluded that there was little difference in aetiology status between the female smokers and non-smokers, although the statistical significance of these differences was not reported. The significance of differences in the mean number of hMG ampoules (75IU) administered was reported, but found to be non-significant ($P>0.05$).

Harrison, Breen and Hennessey (1990) examined oocyte production, fertilisation, clinical pregnancy and miscarriage. Mean number of oocytes retrieved for the non-smokers and 1-10 cig/day, 11-20 cig/day and 21-30 cig/day were 5.1, 5.4, 4.4 and 3.8, respectively ($P>0.05$). Although not stated, it was inferred that the measure used for fertilisation was the number of fertilised oocytes from the total pool of oocytes retrieved from patients within each of the smoking groups (proportion of fertilised oocytes), which were presented as a percentage. A trend, albeit non-significant, of increasing rates of fertilisation with smoking exposure was implied, as the proportion of fertilised oocytes from non-smokers and 1-10 cig/day, 11-20 cig/day and 21-30 cig/day smokers was 66%, 65%, 75% and 79%, respectively ($P>0.05$).

Harrison, Breen and Hennessey (1990) examined the effect of female smoking on miscarriage, which presumably refers to pregnancy loss following diagnosis of a clinical pregnancy. As compared to the clinical pregnancy rate of 22% among the non-smokers, the rates among the 1-10, 11-20 and 21-30 cig/day smoking groups were only 9.7%,

6.5% and 0%, respectively. Of the 119 clinically pregnant non-smokers, 24 experienced a miscarriage (20.2%), as compared to four of the seven 1-10 cig/day smokers (57.1%) and one of the two 11-20 cig/day smokers (50.0%). Collectively, smokers had a significantly lower rate of clinical pregnancy ($P<0.05$) and a significantly higher rate of miscarriage ($P<0.05$) compared to non-smokers.

A study by Elenbogen et al. (1991) involved a sample of 41 female patients who undertook an IVF treatment cycle at a medical centre in Tel Hasomer, Israel. Neither the method or selection criteria for study inclusion were detailed. However, all were younger than 37 years, had tubal infertility and normal ovulatory cycles and the male partner had normal spermatozoa. Gonadotrophin releasing hormone analogue (GnRHa) was administered for pituitary suppression and a combination of hMG, FSH and LH for follicle stimulation, with all patients having had received the same dose of each.

In the Elenbogen et al. (1991) study, smoking status was self-reported and ascertained by questionnaire prior to oocyte retrieval. Twenty-one patients were classified as non-smokers and 20 classified as smokers of >15 cigarettes per day. The mean age of smokers and non-smokers was 33.5 (SD 1.8) and 32.6 (SD 1.6) years, respectively; statistical significance of the difference was not reported. The duration of ovarian follicle stimulation was significantly longer among smokers than non-smokers, mean 11.9 (SD 1.4) vs 8.9 (SD 0.8) days ($P<0.05$). Accordingly, smokers had a significantly higher mean dose of hMG administered than that of non-smokers, 23.3 (SD 1.5) vs 15.6 (SD 1.6) ampoules ($P<0.05$).

Oocyte production, fertilisation and pregnancy were examined by Elenbogen et al. (1991). The mean number of oocytes retrieved from among the smokers and non-smokers was comparable (6.2 (SD 1.5) vs 6.8 (SD 1.4); $P>0.05$). Fertilisation was expressed as the proportion of fertilised oocytes within each smoking group. The fertilisation rate of oocytes from smokers was significantly lower than that from non-

smokers, 40.9% vs 61.7% (P<0.05). One of the 20 smokers and four of the 21 non-smokers achieved a (non-defined) pregnancy, rates of 5.0% and 19.0%, respectively.

In the same year, a retrospective cohort study by Pattinson, Taylor and Pattinson (1991) was reported. It involved data from 447 couples who had IVF treatment for the first time between March 1984 and March 1989 at the University of Calgary Medical Centre in Canada. The female patients received a combination of CC and hMG for ovarian stimulation, and the dose was ovarian response-dependent.

In the Pattinson, Taylor and Pattinson (1991) study, female smoking status was ascertained in the menstrual cycle prior to the cycle of IVF treatment, and included the number of cigarettes smoked per day. Data on smoking exposure was available for 360 of the 447 couples (80.5%). Smokers were defined as having at least one cigarette per day, otherwise classified as non-smokers. One-third of patients smoked (34.4%). Smokers and non-smokers did not differ significantly in term of age, mean 33.1 (SD 3.8) and 32.6 (SD 2.9) years, respectively (P>0.05). Tubal infertility was more prevalent among the female smokers than non-smokers, 93.5% vs 80.0% (P<0.001).

Pattinson, Taylor and Pattinson (1991) examined oocyte production, fertilisation, pregnancy (not defined) and spontaneous abortion. In comparing smokers and non-smokers, neither the mean number of oocytes retrieved (5.1 (SD 2.6) vs 5.4 (SD 3.2); P>0.05), the mean fertilisation rate (65.9% vs 68.5%; P>0.05), nor the pregnancy rate (15.3% vs 19.2%; P>0.05) differed significantly. The incidence of spontaneous abortion, however, was higher among the smokers than the non-smokers, 42.1% (8/19) vs 18.9% (10/50), albeit not of statistical significance (P>0.05). As a result, the rate of delivery among smokers was significantly lower than that of non-smokers, 9.6% (11/124) vs 17.0% (40/236; P<0.01).

Pattinson, Taylor and Pattinson (1991) endeavoured to examine the effect of male smoking on pregnancy by classifying couples by their combined smoking status: wife

only smoked (18.1%), husband only smoked (13.6%), both smoked (33.3%), or neither smoked (35.0%). Pregnancy rates (values derived from figure 3, pp 782) for these respective groups are ~13%, ~28.5%, ~15% and ~20%, differences not being of significance ($P>0.05$). Delivery rates (values derived from figure 3, pp 782) for these same groups are ~7.5%, ~20%, ~8% and ~16%, respectively. As compared to the 'neither smoked' couples, the delivery rate of the 'wife only smoked' and 'both smoked' couples was significantly lower ($P<0.01$). The authors concluded that the husband's smoking habit did not have an independent effect on either the rate of pregnancy or delivery: a conclusion that is not without question.

In the following year, a matched retrospective cohort study by Van Voorhis et al. (1992) was reported. It involved female patients who had undergone IVF at an Iowa University clinic during 1990 or 1991. Eighteen smokers were matched with 36 non-smokers by age (±3 years) and weight (±10%). GnRH agonist was used for pituitary suppression and an ovarian response-dependent dose of FSH and hMG combination was used for follicle stimulation.

In the Van Voorhis et al. (1992) study, female smoking status was ascertained at the first consultation at the clinic. By definition, a smoker consumed ten or more cigarettes per day for at least the previous year. Non-smokers were those who did not smoke nor did their male partner. The age of smokers and non-smokers was similar, mean 32.6 and 32.3 years ($P>0.05$). As compared to non-smokers, tubal infertility was higher among the smokers, 55% vs 31%, and male factor infertility was lower, 22% vs 36%. The statistical significance of these differences in aetiology was not reported. The quantity of FSH and hMG (combined) administered to smokers and non-smokers was comparable, mean ampoules 32 and 31, respectively ($P>0.05$).

Van Voorhis et al. (1992) examined oocyte production, fertilisation and pregnancy. Significantly fewer oocytes on average were retrieved from the smokers than the non-smokers, mean 13.4 (*SD 4.7*) vs 16.4 (*SD 7.8*); $P<0.05$). Fertilisation was expressed as

the proportion of fertilised oocytes. The fertilisation rate of oocytes smokers was similar to that of non-smokers, 78.6% vs 76.6% ($P>0.05$; number of fertilised oocytes/total number of oocytes by smoking group was not reported). In turn, smokers had fewer fertilised oocytes than non-smokers, mean 8.2 (SD 5.1) vs 10.4 (SD 4.8; $P<0.05$). Seven of the 18 smokers and 16 of the 36 non-smokers achieved a clinical pregnancy, 39% vs 44% ($P>0.05$).

A laboratory-based study by Rosevear et al. (1992) involved 45 female patients who were randomly selected from all patients who had had an IVF treatment between 1989 and 1991 at St Michael's Hospital, Bristol, United Kingdom. All patients were younger than 40 years of age, and had either tubal factor or unexplained infertility with normal ovulatory cycles and a normal post-coital test result. Follicle stimulation was achieved with CC and gonadotrophins.

Rosevear et al. (1992) classified patients into smoking categories based on cotinine levels in previously collected and stored samples of follicular fluid. Cotinine is the principal metabolite of nicotine, and is a reliable measure of tobacco smoking exposure (Benowitz 1983). Thirteen of the 45 women (28.9%) had detectable levels of cotinine (lower limit of detection was 20ng/ml), and were classified as smokers. Although the median age of 31 years (range 22-40) was reported for the total sample of patients, the average age of the women in each of the smoking groups was not.

In the Rosevear et al. (1992) study, fertilisation was the clinical outcome of interest, defined as pronucleus formation and progressive cleavage up to the time of embryo transfer (2-3 days post-insemination). Only mature oocytes, those with a well expanded cumulus and a clearly visible radiata were used to calculate the rates of fertilisation. Forty-five oocytes had been collected from the 13 smokers (3.5 per woman) and 116 oocytes from the 32 non-smokers (3.6 per woman). Only 44% of the 45 oocytes from the smokers fertilised, which was significantly lower than 72% of the 116 oocytes that fertilised from the non-smokers ($P<0.01$). Accordingly, the median fertilisation rate was

significantly lower among the female smokers (57%, range 0-100%) than that of the non-smokers (75%, range 0-100%; $P<0.05$).

In the same year, a retrospective study by Rowlands, McDermott and Hull (1992) was published. It involved 71 couples who had had their first IVF treatment cycle at the University of Bristol IVF Unit. Study inclusion was restricted to females younger than 40 years of age who had normal ovulatory cycles and a male partner with normal spermatozoa. There was a 2:1 ratio of non-smoking to smoking females.

Rowlands, McDermott and Hull (1992) determined smoking status in retrospect from patient records. Among the 71 couples, in 15 only the female smoked (21.1%), in 13 only the male smoked (18.3%), in 13 both smoked (18.3%) and in the remaining 30 neither smoked (42.3%). Female age was lowest among the 'female only smoked' couples (mean 30.6 years), which compared with a mean female age of 33.3, 31.3 and 31.8 years among the 'male only smoked', 'both smoked' and 'neither smoked' couples. The statistical significance of these mean age differences was not reported.

In the Rowlands, McDermott and Hull (1992) study, fertilisation was the principal clinical outcome of interest, although oocyte production and pregnancy were also reported. The median number of oocytes retrieved was 14 (range 6-26) for the 'female only smoked' couples, 6 (range 1-11) for the 'male only smoked' couples, 10 (6-29) for the 'both smoked' couples and 8 (2-24) for the 'neither smoked' couples. The differences were not reported to be of statistical significance. Like Rosevear et al. (1992), fertilisation was defined as both normal pronuclear formation and progressive cleavage 2-3 days post-insemination, and included only mature oocytes. The proportion of oocytes that fertilised from the 'female only smoked', 'male only smoked', 'both smoked' and 'neither smoked' couples was 60.3% (123/204), 58.1% (50/86), 59.8% (93/157) and 69.1% (219/317), respectively. Significant differences were reported between the 'female only smoked' and 'neither smoked' couples ($P<0.05$), and between the 'both smoked' and 'neither smoked' couples ($P<0.05$). The 'male only smoked' couples had the

lowest clinical pregnancy rate of 15% (2/13), which compared with 27% (4/15) for the 'female only smoked', 31% (4/13) for 'both smoked' and 37% (11/30) for 'neither smoked' couples. Due to the small sample size, statistical significance of these differences was not assessed.

In 1992, Hughes, YoungLai and Ward (1992) reported a prospective cohort study involving 222 couples who had undertaken 297 IVF treatment at Chedoke-McMaster Hospitals between March 1990 and May 1991. In a subsequent publication, Hughes et al. (1994) reported a prospective cohort study that involved 462 treatment cycles undertaken by 316 couples at Chedoke-McMaster Hospitals between March 1990 and April 1992. It was inferred that the study in the latter publication was an extension of the study in the former, and that couples in the original study were also included in the subsequent study that was published. Therefore, only the study in the latter publication is included in this review. In this study, Hughes et al. (1994) attempted to control for confounding factors by using multivariate statistical methods, the first in this field in which to do so.

In the Hughes et al. (1994) study, data on cigarette smoking were self-reported and collected by questionnaire at the commencement of the treatment cycle and at embryo transfer (ET). Of the 316 patients who undertook the 462 IVF treatment cycles, 30.4% were active smokers (30.9% of the cycles), 32.0% were ex-smokers (32.9% of the cycles), and 37.7% had never smoked (36.3% of the cycles). Patients in the smoking groups did not differ significantly in terms of age; the mean ages of smokers, ex-smokers and never smokers were 33.5 (SD 3.3), 34.3 (SD 4.2) and 34.3 (SD 2.9) years, respectively ($P>0.05$). Smokers consumed the most coffee beverages: mean cups per day for smokers, ex-smokers and never smokers were 3.2 (SD 2.4), 2.0 (SD 1.9) and 1.5 (SD 1.6), respectively, ($P<0.05$). Female smokers' males partners also had significantly higher levels of cigarette consumption than the male partners of the female ex-smokers and never smokers, mean cig/day of 13.5 (SD 12.5), 3.9 (SD 8.4) and 3.7 (SD 8.5), respectively ($P<0.001$). hMG administration for follicle stimulation was shorter in

duration and less in quantity for smokers than ex-smokers and never smokers; mean 9.2 (SD 2.7), 10.4 (SD 6.7) and 10.2 (3.0) days, respectively ($P<0.05$), and mean ampoules administered 19.8 (SD 10.8), 22.8 (SD 13.1) and 24.7 (SD 12.5), respectively ($P<0.05$).

Validation of self-reported smoking status was undertaken by Hughes et al. (1994) in a sub-sample of 52 active smokers and 52 age-matched never smokers. Serum cotinine was measured in the first and last samples of blood taken during each treatment cycle. Among the smokers, a moderate correlation was shown between the number of cigarettes smoked and levels of serum cotinine ($r = 0.68$).

Hughes et al. (1994) examined oocyte production, fertilisation and clinical pregnancy. Clinical pregnancy was defined as ultrasonographic evidence of foetal heart activity ≥28 days post-ET or histologic evidence of chorionic villi after spontaneous abortion or ectopic pregnancy. The number of oocytes retrieved from the smokers, ex-smokers and never smokers was 6.6 (SD 3.8), 7.0 (SD 4.8) and 5.8 (SD 3.7), respectively ($P>0.05$). For these groups, the mean fertilisation rate were 65.7% (SD 37.0), 58.9% (36.4) and 64.3% (36.3), respectively ($P>0.05$). Similar to the results for oocyte production and fertilisation, clinical pregnancy rates also did not differ significantly between the groups. Clinical pregnancy rates for the smokers, ex-smokers and never smokers were 11.3% (13/115), 13.9% (23/165) and 13.7% (25/182), respectively, ($P>0.05$).

Hughes et al. (1994) used logistic regression analysis to examine the effect of male and female smoking on clinical pregnancy. The smoking variable was expressed and examined in a number of different formats: as ever or never smoked; current, past or never smoked; cigarettes per day; pack per year; and early follicular phase cotinine level. None of these options produced results that differed markedly to that of the smoking variable coded as active and non-smoker. Covariates included in the regression analysis were female age, female caffeine consumption, duration of infertility, and treatment cycle number. The interactive effect of female smoking and female age was also examined. Of all the variables investigated, including female and male smoking, only

female age was significant in relation to clinical pregnancy, with the likelihood decreasing with female age ($P<0.05$).

Sharara et al. (1994) reported a novel study in which the objective was to determine whether the reduced fecundity of female smokers could be attributed to the accelerated development of diminished ovarian reserve (DOR). In part one of this two-part study, the incidence of DOR among female smokers and non-smokers was compared. The data were acquired retrospectively from a sample of infertile patients who had previously participated in a clinical trial that evaluated the clomiphene citrate (CC) challenge test as a measure of DOR. This trial had been undertaken at a military tertiary care centre, Maryland, USA, between 1991 and 1992. All of the 210 women had tubal factor infertility and were aged between 35 and 39 years of age, this being the typical age for DOR onset (Scott et al., 1993; cited in Sharara et al., 1994). Patients were classified as either smokers or non-smokers (those who had never smoked), and ex-smokers were excluded. Sixty-five of the 210 women were smokers (31.0%). The mean age of smokers was not significantly different to that of non-smokers, 37.0 (SD 1.1) vs 36.9 years (SD 1.3; $P>0.05$).

Sharara et al. (1994) determined DOR status using the CC challenge test. Serum E_2 and FSH levels were measured on menstrual day 3 (basal), and day 10 after the administration of CC on day 5 and 9. DOR was confirmed if either basal or day 10 FSH concentration exceeded 10IU/L (Scott et al., 1993; cited in Sharara et al., 1994). Basal or day 10 E_2 levels did not differ significantly between the smokers and non-smokers, nor did basal FSH levels. By contrast, smokers had a significantly higher mean day 10 FSH level than the non-smokers (7.9 (SD 0.4) vs 6.8 (SD 0.3) IU/L; $P<0.05$). Therefore, the incidence of DOR was significantly higher among the smokers than the non-smokers, 12.3% (8/65) vs 4.8% (7/145); $P<0.05$). An odds ratio (OR) of 2.8 (95% confidence interval (CI) 1.2 - 8.0) indicated that smokers aged 35-39 were almost three times more likely than non-smokers to have a diminished ovarian reserve.

In the second part of the Sharara et al. (1994) study, the hypothesis that female smokers with no evidence of DOR have similar clinical outcomes following IVF treatment to that of non-smokers with no evidence of DOR was tested. Data were obtained retrospectively from a sample of female IVF patients who had also been treated at the same military tertiary care centre in Maryland, USA. All 102 patients were aged between 35 and 39 years, had tubal factor infertility, and had no evidence of DOR, as confirmed by a CC challenge test administered within one year of the IVF treatment cycle. Gonadotrophins were administered for follicle stimulation. Twenty-nine of the 102 patients were smokers (28.4%). The mean age of smokers (37.8 (SD 1.1)) and non-smokers (37.2 (SD 1.3)) did not differ significantly ($P>0.05$), nor did the mean ampoules of gonadotrophins that were administered, 28.4 (SD 1.1) and 29.3 (SD 0.6), respectively ($P>0.05$).

Sharara et al. (1994) examined oocyte production, fertilisation and pregnancy (not defined). Of these, no differences of significance were evident between the smokers and non-smokers. Specifically, the mean number of oocytes of the smokers and non-smokers was 8.4 (SD 4.2) and 8.6 (SD 4.7), respectively ($P>0.05$). The mean rate of fertilisation was 78% (SD 11) and 82% (SD 14), respectively ($P>0.05$). Eight of the 29 smokers and 21 of the 73 non-smokers achieved a pregnancy (not defined), 27.6% vs 28.8% ($P>0.05$). These data support their hypothesis of similar outcomes between the two groups: smokers and non-smokers, all of which have no evidence of DOR.

A retrospective study by Maximovich and Beyler (1995) included 253 female patients who had IVF treatment between January 1992 and September 1993 at the Beaumont Fertility Center, Michigan. All received GnRH agonist and hMG for follicle stimulation. Data on smoking was obtained by questionnaire at the time of entry into the IVF programme. Forty-three of the 253 patients were classified as smokers (17.0%) and the remaining 210 were non-smokers (83.0%). The mean age of the smokers and non-smokers did not differ significantly, 36.3 (SD 4.5) vs 35.5 (SD 4.4) years ($P>0.05$). Nor

did smokers and non-smokers differ in terms of the mean ampoules of hMG administered (mean and SD values not quoted; $P>0.05$).

Maximovich and Beyler (1995) focused on pregnancy and pregnancy loss as the clinical outcomes of interest. As it was not specifically stated, it was inferred that pregnancy referred to a positive β-hCG pregnancy test at approximately two weeks post -ET. And, by convention, an ongoing pregnancy is that at 20 weeks gestation. Whilst β-hCG pregnancy rates of the smokers and non-smokers did not differ, 34.9% (15/43) vs 31.0% (65/210; $P>0.05$), smokers had significantly lower rates of ongoing pregnancy, 9.3% (4/43) vs 23.3% (49/210; $P<0.05$). Such disparity was a result of the significantly higher rate of pregnancy loss among the smokers, 73.3% (11/15) vs 24.6% (16/65; $P<0.001$). In comparing pregnant smokers who did and did not experience a pregnancy loss, the mean number of cigarettes smoked per day was not significantly different (mean (SD) values not quoted; $P>0.05$). Of interest, the mean weeks of pregnancy at the time of loss was 7.1 weeks (range 5.5-11.0) for the smokers and 8 weeks (range 5.5-13.0) for the non-smokers ($P>0.05$).

In 1995, Zenzes, Wang and Casper (1995) investigated the effect of cigarette smoking on meiotic oocyte maturation. Unfertilised oocytes were obtained from 156 female patients who partook in an IVF programme in Ontario, Canada. For follicle stimulation, patients received ovarian response-dependent doses of 1) CC and hMG, 2) FSH and hMG, or 3) GnRHa and hMG. Of the 156 patients, 21.2% were smokers (12.2% light smokers (<15 cig/day) and 9.0% heavy smokers (≥15 cig/day), 13.5% were passive smokers (female non-smoker, male smoker). Patients in these groups did not differ significantly in terms of age ($P>0.05$). Smoking was associated with tubal factor infertility ($P=0.025$). Indicative of a smoking-related dose effect, 63.7% of non-smokers, 76.2% of passive smokers, 89.6% of light smokers and 92.9% of heavy smokers had tubal factor infertility.

In the Zenzes, Wang and Casper (1995) study, oocyte maturity, determined by cytogenetic analysis, was the principal clinical outcome, although oocyte production and fertilisation were also investigated. Mean number of oocytes retrieved from the non-smokers and passive, light and heavy smokers were 10.0 (SD 6.7), 9.5 (SD 3.5), 8.0 (SD 3.7) and 7.3 (SD 3.8; $P>0.05$), respectively. Whilst not significant, there was a trend of fewer oocytes with increasing levels of smoking exposure. Due to the patient selection criteria, the overall fertilisation rate was low, 39.1% (577/1475. Fertilisation rates for non-smokers and passive, light and heavy smokers were 37.0% (378/1022), 47.0% (94/200), 46.4% (70/151) and 34.3 (35/102), respectively ($P<0.01$). Although fertilisation was associated with female smoking status, the relationship was not exposure-dependent.

In the following year, Gustafson, Nylund and Carlström (1996) reported a study in which female IVF patients were compared based on smoking status. A total of 253 IVF treatment cycles had been undertaken during the period October 1985 to January 1989 at the Huddinge University Hospital in Sweden. From these patient records, data from 50 female smokers (median age 33 years, range 25-37) and 50 female non-smokers (median age 34 years, range 25-37) was acquired retrospectively, including data on smoking habits. The selection process was not detailed. However, for inclusion in the study, the treatment cycle had to result in at least one cleaved and transferred embryo and at least one unfertilised oocyte, and smokers consumed ≥10 cig/day (median 14, range 10-60). All had regular spontaneous ovulatory cycles and a male partner who had produced at least one normal spermatozoa sample. Follicle stimulation was with CC and hMG, a fixed daily dose with duration ovarian response-dependent.

Gustafson, Nylund and Carlström (1996) examined oocyte production, fertilisation and pregnancy. The latter was defined as a sonographically verified foetal heart activity. The median number of oocytes retrieved from smokers and non-smokers did not differ, 6 (range 2-15) vs 5 (range 2-28), respectively ($P>0.05$). The median fertilisation rate for smokers was lower than that of non-smokers, 57% (range 11-89%) vs 68% (range 17-

84%), albeit non-significant ($P>0.05$). The pregnancy rate, however, was significantly lower for the smokers than the non-smokers, 10% vs 36% ($P<0.01$).

In 1996, a prospective cohort study by Sterzik et al. (1996) was reported. It involved 197 female patients who had undergone IVF treatment at the Women's University Hospital of the University of Ulm, Germany. All were younger than 40 years of age, had tubal factor infertility and a male partner with a normal spermatozoa sample. hMG was administered for follicle stimulation. Smoking exposure was determined from the cotinine levels of follicular fluid collected during oocyte retrieval. Female patients were classified as either active smokers (cotinine concentration >50ng/mL), passive smokers (20≤50 ng/mL) or non-smokers (≤20ng/mL). Of the 197 women, 52.3% were active smokers, 13.2% were passive smokers, and only 34.5% were non-smokers. Age did not differ significantly between the active, passive and non-smokers, with mean ages of 32.4 (SD 4.3), 32.7 (SD 4.4) and 32.5 (SD 4.1) years, respectively ($P>0.05$). Similarly, hMG administration did not differ between active, passive and non-smokers: mean ampoules of 22 (SD 3.1), 22 (SD 3.5) and 21 (SD 3.2), respectively ($P>0.05$).

Sterzik et al. (1996) examined oocyte production, fertilisation and clinical pregnancy. The mean number of oocytes retrieved from active, passive and non-smokers was comparable, 5.5 (SD 2.7), 5.4 (SD 2.9) and 5.4 (SD 3.3), respectively ($P>0.05$). Although not specifically stated, it was inferred that fertilisation was expressed as the proportion of oocytes that fertilised from the total pool of oocytes retrieved from women within each smoking group. Oocytes from passive smokers had the lowest rate of fertilisation at 57.7% as compared to active and non-smokers at 67.9% and 67.6% ($P>0.05$). Clinical pregnancy rates for active, passive and non-smokers were similar, 32.9% (23/103), 33.3% (5/26) and 32.6% (15/68), respectively ($P>0.05$).

Also in 1996, a further study by Van Voorhis et al. (1996) was reported. This retrospective cohort study involved female patients who had their first attempt at either IVF, GIFT or zygote intra-Fallopian transfer (ZIFT) between January 1989 and July

1994 at the University of Iowa. No age restriction applied. For follicle stimulation, all received GnRH agonist, FSH and hMG, with dose ovarian-response dependent.

Van Voorhis et al. (1996) ascertained smoking status retrospectively. Seven hundred and eighty-nine female patients were mailed a questionnaire. A second questionnaire was sent to those who did not respond to the first. The response rate was 78% (613/789). Excluding cycles involving donor oocytes, 499 of these 613 women had a treatment cycle that proceeded to a transfer, either ET, GIFT or ZIFT. Only 7.4% of these patients were smokers, 22.3% had smoked in the past and 70.3% had never smoked. Female smokers were on average younger than the past and non-smokers, mean age 31.5 (SD 3.6), 33.8 (SD 3.6) and 32.9 (SD 4.2) years, respectively (P=0.004). As compared to 34% of non-smokers, a significantly higher proportion of smokers and past smokers had tubal disease, 62% and 57%, respectively (P<0.001). This, in part, accounted for the significantly higher proportion of female smokers (70%) and past smokers (63%) that had treatment by IVF (vs GIFT and ZIFT) as compared to 41% of non-smokers (P<0.001).

Van Voorhis et al. (1996) examined oocyte production, fertilisation and pregnancy. Oocyte production included 1) total number of oocytes retrieved and 2) number of mature oocytes (metaphase I and II). The number of oocytes retrieved did not differ between smokers, past smokers and non-smokers: means of 14.0 (SD 6.2), 15.1 (SD 9.7) and 15.4 (SD 9.0), respectively (P>0.05). No differences of significance were evident between the mean fertilisation rate for smokers, past smokers and non-smokers of 49.9% (SD 25.4), 54.1% (SD 24.0) and 54.1% (SD 25.0), respectively (P>0.05). Statistical adjustment for age was undertaken, but differences in oocyte production and fertilisation between the female smoking groups remained non-significant.

Van Voorhis et al. (1996) also calculated and used cigarette pack-years as a measure of smoking exposure. Adjusting for age, oocyte production decreased with cigarette-pack years (P<0.05). Specifically, 2.5 fewer mature oocytes on average were retrieved for

every 10 pack-years of cigarette smoking. Smokers experienced significantly lower rates of pregnancy. For smokers, past and non-smokers, clinical pregnancy rates were 21.6%, 37.8% and 40.2% ($P<0.05$), and ongoing pregnancy rates were 16.2%, 33.3% and 35.3% ($P<0.05$), respectively. Age-adjusted clinical and ongoing pregnancy odds ratios for smokers (as compared to past and non-smokers collectively) were 0.38 (95% CI 0.17-0.86) and 0.32 (95% CI 0.13-0.79).

A further study by Zenzes, Reed and Casper (1997) investigating the effect of smoking on oocyte maturity and fertilisation was reported. This study involved 234 female patients who had an IVF treatment cycle between June 1995 and April 1996 within a hospital-based IVF programme in Ontario, Canada. Either hMG or FSH was used for follicle stimulation, the dose being ovarian response-dependent.

Zenzes, Reed and Casper (1997) used as the measure of smoking exposure cotinine levels in follicular fluid samples collected at the time of oocyte retrieval. Due to non-normality, cotinine values were logarithm (to base 10) transformed (log cotinine). Female patients were also classified by self-reported smoking status: 31.6% were active smokers (female smoker; male smoker or non-smoker), 12.8% passive smokers (female non-smoker; male smoker) and 55.6% non-smokers (female non-smoker; male non-smoker). Differences in the mean ages of these three groups were not significant 33.0 (SE 0.4), 33.9 (SE 0.8) and 34.4 years (SE 0.3), respectively ($P>0.05$).

In an examination of oocyte production, Zenzes, Reed and Casper (1997) considered both the total and mature number of oocytes retrieved. A mature oocyte was defined as an expanded cumulus and well dispersed radiating corona, evenly distributed around the oocyte. With or without age in the regression analysis, follicular fluid log cotinine did not correlate with the total number of oocytes aspirated, nor with the number of mature oocytes.

Zenzes, Casper and Reed (1997) examined in detail the effect of female smoking on fertilisation. After excluding 30 couples with male factor infertility, data on women <35 years and ≥35 years of age were analysed separately. Within each age stratum, patients were categorised by log follicular fluid cotinine concentration, <-0.4, -0.4 to1.2 and >1.2. Chi square analysis was used to determine the significance of differences in the percentage of the oocytes that fertilised from women within each log follicular fluid cotinine stratum. Significant differences were identified among the women aged ≥35 years, but not those aged <35 years. Among the women aged ≥35 years, the proportion of oocytes that fertilised within the <-0.4, -0.4 to 1.2 and >1.2 log follicular fluid cotinine strata were 67.5% (226/335), 61.3% (174/284) and 78.1% (121/155), respectively (P=0.0016).

Zenzes, Casper and Reed (1997) also examined fertilisation by regressing the individual proportion of oocytes that fertilised, weighted by the number of oocytes retrieved, on age and the follicular fluid log cotinine levels. Both female age and log follicular fluid cotinine level entered positively into the regression equation, P<0.0001 and P=0.0065, respectively. In effect, after adjusting for female age, fertilisation increased with female smoking exposure. The interactive effect of these two factors, female age and log follicular fluid cotinine levels, on fertilisation was examined, but was not significant.

In a meta-analysis reported by Feichtinger et al. (1997) previously unpublished data on 541 couples was presented. All had undertaken IVF treatment for the first time at an Austrian medical institute during 1996 to 1997. Data from these 541 patients were examined retrospectively, including smoking status. At least one in four of the female patients in this sample smoked, 26.3% (142/541). The clinical pregnancy rate of smokers and non-smokers was not statistically different, 28% (40/102) and 32% (126/399), respectively (P>0.05).

A preliminary study by the Investigator to the study presented here was published in the following year (Joesbury et al. 1998; Appendix B). In this retrospective cohort, data

from 465 IVF treatment cycles from 385 couples was collected between January 1994 and December 1995 at PIVET Medical Centre in Perth, Western Australia. The sample of patients included in this previous study was different to that sampled in this current study.

In this previous study, male and female smoking status were ascertained at the initial consultation at the clinic (Joesbury et al. 1998). Patients were classified as either smokers or non-smokers, the latter of which include ex- and never smokers. Smokers accounted for 15.9% of female patients and 20.3% of the males. Female smoking was associated with male smoking ($P<0.001$), with over one-half of the female smokers (56.5%) having a male partner who also smoked. Female smokers were younger than non-smokers, with mean ages of 33.1 (SD 4.7) and 34.6 (SD 4.7) years, respectively ($P<0.05$). A similar proportion of male smokers and non-smokers had intra-cytoplasmic sperm injection (ICSI) for fertilisation.

Joesbury et al. (1998) examined oocyte production, fertilisation and pregnancy. The mean number of oocytes retrieved from the smokers and non-smokers was not significantly different, 13.1 (SD 7.7) vs 12.2 (SD 6.8; $P>0.05$). Of significance, the age-related reduction in oocyte production was more pronounced in smokers than non-smokers ($P<0.05$). For every one year increase in age, oocyte production decreased by 0.75 (95% CI 0.40-1.10) oocytes among smokers, and by 0.55 (95% CI 0.42-0.68) oocytes among non-smokers. In relation to fertilisation, the mean proportion of oocytes that fertilised among female smokers and non-smokers was comparable, 0.60 (SD 0.2) vs 0.61 (SD 0.2; $P>0.05$), as was that between male smokers and non-smokers, 0.61 (SD 0.2) vs 0.61 (SD 0.2; $P>0.05$).

Multiple logistic regression analysis was undertaken in this previous study to examine the effect of female and male smoking on a 12-week ongoing pregnancy (Joesbury et al. 1998). Covariates in the regression analysis included embryo quality (modified cumulative embryo score (mCES)), female and male age, ICSI, female tubal factor

infertility and endometrial grade and thickness. Embryo quality, female tubal factor infertility and male smoking, but not female smoking, were shown to significantly affect the likelihood of a 12-week pregnancy ($P<0.05$). On further examination, a significant interaction between male smoking and male age on 12-week pregnancy was found. More specifically, the chance of a 12-week pregnancy among partners of male smokers decreased by 2.4% for every one-year increase in male age. No such effect of male age was apparent among male non-smokers. When clinical pregnancy was the outcome (dependent variable in the multiple logistic regression analysis), no adverse effect of male smoking was evident.

A retrospective cohort by El-Nemr et al. (1998) was also reported in 1998. It included data from 173 consecutive IVF treatment cycles undertaken between June 1995 and February 1996 at the Royal Hospitals Trust Fertility Centre, London. Treatment cycles that involved ICSI were not included. Ovarian follicle stimulation was with either hMG or FSH, the dose being ovarian response-dependent.

In the El-Nemr et al. (1998) study, female smoking status was ascertained at the first consultation. Women were classified as either a smoker or a non-smoker. Sixty-five of the 173 cycles investigated involved women who were smokers (37.6%). The mean age of female smokers and non-smokers did not differ significantly, 32.3 (SD 4.5) and 33.2 (SD 4.5) years, respectively ($P>0.05$). The smokers mean serum basal FSH level (menstrual cycle day 2-4) was significantly higher than that of the non-smokers, 7.5 (SD 2.9) vs 4.7 (SD 1.9) IU/l ($P<0.0001$), as was the mean ampoules of gonadotrophins administered for follicle stimulation, 48.1 (SD 15.6) vs 38.9 (SD 13.6; $P<0.0001$).

The clinical outcomes examined by El-Nemr et al. (1998) included oocyte production, fertilisation and pregnancy. Female smokers had significantly fewer oocytes retrieved than non-smokers, mean 6.2 (SD 3.4) vs 11.1 (SD 6.3) oocytes ($P<0.0001$). A higher proportion of the smokers than the non-smokers experienced complete failure of all

oocytes (failed fertilisation), 18.5% vs 8.3% (P=0.1). Eleven of the 65 female smokers (16.9%) and 23 of the 108 non-smokers (21.3%) attained a clinical pregnancy (P>0.05).

El-Nemr et al. (1998) repeated the data analysis only among couples undertaking their first IVF cycle: 101 of the original 173 cycles (58.4%). In approximately one-third of these 101 couples (32.7%), the female was a smoker. The significance of differences between female smokers and non-smokers was not dissimilar to that reported among all 173 cycles. One exception was the high proportion of smokers with an elevated basal FSH level (>8IU/l), 36.4% as compared to only 7.4% among non-smokers (P=0.001).

In the following year, a study by Weigert et al. (1999) was reported. Data from 834 female patients who had an IVF treatment cycle at a Viennese institute, Austria were examined retrospectively. Patients had one of three different follicle stimulation regimes 1) CC and hMG, 2) GnRHa and FSH ("flare-up") or 3) GnRHa and FSH ("down-regulation"). Based on self-reported smoking habits ascertained by questionnaire at the commencement of treatment, 24.0% of the female patients were classified as smokers and 76.0% as non-smokers. Female smokers were significantly younger than non-smokers, 33.3 (SD 4.9) vs 34.2 (SD 5.3) years (P=0.0195).

Weigert et al. (1999) examined oocyte production, fertilisation and pregnancy. Female smokers and non-smokers did not differ significantly in terms of the number of oocytes retrieved, 6.4 (SD 3.7) vs 7.0 (SD 4.1; P>0.05), nor the number of fertilised oocytes, 4.3 (SD 2.8) vs 3.9 (SD 2.6; P>0.05). Although smokers had a slightly lower rate of pregnancy (not defined) than the non-smokers, 24.3% vs 30.6%, the difference was not statistically significant (P>0.05).

More recently, a prospective cohort study by Klonoff-Cohen, et al. (2001) was reported. It involved 221 couples who underwent either IVF or GIFT during July 1993 to June 1998 at one of several centres in Los Angeles, Orange and San Diego. The selection criterion was relatively non-restrictive, and as a result, the authors' report that the sample

is representative of couples enrolling in IVF and GIFT programmes in Southern California.

Klonoff-Cohen et al. (2001) ascertained both male and female smoking status/history by questionnaires at various times throughout treatment: initial consultation (males and females), ET and the final pregnancy (females) and semen collection (males). Of the 221 females, 50.2% had never smoked, 47.1% had smoked during their lifetime or still do, and smoking status/history of the remaining 2.7% was unknown. Only 9% of women smoked within the year prior to the IVF or GIFT treatment cycle, 7% within the month, and <4% within the week or during the cycle. Fifty-five of the 221 males refused to answer questions regarding lifestyle. Of the 166 male respondents, 42.1% had never smoked, 42.5% had smoked in their lifetime or still do, and in 15.4% smoking status/history was unknown. In relation to the IVF or GIFT treatment, only 7% of males smoked the year before, 5% the month before, and 6% during the week or day prior, or during the procedure.

Couples were also defined by Klonoff-Cohen et al. (2001) based on their combined smoking status/history of both partners (couple smoking status). A smoking couple was defined as either one or both partners smoking at a given point in time or at any time over a specified period (eg, lifetime, within one year, month, week or day of or during treatment). For couples, 62% had ever smoked in their lifetime, 22% never smoked, and this information was unknown for 16%. In relation to the treatment of IVF or GIFT, 15% of couples smoked the year before, 11% smoked the month before, and <9% during the week or day prior, or during the procedure.

Klonoff-Cohen et al. (2001) reported the effect of female, male and couple smoking on oocyte production and pregnancy. In relation to oocyte production, female patients who smoked during treatment had a 45% reduction in the number of oocytes retrieved (log coefficient = -0.62, P=0.01). Clinical pregnancy was defined as elevated serum hCG levels and an ultrasound verified gestational sac, and excluded ectopic and biochemical

pregnancies. Relative to female non-smokers, the adjusted-relative risk of not achieving a pregnancy among patients had ever smoked was 2.7 (95% CI 1.4 - 5.4), and the risk increased by 9.0% per every one year that she smoked (smoke-year; RR =1.09, 95% CI 1.01-1.16). The adjusted-RR of no pregnancy for female patients who smoked >5 years was 4.9 (95% 1.8-13.3). Relative to non-smoking couples, the adjusted-RR of no pregnancy was 2.4 (95% CI 1.1-5.4) for smoking couples, and 4.3 (95% CI 1.5-12.0) for couples whom smoked >5 years. Male smoking, independent to that of the female, was not shown statistically to affect the likelihood of a pregnancy outcome.

Also in 2001, Crha et al. (2001) reported a prospective cohort study that involved 159 couples who undertook IVF treatment at the Centre for Assisted Reproduction in the Faculty Hospital of the 1st Dept of Gynaecology and Obstetrics in Brno, Czech Republic between 1997 and 1999. Female patients received hMG and FSH for follicle stimulation, with doses varying between 150-225IU per day.

In the Crha et al. (2001) study, female smoking status was self-reported by questionnaire, which patients completed at the initial examination prior to IVF treatment. It also included questions regarding smoking history, partner's smoking status and exposure to passive smoke in the home and workplace. Urinary cotinine levels were measured for verification of self-reported smoking status. Of the 159 women, 24 were smokers (2-20 cig/day; 15.1%), 16 were occasional smokers (0-1 cig/day; 10.1%), 29 were ex-smokers (18.2%) and 90 were non-smokers (56.6%). Female and male smoking status were associated; nearly 70% of female smokers' male partners smoked as compared to only one-third of female non-smokers' male partners.

Female patients in the Crha et al. (2001) study were younger on average than those involved in former studies, with a mean of 29.4 (SD 4.3) years. Based on mean values, the age of women in the different smoking groups did not differ significantly (specific values not reported). Nor did the total mean ampoules of FSH required for follicle

stimulation differ between smokers, ex-smokers and non-smokers, 32.2 (SD 14.5), 28.5 (SD 13.9) and 31.5 (SD 14.4), respectively (P>0.05).

Crha et al. (2001) examined oocyte production, fertilisation and pregnancy. Oocyte production was examined by the mean number of oocytes retrieved and by the proportion of >15mm ovarian follicles eliciting an oocyte. The latter being age stratified by women ≤35 and >35 years of age. Female smokers had a lower mean number of oocytes retrieved than non-smokers, 7.3 (SD 4.8) vs 10.9 (SD 5.6), but the difference was not statistically significant (P>0.05). Among women >35 years of age, the percentage of smokers' follicles that yielded an oocyte was significantly less on average than that of never smokers and ex-smokers (P<0.05; values not specified). No such difference was evident between the smoking groups among women aged ≤35 years.

Fertilisation was examined by Crha et al. (2001) in a number of ways: mean fertilisation rate, the proportion of couples who experienced failed fertilisation (0% fertilisation) and the proportion whose rate of fertilisation was ≥60%. Female smokers had a significantly lower mean fertilisation rate than non-smokers, 47.8 (SD 40.3)% vs 68.2 (SD 33.2%; P<0.01). Only 38.2% of female smokers achieved a ≥60% fertilisation rate as compared to 68.0% of ex-smokers and 60.8% of non-smokers. Accordingly, a significantly higher proportion of female smokers experienced failed fertilisation as compared to rates of 2.0% and 1.3% among the ex-smokers and non-smokers, respectively (P<0.01). Although differences were not found to be statistical significant, pregnancy (not defined) rates for the smokers, occasional smokers, ex-smokers and never smokers were 0%, 12.5%, 15.4% and 28.8%, respectively.

Although the relationship between exposure to tobacco smoke and oxidative stress markers was the focus of the study by Paszkowski, Clarke and Hornstein (2002), oocyte production was also reported. In total, 108 women underwent IVF for infertility due to male or tubal factor infertility at the Brigham and Women's Hospital IVF Program in

Boston, USA. All patients underwent a standard controlled ovarian stimulation with hMG and GnRH.

Cotinine levels in follicular fluid (FF-C) were used as an index of the tobacco smoke exposure. Paszkowski, Clarke and Hornstein (2002) applied the same criteria reported by Sterzik et al. (1996): non-smoker, FF-C <20ng/ml); passive smoker, FF-C 20-50ng/ml; active smoker, FF-C >50ng/ml. The age of women in these three groups did not differ significantly, with median years of 36 (range 25-39), 35 (range 26-38) and 37 (range 24-39), respectively ($P>0.05$). However, active smokers had significantly few oocytes retrieved (median 8, range 2-27) as compared to passive smokers (median 12, range 2-52) and non-smokers (median 16, range 7-38; $P=0.010$). Of interest, evidence of oxidative stress increased ($P<0.001$) and total antioxidative potential decreased ($P=0.004$) in follicular fluid with increasing levels of cotinine.

Appendix B

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Evidence that male smoking affects the likelihood of a pregnancy following IVF treatment: application of the modified cumulative embryo score

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Female cigarette smoking has been implicated as having a detrimental effect on in-vitro fertilization (IVF) outcomes mediated through: (i) a diminished ovarian reserve (DOR), and (ii) an elevated pregnancy loss. Research is sparse regarding the effect of male smoking. The objective of this retrospective cohort study was to investigate the effect of male and female smoking on: (i) the collective quality of embryos selected for uterine transfer, and (ii) the likelihood of achieving an ongoing pregnancy at 12 weeks. A total of 498 consecutive IVF treatment cycles were analysed. Female smokers were significantly younger ($P < 0.05$) and achieved a better modified cumulative embryo score (mCES) ($P < 0.05$) than female non-smokers. Female age correlated inversely with the number of oocytes collected ($r = -0.42$, $P < 0.01$) and the number of oocytes in turn was important in terms of predicting mCES. The decreasing number of oocytes aspirated with increasing age was of a significantly stronger magnitude for female smokers than for female non-smokers ($P < 0.05$). Multiple logistic regression was used to determine whether smoking affected the likelihood of achieving a 12-week pregnancy. The mCES, tubal infertility and male smoking were found to be significant. Male smoking interacted with male age ($P = 0.0164$), indicating for male smokers a decrease of 2.4% in the likelihood of achieving a 12-week pregnancy with every 1-year increase in age. This is the first study to show that male smoking has a deleterious effect on pregnancy outcome among IVF patients. Our study supports the increased risk of DOR but fails to support the elevated incidence of pregnancy loss among female smokers. A reduced pregnancy rate was associated with male smoking possibly through pre-zygotic genetic damage. The growing realization of a paternal component of reproductive impairment suggests that studying the male is necessary.

Key words: embryo quality/IVF/mutagenicity/pregnancy loss/smoking

Introduction

The impact of smoking on the clinical outcomes of in-vitro fertilization (IVF) treatment has yet to be resolved. Collectively the studies indicate that female smoking has a small but significant detrimental effect (Hughes and Brennan, 1996). The evidence implicates two mechanisms whereby female smoking may exert this effect: a diminished ovarian reserve resulting in the production of fewer oocytes (Sharara *et al.*, 1994; Van Voorhis *et al.*, 1996), and an elevated incidence of pregnancy loss (Harrison *et al.*, 1990; Pattinson *et al.*, 1991; Maximovich and Beyler, 1995). Research is sparse regarding the effect of male smoking but what is available suggests that male smoking does not significantly affect IVF outcomes (Pattinson *et al.*, 1991; Hughes *et al.*, 1994).

The objective of this study was to investigate the effect of male and female smoking on: (i) the collective quality of embryos selected for uterine transfer, and (ii) the likelihood of achieving an ongoing pregnancy at 12 weeks.

Materials and methods

This retrospective cohort study includes 498 consecutive IVF treatment cycles from 385 couples performed between January 1994 and December 1995. The data were obtained from patient clinical outcome records and patient files. In-vitro fertilization–embryo transfer (IVF–embryo transfer) and intracytoplasmic sperm injection–embryo transfer (ICSI–embryo transfer) cycles were included in the study. Treatment cycles involving donor sperm, donor oocytes or donor embryos were excluded.

Smoking status

Female and male smoking status was obtained from the patients' files, which was typically recorded at the patient's first consultation at the clinic. Smoking status was coded for analysis as 'smoker' or 'non-smoker'. Non-smokers included 'never' and 'ex-smokers'. The average number of cigarettes smoked per day was missing for a high proportion of the patients recognized as smokers and this precluded the use of such a smoking exposure variable in the data analysis.

Female patients received subcutaneous injections of gonadotrophin-releasing hormone (GnRH) analogue, leuprolide acetate (Lucrin; Abbott, Kurnell, NSW, Australia), 1 mg (20 IU) daily, either from day 21 of the previous menstrual cycle (pituitary down-regulation) or from day 1 of the treatment cycle ('flare' effect). Human menopausal gonadotrophin (Pergonal; Serono, Frenchs Forest, NSW) was given to all the female patients from day 3 of the treatment cycle, with an increase in the dosage depending on the oestradiol rise. Ovulation was triggered with 10 000 IU of human chorionic gonadotrophin (HCG) and oocytes were aspirated transvaginally by ultrasound guidance 35 h after the HCG trigger.

Oocytes and embryos were cultured in human tubal fluid medium supplemented with 10% heat-inactivated patient's serum. Insemination was carried out 4–6 h after oocyte recovery with 50 000–100 000 spermatozoa/ml. The presence of two pronuclei 18–20 h after insemination confirmed that normal fertilization had occurred.

The texture and thickness of the endometrium was assessed on the

day of or within 1 or 2 days of the HCG trigger by transvaginal ultrasound. The maximum thickness of the endometrium on both sides of the midline was measured in the plane through the central longitudinal axis of the uterine body (Gonen and Casper, 1990). The endometrium was classified as: A, an entirely homogeneous, hyperechogenic endometrium; B, an intermediate type characterized by the same reflectivity of ultrasound as the myometrium, with a non-prominent or absent central echogenic line; or C, a multilayered endometrium consisting of prominent outer and midline hyperechogenic lines and inner hypoechogenic regions (Gonen and Casper, 1990).

Embryo quality

Embryos were graded on the second day following insemination just prior to transfer. Grading was based on granularity and symmetry of the blastomeres, fragmentation and rate of development. A hypothetically perfect embryo was graded a maximum of 4.0 points with 0.5 or 1.0 point deducted in accordance with the degree of deviation from the optimum for each morphological parameter (Yovich and Lower, 1991). The highest scoring embryos were selected for uterine transfer.

The collective quality of embryos selected for transfer was based on the cumulative embryo score (CES) devised by Steer *et al.* (1992), which entailed multiplying the grade of each embryo selected for transfer by the number of cells and then summing these values. However, embryos developing faster than the 'ideal' growth rate (Trounson *et al.*, 1982; Cummins *et al.*, 1986) have been shown to have pregnancy outcomes that are poorer than embryos that exhibit a 'normal' growth pattern (Cummins *et al.*, 1986; Giorgetti *et al.*, 1995). To account for the potential inferiority of fast-developing embryos, we modified the manner in which the CES was calculated. For embryos at the 5-cell stage, we awarded 3 points for number of cells and for 6-, 7-, or 8-cell embryos, we awarded 2 points for number of cells. This modified cumulative embryo score (mCES) was used as a measure of the collective quality of the embryos selected for uterine transfer.

Pregnancy outcome

The principal pregnancy outcome variable was an ongoing pregnancy at 12 weeks, which included pregnancies that had reached 12 weeks of gestation from the commencement of the last menstrual period and excluded any prior pregnancy losses including biochemical, blighted ovum, ectopic pregnancy and spontaneous miscarriage. Other pregnancy outcomes examined were a positive β-HCG pregnancy test, clinical pregnancy and a live birth. A positive β-HCG test was characterized by an elevated serum β-HCG level on or after day 16 of the luteal phase, with a significant rise at least 3 days thereafter (Yovich *et al.*, 1986). The assay for β-HCG had a minimum detection level of 2.5 IU/l and all levels of 25 IU/l were standardized against the 2nd International Standard (61/6). Serum oestradiol and progesterone were also required to be in the appropriate pregnancy range of >550 pmol/l and >37 nmol/l, respectively. A clinical pregnancy was defined as ultrasonographic evidence of fetal heart activity or chorionic villi evidence after a spontaneous abortion or an ectopic pregnancy. A live birth included all pregnancies with at least one live birth of which an infant was alive one month post-delivery.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) for Windows (Release 7.0). The treatment cycle was treated statistically as an independent event. We acknowledge that this practice is open to question and needs to be addressed in future studies involving IVF patients. Independent sample *t*-tests were used to compare the means of two groups. The Pearson χ^2 was used to test the difference between two proportions and between two categorical variables. The Student's *t*-test was used to compare the difference between the simple linear regression coefficients (Zar, 1974). To test the effect of multiple factors on a continuous outcome variable, multiple linear regression was applied. Logistic regression was used to investigate the effect of multiple factors on pregnancy outcomes. Backward elimination was conducted to first construct a model which comprised only the significant independent variables. For each of these variables, interaction terms were constructed from all the original independent variables. Each interaction term was examined in the final model in a forward fashion.

Results

Of the 498 treatment cycles, smoking status was ascertained for 465 of the females (93.4%) and for 422 of the males (84.6%). Based on this, 76 of the female patients (15.9%) and 101 of the male patients (20.3%) were smokers. Smoking status was determined for 415 of the couples (83.3%): for 287 couples both partners were non-smokers (69.2%), for 30 couples only the female smoked (7.2%), for 59 couples only the male smoked (11.8%), and for 39 couples both partners were smokers (9.4%). With regard to the number of embryos transferred, 82.7% of cycles involved the transfer of three embryos, 11.6% two embryos, 3.2% one embryo, and in 2.4% of treatment cycles, four embryos were transferred.

Smokers versus non-smokers

Female smokers were compared with female non-smokers in terms of age, serum oestradiol levels, number of oocytes retrieved, proportion of oocytes fertilized and mCES (Table I). Female smokers were shown to be significantly ($P < 0.05$) younger than female non-smokers by an average of 18 months. No significant differences were demonstrated among the other female factors examined. For males, age, fertilization rates and mCES were not significantly different among smokers and non-smokers.

Differences in infertility aetiology were examined by smoking status and sex (Table II). The difference in the proportion of tubal infertility cases among the female smokers and non-smokers was not of significance. The proportion of ICSI cycles undertaken by male smokers was not significantly different from that of male non-smokers. No difference was noted with regard to the grade or thickness of the endometrium between female smokers and female non-smokers (Table II). Female smoking was significantly associated with male smoking ($\chi^2 = 49.68$, df 1, $P < 0.001$) with over half of the female smokers (56.5%) having a male partner that smoked.

Smoking and the mCES

The number of oocytes retrieved, the percentage of oocytes that fertilized, the number of embryos transferred, female smoking and the treatment cycle number were shown to be significant predictors of the mCES (Table III). The adjusted R^2 was 45.0% which shows that almost half of the variability in the mCES was explained by the collective variability of the significant independent variables in the final model.

The β-coefficient for the number of oocytes retrieved was 0.34 [95% confidence interval (CI): 0.24–0.43] indicating that,

Table I. Continuous variables, mean ± SD by sex and smoking status

	Variable	Smokers	Non-smokers	Total
Female	Age (years)	33.1 ± 4.7[a]	34.6 ± 4.7	34.1 ± 4.7
	Serum oestradiol level (pmol/l)[b]	9250 ± 4143	9019 ± 4162	9039 ± 4124
	No. of oocytes retrieved	13.1 ± 7.7	12.2 ± 6.8	12.3 ± 6.7
	Individual proportion of oocytes fertilized	0.60 ± 0.2	0.61 ± 0.2	0.60 ± 0.2
	mCES	29.7 ± 9.1	27.4 ± 9.0	27.6 ± 9.3
	Number	74	391	465
Male	Age (in years)	36.2 ± 5.7	36.5 ± 6.3	36.6 ± 6.1
	Individual proportion of oocytes fertilized	0.61 ± 0.2	0.61 ± 0.2	0.60 ± 0.2
	mCES	28.5 ± 8.1	26.9 ± 9.3	27.6 ± 9.3
	Number	101	321	422

[a]$P < 0.05$ level.
[b]On the day of or within 1 day of the human chorionic gonadotrophin administration/trigger.
mCES = modified cumulative embryo score.

Table II. Categorical variables and distribution by sex and smoking status

	Variable	Levels (%)	Smokers (%)	Non-smokers (%)	Total
Female	Female tubal infertility aetiology	Tubal only	28.4	25.6	26.0
	Grade of endometrium	A	21.7	19.4	19.8
		B	71.0	68.7	69.0
		C	7.2	12.0	11.2
	Thickness of endometrium	<10 mm	20.0	23.6	23.0
		10–12 mm	55.7	53.8	54.2
		13+ mm	24.3	22.5	22.8
Male	Type of treatment cycle	ICSI	22.8	24.9	24.4

ICSI = intracytoplasmic sperm injection.

Table III. Final multiple linear regression model evaluating the factors affecting the mCES[a]

Variable parameters in final model		Regression coefficient	95% CI (LCL, UCL)	Significance (*P*-value)
No. of oocytes retrieved		0.34	0.24, 0.43	<0.001
Proportion of oocytes that fertilized		10.55	7.56, 13.53	<0.001
No. of embryos transferred:	1 vs 3	–20.2	–23.7, –16.7	<0.001
	2 vs 3	–8.5	–10.6, –6.5	<0.001
	4 vs 3	6.0	2.0, 10.0	0.003
Female smoking (no/yes)		2.38	0.68, 4.09	0.006
Treatment cycle no.		–0.47	–0.04, –0.91	0.034

[a]Covariates available to the model included female age (years), male age (years), in-vitro fertilization (IVF)–embryo transfer versus IVF-embryo transfer-intracytoplasmic sperm injection treatment cycles, female diagnosis of tubal infertility only versus all other conditions including tubal plus an additional condition, number of oocytes retrieved, proportion of oocytes that fertilized, treatment cycle number, day of human chorionic gonadotrophin trigger, female smoking status (non-smokers versus smokers), male smoking status (non-smokers versus smokers) and number of embryos transferred.
mCES = modified cumulative embryo score; CI = confidence interval; LCL = lower confidence limit; UCL = upper confidence limit.

for every three additional oocytes collected, there was an incremental increase in the mCES of approximately 1 unit when all other variables were held constant. The β-coefficient for the proportion of oocytes fertilized was 10.55 (95% CI: 7.56–13.53) demonstrating that, for every 10% of additional oocytes fertilized, there was on average an increase in the mCES of 1 point. There was an inverse relationship between the mCES and number of treatment cycles in that, on average, there was a decrease of ~0.5 points in the mCES for every additional treatment cycle attempted. The number of embryos transferred significantly affected the mCES score. In relation to a transfer of three embryos, the mCES of a two-embryo transfer was on average 8.5 points lower, and a one-embryo transfer was on average 20.2 points lower. By contrast, a transfer of four embryos was on average 6.0 points higher than a three-embryo transfer. This interpretation assumes that

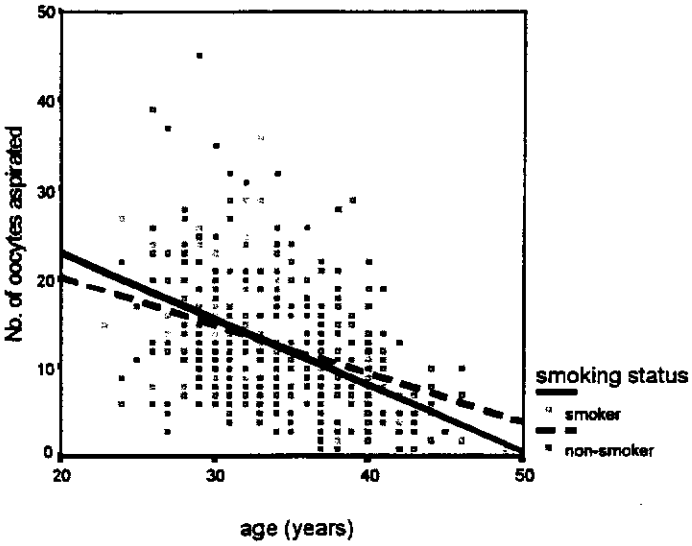

Figure 1. Relationship between female age and number of oocytes aspirated by smoking status.

the number of embryos transferred is independent of embryo quality. In practice, this may not be the case as the number of embryos selected for transfer may be dependent upon the quality of the embryos.

Female smokers on average had a mCES which was 2.4 (95% CI: 0.7–4.1) points higher than that of female non-smokers. Male smoking status was shown to be of no significance in relation to the mCES. Also of note was the non-significant effect of female age on the mCES. On further investigation it was demonstrated that female age was inversely correlated with number of oocytes collected ($r = -0.42$, $P < 0.01$). This relationship was of a stronger magnitude for female smokers than for female non-smokers (Figure 1). Simple linear regression analysis for female smokers and non-smokers was performed to assess the relationship between age and number of oocytes retrieved. The regression coefficient represents the linear relationship between female age and number of oocytes retrieved (the slope of the line). The regression coefficient for the female smokers was shown to differ significantly from that of the non-smokers ($P < 0.05$). For female smokers, there was a significant ($P < 0.001$) reduction of 0.75 (95% CI: –0.40 to –1.10) oocytes per 1-year increase in age compared with 0.55 (95% CI: –0.42 to –0.68) oocytes per 1-year increase in age for female non-smokers ($P < 0.001$).

The crude pregnancy rates for positive β-HCG pregnancy test, ongoing pregnancy at 12 weeks and live birth by smoking status are shown in Figure 2. For males and females, no significant difference ($P > 0.05$) between the pregnancy rates of smokers and non-smokers was evident.

Smoking and pregnancy

The mCES, tubal infertility and male smoking were shown to be significant factors in relation to the likelihood of achieving an ongoing pregnancy of 12 weeks (Table IV). Moreover, male smoking was shown to interact with male age. The final logistic model was as follows:

$$P = 1/1 + \exp^{-(u)}$$

where $u = -3.7719 + (0.0742 \times \text{mCES}) + (0.6294 \times \text{tubal}) - (0.0247 \times \text{mas})$; P = probability of a pregnancy at ≥12 weeks; mCES = modified cumulative embryo score; tubal = female tubal infertility aetiology (1 when the reason for female infertility was only tubal, and 0 when the reason for female infertility was 'other' including tubal infertility plus an additional reason); and mas = male age×male smoking (1 when the male was a 'smoker' and 0 when the male was a 'non-smoker').

The odds ratio for mCES was 1.077 (95% CI: 1.040–1.115), which indicates that, for an increase of 1 point in the mCES, the probability of pregnancy increases by 8% when all other factors are held constant. For women with tubal infertility, the odds ratio of achieving a 12-week pregnancy was 1.876 (95% CI: 1.052–3.346). Therefore, women with tubal infertility are nearly two times more likely to reach a 12-week pregnancy than women with any other classification of infertility.

The odds ratio for the interaction between male smoking and male age was 0.976 (95% CI: 0.956–0.9955). This means that, for couples where the male partner is a smoker, for every 1-year increase in the age of the male, there is a decrease of 2.4% in the likelihood of achieving a 12-week pregnancy. In other words, a 31-year-old male smoker's partner has a likelihood of achieving a 12-week pregnancy which is 97.6% of that of a 30-year-old male, or a 40-year-old male smoker's partner is 24% less likely to obtain a 12-week pregnancy than a 30-year-old male smoker's partner. For male non-smokers, male age does not affect the likelihood of their partner achieving a 12-week pregnancy.

Logistic regression analysis was repeated using clinical pregnancy as the dependent variable. The mCES and tubal infertility were found to be significant ($P < 0.001$ and $P = 0.0057$, respectively), however, male and female smoking were non-significant. Female smoking was removed from the model with a P-value of 0.99 and male smoking with a P-value of 0.54.

Discussion

Smoking and the mCES

We modified the CES to account for the potential inferiority of fast-developing embryos. The mCES was shown to be significantly associated with female but not male smoking. Female smokers experienced the transfer of significantly better-quality embryos than female non-smokers by an average of 2.4 points. However, female smokers were significantly younger ($P < 0.05$) than their non-smoking counterparts. In clinical practice, a proportion of treatment cycles are cancelled prior to embryo transfer. Approximately one-third of the 3093 treatment cycles commenced in the study by Haan *et al.* (1991) failed to result in an embryo transfer. It is possible that female smokers are over-represented among these cancelled cycles, especially older women who have long-term smoking histories. This age discrepancy supports our hypothesis that older female smokers are less likely to achieve an embryo transfer and are under-represented in our study population.

Cigarette smoking appears to interfere with ovarian follicular

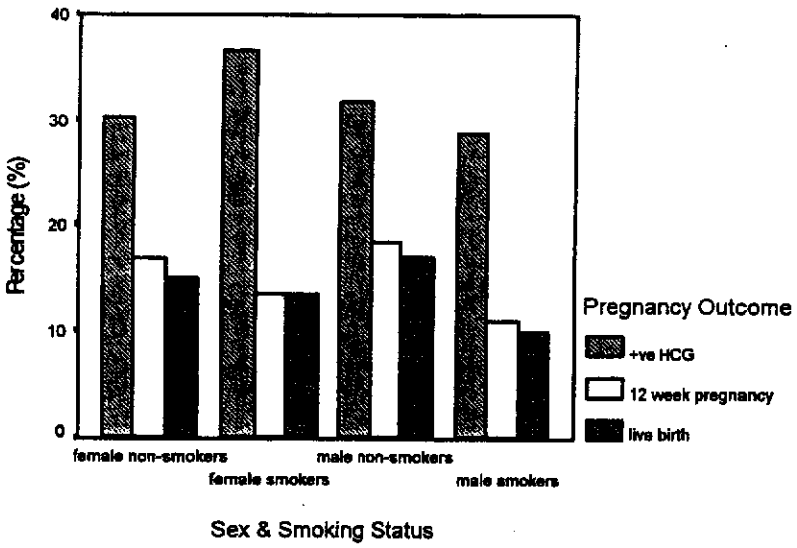

Figure 2. Crude pregnancy rates by sex and smoking status.

Table IV. Final logistic regression model evaluating the factors affecting 12-week ongoing pregnancy

Variable parameters in final model	Odds ratio	95% CI (LCL, UCL)	Significance (*P*-value)
mCES	1.077	1.040, 1.115	<0.0001
Female tubal infertility only (no/yes)	1.876	1.052, 3.346	0.0329
Male smoking (no/yes) by male age (years)	0.976	0.956, 0.996	0.0164

Covariates available to the model included mCES, female age (years), male age (years), in-vitro fertilization (IVF)–embryo transfer versus IVF–embryo transfer-intracytoplasmic sperm injection treatment cycles, female diagnosis of tubal infertility only versus all other conditions including tubal plus an additional condition, oestradiol levels on day of human chorionic gonadotrophin (HCG) trigger or within 1 day of HCG trigger, vascular grade of endometrium (A, B, C), endometrium thickness (<10 mm, 10–12 mm, ⩾13 mm), female smoking status (non-smokers versus smokers), and male smoking status (non-smokers versus smokers). mCES = modified cumulative embryo score; CI = confidence interval; LCL = lower confidence limit, UCL = upper confidence limit.

maturation and oocyte production, as female smokers tend to produce fewer oocytes than female non-smokers (Harrison *et al.*, 1990; Van Voorhis *et al.*, 1992). Van Voorhis *et al.* (1996) showed that for every 10-pack-years of cigarette smoking, 2.5 fewer mature oocytes and two less embryos were obtained from female smokers. Pack-years were defined as the number of packs of cigarettes smoked per day times the number of years a women smoked. Sharara *et al.* (1994) reported that the incidence of diminished ovarian reserve (DOR) is higher among female smokers than non-smokers. DOR is characterized by elevated levels of follicle stimulating hormone resulting in a decreased ovarian response (Navot *et al.*, 1987). Consistent with these findings, we demonstrated that the decline in the number of oocytes with age occurs at a significantly faster rate among smokers than non-smokers. For female smokers, there was a reduction in oocytes of 0.75 (95% CI: 0.4–1.1) for every 1-year increase in age compared with a reduction of 0.55 (95% CI: 0.4–0.7) per year for non-smokers. Our study, like these other studies, supports the association between female smoking and early menopause (Jick *et al.*, 1977).

Smoking and pregnancy

When investigating the effect of smoking on pregnancy outcome, it is important to consider possible biological mechanisms that may account for an effect and to choose a pregnancy outcome accordingly. It has been postulated that cigarette smoke may interfere with the DNA of the gametes and in turn may affect the developing embryos (Wyrobek, 1993). We selected ongoing pregnancies at 12 weeks as the pregnancy outcome, as over 90% of the pregnancy losses in our sample occurred within the first 12 weeks after the last menstrual period. Therefore, a pregnancy that reaches the 12-week stage has a good chance of proceeding to a live birth.

Female smoking was not shown to reduce the likelihood of achieving a 12-week pregnancy. However, others have shown a reduction in crude pregnancy rates among female non-smokers compared with those of female non-smokers (Harrison *et al.*, 1990; Pattinson *et al.*, 1991). The findings of these studies are difficult to interpret as crude pregnancy rates do not take into account important group differences such as age, embryo quality and infertility status. A few other studies have used multivariate analysis techniques to assess the effects of smoking. A cohort study involving 499 female IVF patients reported a reduction of 50% in the ongoing pregnancy rate of female smokers in relation to female non-smokers (Van Voorhis *et al.*, 1996). By contrast, Hughes *et al.* (1994) found that neither male nor female smoking affected the likelihood of a clinical pregnancy. The issue as to whether female smoking

impacts on pregnancy outcome, in addition to the alleged reduction of oocytes produced, needs further clarification.

Male smoking was shown to have a significant negative effect on pregnancy outcome, and the effect was dependent on the age of the male smoker. For male smokers, every 1-year increase in age was associated with a reduction of 2.4% in the probability of their partner achieving a 12-week pregnancy. It is plausible that male age is a surrogate measure of duration of smoking exposure in that it is an indirect measure of the number of years the male has been a smoker. This finding suggests that there is a dose response relationship between male smoking and pregnancy outcome, thus supporting the potential causality of the relationship.

To the authors' knowledge, this is the first study to implicate male smoking as having a detrimental effect on pregnancy outcome following IVF treatment. The smoking status of the male partner was not controlled for in the study by Van Voorhis *et al.* (1996), who reported a 50% reduction in the pregnancy rate of female smokers. We found, as did Hughes *et al.* (1994), that male smoking was associated with female smoking and this potentially confounding effect cannot be eliminated as an explanation for the observed difference in pregnancy rates between female smokers and non-smokers in the Van Voorhis *et al.* (1996) study. Hughes *et al.* (1994) reported that male smoking did not affect the rate of clinical pregnancies. A limitation of their study was the choice of clinical pregnancies as the pregnancy outcome. Pregnancy losses following clinical pregnancy detection may be linked to male smoking. We found that male smoking was not significant in relation to clinical pregnancies. This raises the suggestion that male smoking may be associated with pregnancy loss following early clinical detection of a pregnancy.

Biological plausibility

Over 60 chemical compounds have been identified in tobacco smoke which are carcinogenic and/or mutagenic (Bos and Henderson, 1984). Cotinine, a tobacco specific metabolite, has been detected in the follicular fluid of female smokers (Weiss and Eckert, 1989; Zenzes *et al.*, 1996). More recently, cotinine was shown to accumulate in the maturing follicles and to interact with and incorporate into the follicular cells (Zenzes *et al.*, 1997). Extracts of cigarette smoke have been shown to inhibit the conversion of androstenedione to oestradiol in cultures of human granulosa cells (Barbieri *et al.*, 1986). This in part may explain the higher incidence of immature oocytes observed among female smokers (Zenzes *et al.*, 1995). Diploid oocytes are immature and possibly result from prevention of the first polar body extrusion (Edirisinghe *et al.*, 1992; Zenzes *et al.*, 1995) or as a result of oocyte arrest at syngamy after sperm entry (Edirisinghe *et al.*, 1992). Whilst Zenzes *et al.* (1995) found that female smokers were more likely to produce immature diploid oocytes, they were unable to show any difference in the occurrence of other cytogenetic abnormalities, specifically aneuploid (19–22 and 24–27) and haploid (23) chromosome complements among female smokers. A high proportion of abortuses exhibit chromosomal abnormalities (Plachot, 1989; Yovich and Lower, 1991) of which aneuploidy has been identified as being the most common (Hassold *et al.*, 1980).

Chemical components of tobacco smoke may induce alkylation of DNA in the oocyte (Bos *et al.*, 1989). Bos *et al* (1989) used the *Salmonella* microsome assay to compare follicular fluid mutagenicity of female smokers and female non-smokers. They were unable to show that the mutagenicity differed significantly even though control samples of urine did show marked elevation of mutagenicity among the female smokers. They concluded that a measurable amount of tobacco smoke mutagens is absent in follicular fluid, possibly due to the difficulty mutagens have of reaching and/or penetrating the protective surrounding layers of the follicle, theca interna, basement membrane and granulosa cells.

The opportunities for mutation to occur in the maternal germ cells differ from those of the paternal germ cells. In males, spermatogenesis occurs from adolescence to an advanced age resulting in a prolonged 'window' of exposure (Little and Vainio, 1994). By contrast, the opportunities for mutation to occur in maternal germ cells may be restricted by the fact that the germ cells are almost fully mature in the ovaries of newborn females (Little and Vainio, 1994) and remain so until fertilization. Therefore, mutant genes are less likely to accumulate in the female than in the male (Cavalli-Sforza and Bodmer, 1971). The interaction between male smoking and age observed in our study lends support to this theory that mutations may arise in the paternal gametes during the preconceptional period and accumulate with time (Little and Vainio, 1994).

Our view is consistent with Wyrobek (1993) in that male reproductive health is related to reproductive potential and the health of the resultant offspring. Wyrobek (1993) proposed that there are a number of avenues whereby exposure to toxic agents may impair male reproduction. Paternally transmitted genetic defects, mutations or non-mutational alterations could result in fetal loss or abnormal development *in utero*. Reproductive potential may also be related to the spermatogonial cells' and spermatids' ability to repair DNA lesions (Zenzes, 1995) and to the capacity of the fertilized egg to repair DNA lesions in the spermatozoon (Matsuda and Tobari, 1989; Genesca *et al.*, 1992). The frequency of genetic defects, non-mutational alterations and/or sperm lesions may be related to paternal age, as may the capacity to repair sperm lesions. This in part may account for the reduction in the likelihood of a successful outcome with increasing age of male smokers.

A recent study reported that at least one-quarter of semen samples contain between 5% and 40% of spermatozoa with fragmented DNA, and that male smokers had a significantly higher percentage of DNA-fragmented spermatozoa than non-smokers (Sun *et al.*, 1997). DNA fragmentation was negatively correlated with sperm motility, morphology and count, and was associated with a reduced rate of fertilization and embryo cleavage. By contrast, spermatozoa with DNA anomalies have also been shown to have a similar fertilization potential to that of spermatozoa with normal DNA (Peluso *et al.*, 1992). In our study, the rate of fertilization did not differ between male smokers and male non-smokers, which is consistent with the findings of Peluso *et al.* (1992). This implies that the process

of fertilization is not impaired in spermatozoa with potential tobacco smoke-induced DNA damage. However, if fertilization is initiated by a spermatozoon with defective DNA, it is likely that the resulting zygote would have limited capacity for normal embryonic development. The zygote genome begins to control embryonic development around the 8- to 16-cell stage, and therefore any defect in the DNA will not be evident if the transfer occurs on the second or third day after insemination (Peluso *et al.*, 1992).

Study limitations

We acknowledge that our data on smoking status obtained at the first patient consultation may not accurately reflect cigarette consumption at the time of IVF treatment (Hughes *et al.*, 1992). The average number of daily cigarettes consumed for smokers was not readily available from the patients' files. Misclassification of smoking exposure, as may be the case in our study, is likely to generate results that favour the null hypothesis of no effect. Despite this, a negative effect of male smoking on pregnancy outcome was shown. This implies that the observed risk is an underestimate of the true effect. We are confident that our male smoking variable does represent at least past tobacco exposure even if the male had ceased smoking upon commencing treatment. Given the current public health awareness of the effects of smoking on female reproduction, smoking cessation or reduction is more likely among female patients. It is less likely that male patients will modify their smoking behaviour as little information exists to show that male smoking has a detrimental effect on reproduction, especially in relation to pregnancy loss. Despite failing to demonstrate an effect of female smoking on pregnancy, we cannot eliminate the possibility that misclassification of female smoking status obscured a true negative effect.

Conclusion

Our study supports the increased risk of DOR but fails to support a reduction in the likelihood of a 12-week pregnancy among female smokers. Rather, we found that pregnancy outcome was negatively associated with male smoking. The findings of this study add to a growing body of knowledge that shows that male smoking may cause pre-zygotic DNA damage that in turn is linked to the failure of an embryo/fetus to develop *in utero*. Reproductive problems such as fetal loss have been associated traditionally with the female partner. Consequently, research on the effect of smoking has been focused on women. The increasing awareness of a paternal component of reproductive impairment suggests that studying the male is necessary (Stillman *et al.*, 1986). We are currently undertaking a prospective cohort study investigating the effect of lifestyle factors on the clinical outcomes of IVF treatment. Specific attention is being given to the quantification of tobacco (past and present), caffeine and alcohol consumption of both the male and female partner. We anticipate that this will contribute towards clarifying whether female and male smoking affects the outcomes of IVF, and reproduction in general.

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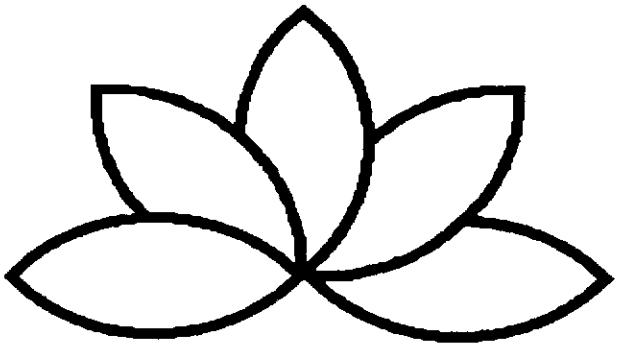
Received on September 12, 1997; accepted on March 17, 1998

Appendix C

Female Lifestyle Questionnaire and Diary



PIVET MEDICAL CENTRE

LIFESTYLE & IVF OUTCOMES STUDY

Female Questionnaire

PIVET Medical Centre
thank you for taking the time and effort
to complete this diary and questionnaire.

Please return this booklet to PIVET
when you have completed the diary.

46148-2-98

For office use only

Reference No._____

If in the event you misplace this document,
please do not hesitate to ask for a replacement.

Please answer each question by placing a tick (√) in the most appropriate box or writing in the space provided.

1. Were you born in Australia? yes ☐1 → go to Qn 5
   no ☐0

2. In which country were you born? _____

3. Are you a permanent resident in Australia? no ☐0 → go to Qn 5
   yes ☐1

4. What year did you arrive in Australia to live? 19____

5. Which one best describes the highest level of education that you have completed?
   - Left high school before year 10 (3rd year) ☐0
   - Completed year 10 (3rd year) of high school ☐1
   - Completed year 12 (5th year) of high school ☐2
   - Certificate, Diploma or TAFE course qualification ☐4
   - Tertiary qualification ☐5

   Other, please specify_____

6. In relation to diet, are you a vegetarian? no ☐0 → go to Qn 8
   yes ☐1

7. Which one best describes your vegetarian diet?
   - semi-vegetarian (no red meat but consume poultry & fish) ☐0
   - lacto-ovo vegetarian (no animal flesh but consume eggs & dairy products) ☐1
   - lacto-vegetarians (no animal flesh and egg but consume dairy products) ☐2
   - vegans (avoid all food of animal origin) ☐3
   - other, please specify_____

8. What is your:
   height _____cms (or ft & inches)
   weight _____kgs (or stones & lbs)

9. Is your workplace 'smoke-free'? yes ☐1 → go to Qn 11
no ☐0

10. Which **one** best describes your workplace?

small office	☐1	small factory	☐3	outdoors	☐0
large office	☐2	large factory	☐4	small shop	☐5
				large shop	☐6

other, please specify_____

11. Is your home 'smoke-free' (in-doors)? yes ☐1 → go to Qn 13
no ☐0

12. Which is correct regarding smoking within your home? (tick **more** than one, if necessary)

one occupant (someone who lives there) is a regular smoker..... ☐1
two occupants are regular smokers..... ☐2
more than two occupants are regular smokers..... ☐3
visitors smoke in my home..... ☐4

The next part of this questionnaire is designed to establish your <u>usual</u> lifestyle behaviours.

13. Do you drink coffee beverages? no ☐0 → go to Qn 20
yes ☐1

The following section is designed to establish the coffee beverage you most frequently drink. This coffee beverage will be termed your <u>usual coffee beverage</u>.

14. Which **one** best describes your **usual coffee beverage**? regular ☐1
decaffeinated ☐0

15. Which **one** best describes the brewing method of your **usual coffee beverage**?

instant ☐1 → go to Qn 16
plunge ☐2 → go to Qn 17
drip/filtered ☐3 → go to Qn 17
percolated ☐4 → go to Qn 17

16. How many teaspoons (tspn) of instant coffee do you usually use per serving of coffee?

½ tspn ☐0 → go to Qn 18
¾ tspn ☐1 → go to Qn 18
1½ tspn ☐4 → go to Qn 18
1 level tspn ☐2 → go to Qn 18
1 heaped tspn ☐3 → go to Qn 18
2 tspn ☐5 → go to Qn 18

other, please specify_____

17. Which **one** best describes how much ground coffee you usually use per serving of coffee?

1 teaspoon ☐1
2 teaspoons ☐2
1 dessert spn ☐3
2 dessert spns ☐4
recommended coffee scoop ☐5
other, please specify_____

18. Which **one** best describes your **usual** coffee drink container?

cup ☐0
mug ☐1

19. Do you **usually** drink most (at least ¾) of the coffee in your cup/mug?

no ☐0
yes ☐1

20. Do you drink **tea** beverages?

no ☐0 → go to Qn 26
yes ☐1

The next section is designed to establish the tea beverage you most frequently drink. This tea beverage will be termed your <u>usual tea beverage</u>.

21. Is there **one** brand of tea that you **usually** drink?

yes ☐1 → Please state which one_____
no ☐0

22. Which **one** best describes the **usual** type of tea beverage that you drink?

tea bags ☐0 instant tea ☐1

brewed tea ☐2 herbal tea ☐3 → Please specify_____

23. Which **one** best describes your **usual** tea beverage? regular ☐1

decaffeinated ☐0

24. Which **one** best describes your **usual tea beverage** drink container? cup ☐0

mug ☐1

25. Do you **usually** drink most (at least ¾) of the tea in your cup/mug? no ☐0

yes ☐1

The following section is designed to determine your smoking history.

26. Which **one** best describes you?

I have never smoked tobacco ☐0 → **go to the end of the page**

I have smoked tobacco in the past ☐1 → go to Qn 27

I smoke tobacco occasionally ☐2 → go to Qn 29

I smoke tobacco regularly ☐3 → go to Qn 29

27. How long ago did you quit smoking?

_____**days or** _____**months or** _____**years**

28. Before you quit, how many years did you smoke? _____**years** → **go to the end of the page**

29. How many years have you smoked? _____**years**

30. Which **cigarette/tobacco brand** do you usually buy?_____

For example: WINFIELD Super Mild or PETER JACKSON Menthol Mild

THE END of the questionnaire
THANK YOU

DIARY

DIARY - DAY 4

Work Commitments

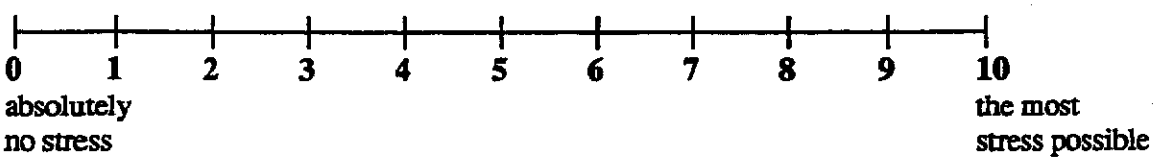
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

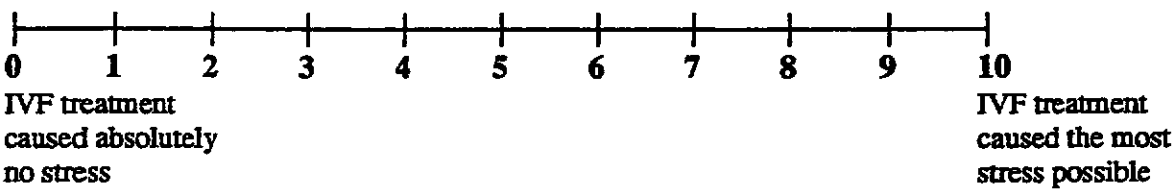
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐$_0$ → go to Qn 16
yes ☐$_1$

14. Did you consume any of your **usual coffee beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

15. Did you consume any other coffee beverages today?
no ☐$_0$ → go to Qn 16
yes ☐$_1$ → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$

17. Did you consume any of your **usual tea beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$ → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (**R**) or decaffeinated (**D**)	Quantity (**mls**) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 5

Work Commitments

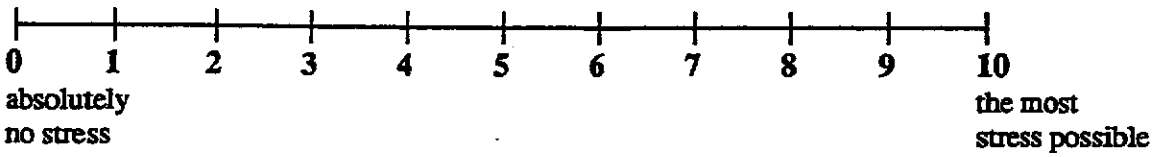
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

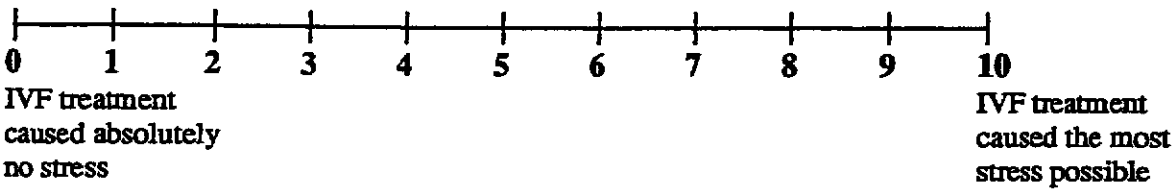
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐0 → go to Qn 6

   yes ☐1 → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐0 → go to Qn 7

   yes ☐1 → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐$_0$ → go to Qn 16
yes ☐$_1$

14. Did you consume any of your **usual coffee beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

15. Did you consume any other coffee beverages today?
no ☐$_0$ → go to Qn 16
yes ☐$_1$ → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$

17. Did you consume any of your **usual tea beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$ → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐$_0$ → go to Qn 20

yes ☐$_1$ → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐$_0$

yes ☐$_1$ → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐$_1$ → How many?_____

no ☐$_0$

22. Did you smoke any other tobacco products today?

no ☐$_0$ → **Please go to the end of the page**

yes ☐$_1$ → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 6

Work Commitments

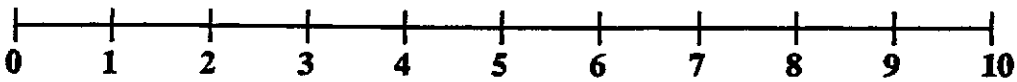
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

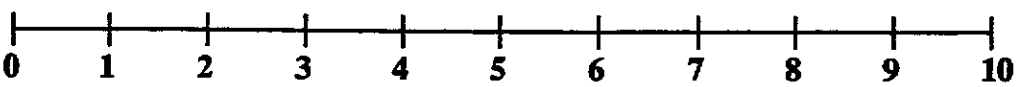
Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



0 absolutely no stress — 10 the most stress possible

4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



0 IVF treatment caused absolutely no stress — 10 IVF treatment caused the most stress possible

5. Did you engage in any activity today for the specific purpose of reducing stress?

no ☐0 → go to Qn 6

yes ☐1 → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

no ☐0 → go to Qn 7

yes ☐1 → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 7

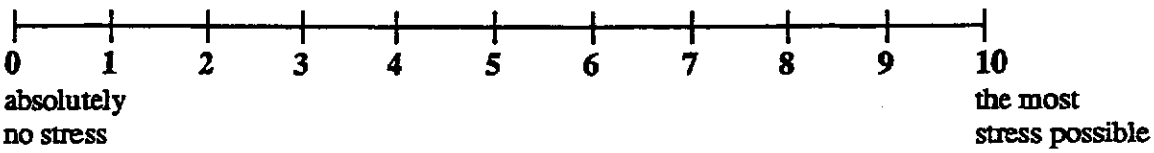
Work Commitments Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

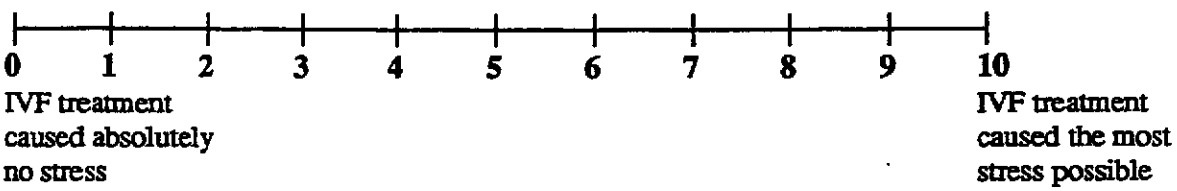
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

no ☐₀ → go to Qn 6

yes ☐₁ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

no ☐₀ → go to Qn 7

yes ☐₁ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 8

Work Commitments

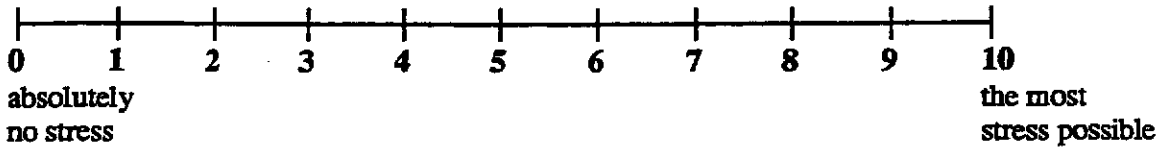
Date ____/____/____

1. How many hours of **paid** work did you do today? _____**hours**

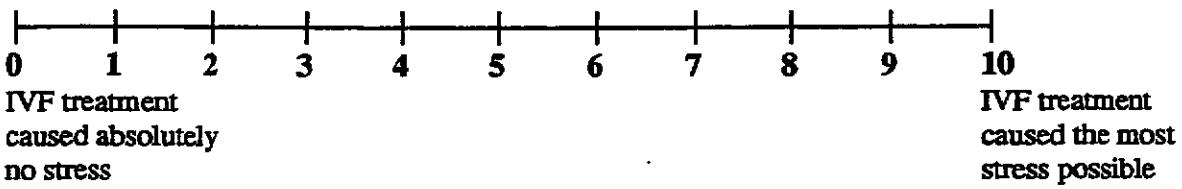
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐₀ → go to Qn 6

   yes ☐₁ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐₀ → go to Qn 7

   yes ☐₁ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Browns Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 9

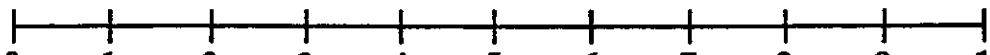
Work Commitments Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

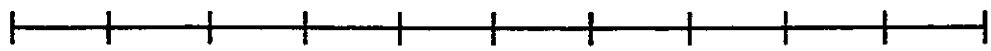
3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



0 1 2 3 4 5 6 7 8 9 10

0 absolutely no stress — 10 the most stress possible

4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



0 1 2 3 4 5 6 7 8 9 10

0 IVF treatment caused absolutely no stress — 10 IVF treatment caused the most stress possible

5. Did you engage in any activity today for the specific purpose of reducing stress?

no ☐0 → go to Qn 6

yes ☐1 → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

no ☐0 → go to Qn 7

yes ☐1 → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → **Please go to the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 10

Work Commitments

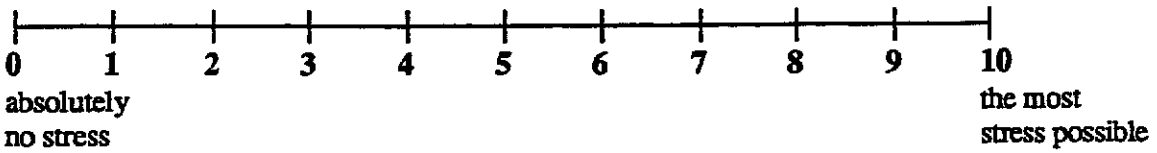
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

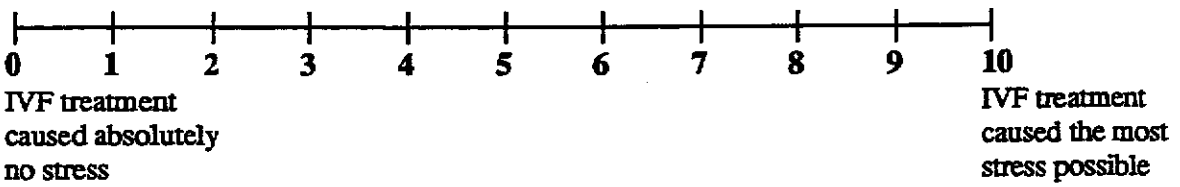
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐0 → go to Qn 6

   yes ☐1 → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐0 → go to Qn 7

   yes ☐1 → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

   no ☐0 → go to Qn 8

   yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

   no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

   no ☐0 → go to Qn 9

   yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

   no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

   yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

   no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

    no ☐0

11. Did you eat any vegetables today?

    yes ☐1 → How many servings (see back page)_____

    no ☐0

12. Did you visit PIVET today?

    yes ☐1 → What time did you arrive at PIVET_____am/pm

    no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced tea**?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐$_0$ → go to Qn 20

yes ☐$_1$ → Please complete the table below:

Brand Name eg. Diet Pepsi or Browns Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐$_0$

yes ☐$_1$ → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐$_1$ → How many?_____

no ☐$_0$

22. Did you smoke any other tobacco products today?

no ☐$_0$ → Please go to **the end of the page**

yes ☐$_1$ → Please complete the table below:

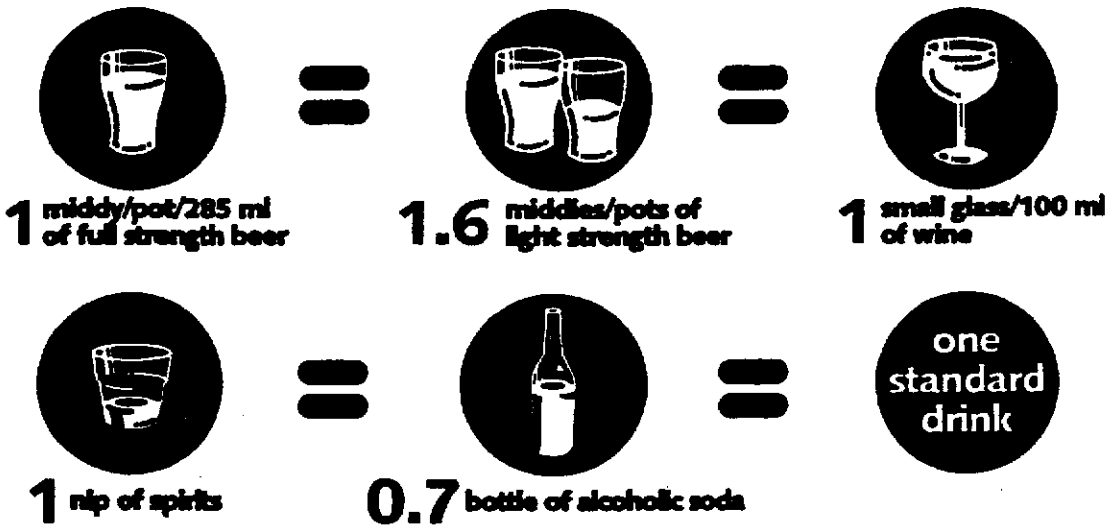
Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of the diary, THANK YOU

We appreciate that it is sometimes difficult to know your exact consumption of alcohol, fruit and vegetable consumption, therefore, we request you provide your best approximation based on the following.

Standard Alcohol Drinks

Any drink containing 10 grams of alcohol is a standard drink. These are standard drinks:



You can also check for standard drink information on bottles and cans.

Fruit

(fresh, frozen, canned)

1 serve =

- 1 medium piece of fruit
- 1 cup of diced pieces
- 3 pieces of smaller sized fruit (apricots, plums, kiwi-fruit, figs)
- ¼ medium sized melon (rockmelon, honeydew)
- 4-6 pieces of dried fruit
- 2tbs sultanas and/or raisins
- 1 cup berries
- 20 grapes
- 10 strawberries

Vegetables

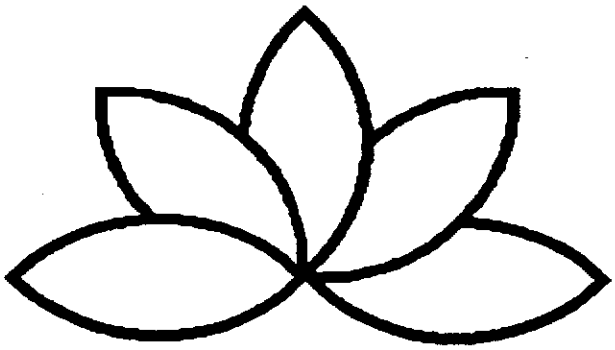
(fresh, frozen, canned)

1 serve =

- 1 small to medium potato
- ½ cup of cooked vegetables
- ½ to 1 cup of salad

Appendix D

Male Lifestyle Questionnaire and Diary



PIVET MEDICAL CENTRE

LIFESTYLE & IVF OUTCOMES STUDY

Male Questionnaire

PIVET Medical Centre
thank you for taking the time and effort
to complete this diary and questionnaire.

Please return this booklet to PIVET
when you have completed the diary.

46147 2-98

For office use only

Reference No._____

If in the event you misplace this document,
please do not hesitate to ask for a replacement.

Please answer each question by placing a tick (√) in the most appropriate box or writing in the space provided.

1. Were you born in Australia? yes ☐1 → go to Qn 5
   no ☐0

2. In which country were you born? _____

3. Are you a permanent resident in Australia? no ☐0 → go to Qn 5
   yes ☐1

4. What year did you arrive in Australia to live? 19____

5. Which **one** best describes the highest level of education that you have completed?

   Left high school before year 10 (3rd year) ☐0
   Completed year 10 (3rd year) of high school ☐1
   Completed year 12 (5th year) of high school ☐2
   Certificate, Diploma or TAFE course qualification ☐4
   Tertiary qualification ☐5

   Other, please specify_____

6. In relation to diet, are you a vegetarian? no ☐0 → go to Qn 8
   yes ☐1

7. Which **one** best describes your **vegetarian diet**?

   semi-vegetarian (no red meat but consume poultry & fish) ☐0
   lacto-ovo vegetarian (no animal flesh but consume eggs & dairy products) ☐1
   lacto-vegetarians (no animal flesh and egg but consume dairy products) ☐2
   vegans (avoid all food of animal origin) ☐3

   other, please specify_____

8. What is your: height _____cms (or ft & inches)

   weight _____kgs (or stones & lbs)

9. Is your workplace 'smoke-free'? yes ☐1 → go to Qn 11
no ☐0

10. Which one best describes your workplace?

				outdoors	☐0
small office	☐1	small factory	☐3	small shop	☐5
large office	☐2	large factory	☐4	large shop	☐6

other, please specify_____

11. Is your home 'smoke-free' (in-doors)? yes ☐1 → go to Qn 13
no ☐0

12. Which is correct regarding smoking within your home? (tick **more** than one, if necessary)

one occupant (someone who lives there) is a regular smoker..... ☐1
two occupants are regular smokers..... ☐2
more than two occupants are regular smokers..... ☐3
visitors smoke in my home..... ☐4

The next part of this questionnaire is designed to establish your <u>usual</u> lifestyle behaviours.

13. Do you drink coffee beverages? no ☐0 → go to Qn 20
yes ☐1

The following section is designed to establish the coffee beverage you most frequently drink. This coffee beverage will be termed your <u>usual coffee beverage</u>.

14. Which one best describes your **usual coffee beverage**? regular ☐1
decaffeinated ☐0

15. Which **one** best describes the brewing method of your **usual coffee beverage**?

instant ☐1 → go to Qn 16
plunge ☐2 → go to Qn 17
drip/filtered ☐3 → go to Qn 17
percolated ☐4 → go to Qn 17

16. How many teaspoons (tspn) of instant coffee do you usually use per serving of coffee?

½ tspn ☐0 → go to Qn 18
¾ tspn ☐1 → go to Qn 18
1½ tspn ☐4 → go to Qn 18
1 level tspn ☐2 → go to Qn 18
1 heaped tspn ☐3 → go to Qn 18
2 tspn ☐5 → go to Qn 18

other, please specify_____

17. Which **one** best describes how much ground coffee you usually use per serving of coffee?

1 teaspoon ☐1
2 teaspoons ☐2
1 dessert spn ☐3
2 dessert spns ☐4
recommended coffee scoop ☐5
other, please specify_____

18. Which **one** best describes your **usual** coffee drink container?

cup ☐0
mug ☐1

19. Do you **usually** drink most (at least ¾) of the coffee in your cup/mug?

no ☐0
yes ☐1

20. Do you drink **tea** beverages?

no ☐0 → go to Qn 26
yes ☐1

The next section is designed to establish the tea beverage you most frequently drink. This tea beverage will be termed your <u>usual tea beverage</u>.

21. Is there **one** brand of tea that you **usually** drink?

yes ☐1 → Please state which one_____
no ☐0

22. Which **one** best describes the **usual** type of tea beverage that you drink?

tea bags ☐0 instant tea ☐1

brewed tea ☐2 herbal tea ☐3 → Please specify_____

23. Which **one** best describes your **usual** tea beverage? regular ☐1

decaffeinated ☐0

24. Which **one** best describes your **usual tea beverage** drink container? cup ☐0

mug ☐1

25. Do you **usually** drink most (at least ¾) of the tea in your cup/mug? no ☐0

yes ☐1

The following section is designed to determine your smoking history.

26. Which **one** best describes you?

I have never smoked tobacco ☐0 → **go to the end of the page**

I have smoked tobacco in the past ☐1 → go to Qn 27

I smoke tobacco occasionally ☐2 → go to Qn 29

I smoke tobacco regularly ☐3 → go to Qn 29

27. How long ago did you quit smoking?

_____**days or** _____**months or** _____**years**

28. Before you quit, how many years did you smoke? _____**years** → **go to the end of the page**

29. How many years have you smoked? _____**years**

30. Which **cigarette/tobacco brand** do you usually buy?_____

For example: WINFIELD Super Mild or PETER JACKSON Menthol Mild

THE END of the questionnaire
THANK YOU

DIARY

DIARY - DAY 4

Work Commitments

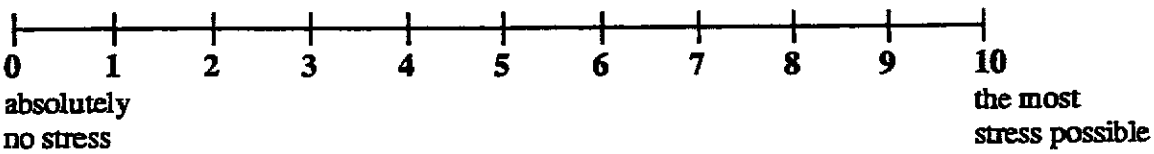
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

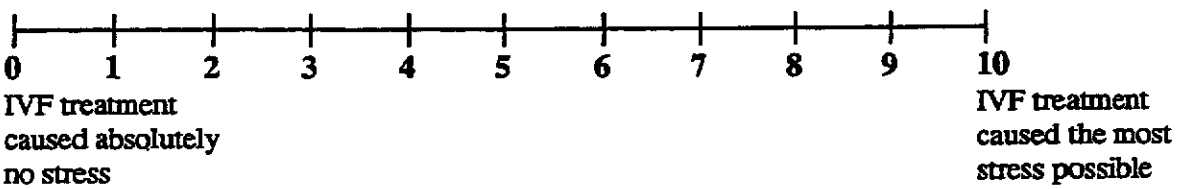
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐$_0$ → go to Qn 20

yes ☐$_1$ → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐$_0$

yes ☐$_1$ → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐$_1$ → How many?_____

no ☐$_0$

22. Did you smoke any other tobacco products today?

no ☐$_0$ → Please go to **the end of the page**

yes ☐$_1$ → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 5

Work Commitments

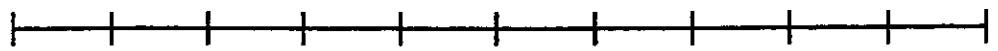
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

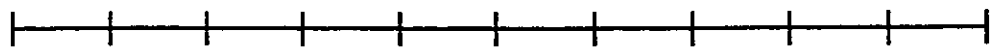
Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (**X**) on the stress scale below:



0 absolutely no stress — **10** the most stress possible

4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (**X**) on the stress scale below:



0 IVF treatment caused absolutely no stress — **10** IVF treatment caused the most stress possible

5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐$_0$ → go to Qn 16
yes ☐$_1$

14. Did you consume any of your **usual coffee beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

15. Did you consume any other coffee beverages today?
no ☐$_0$ → go to Qn 16
yes ☐$_1$ → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$

17. Did you consume any of your **usual tea beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$ → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐$_0$ → go to Qn 20

yes ☐$_1$ → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (**R**) or decaffeinated (**D**)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐$_0$

yes ☐$_1$ → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐$_1$ → How many?_____

no ☐$_0$

22. Did you smoke any other tobacco products today?

no ☐$_0$ → Please go to **the end of the page**

yes ☐$_1$ → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 6

Work Commitments

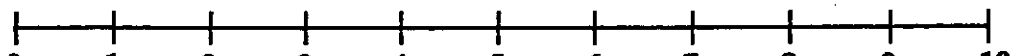
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

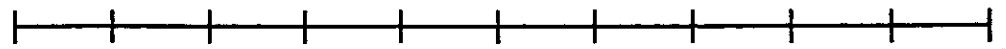
Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



0 absolutely no stress — **10** the most stress possible

4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



0 IVF treatment caused absolutely no stress — **10** IVF treatment caused the most stress possible

5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 7

Work Commitments

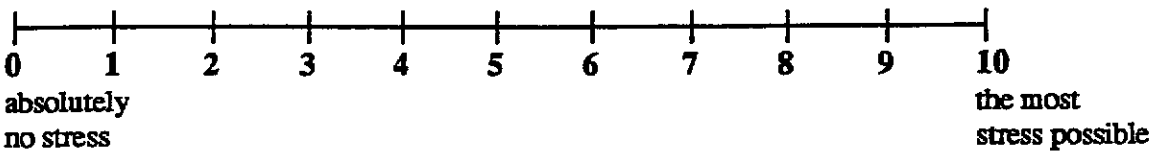
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

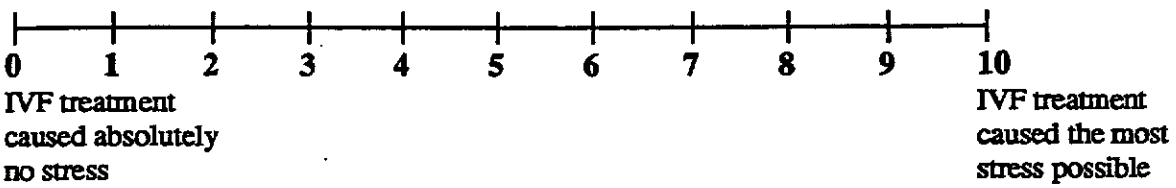
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 8

Work Commitments

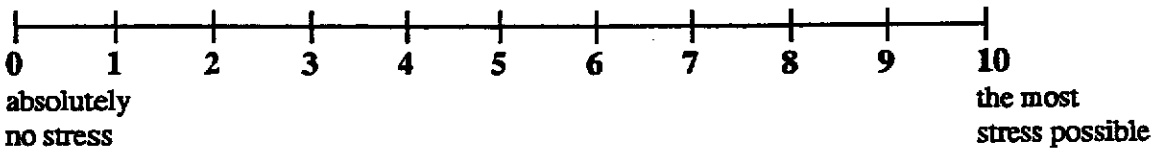
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

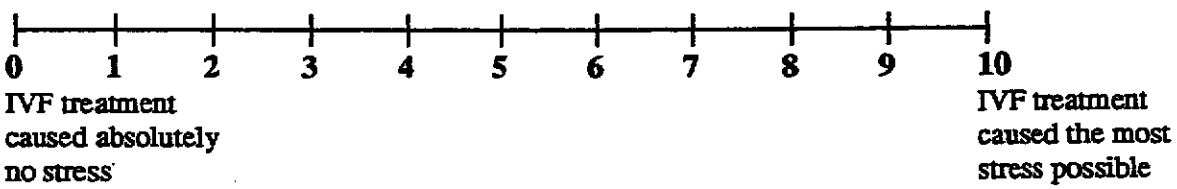
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

   no ☐0 → go to Qn 8

   yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

   no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

   no ☐0 → go to Qn 9

   yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

   no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

   yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

   no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

    no ☐0

11. Did you eat any vegetables today?

    yes ☐1 → How many servings (see back page)_____

    no ☐0

12. Did you visit PIVET today?

    yes ☐1 → What time did you arrive at PIVET_____am/pm

    no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐$_0$ → go to Qn 16
yes ☐$_1$

14. Did you consume any of your **usual coffee beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

15. Did you consume any other coffee beverages today?
no ☐$_0$ → go to Qn 16
yes ☐$_1$ → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$

17. Did you consume any of your **usual tea beverages** today?
yes ☐$_1$ → How many?_____
no ☐$_0$

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐$_0$ → go to Qn 19
yes ☐$_1$ → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola** drinks or **iced coffee drinks** today?

no ☐$_0$ → go to Qn 20

yes ☐$_1$ → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (**mls**) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐$_0$

yes ☐$_1$ → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐$_1$ → How many?_____

no ☐$_0$

22. Did you smoke any other tobacco products today?

no ☐$_0$ → **Please go to the end of the page**

yes ☐$_1$ → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 9

Work Commitments

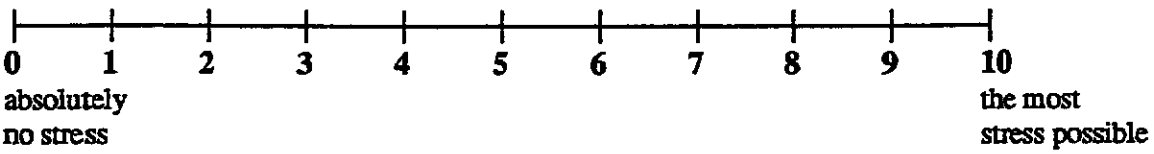
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

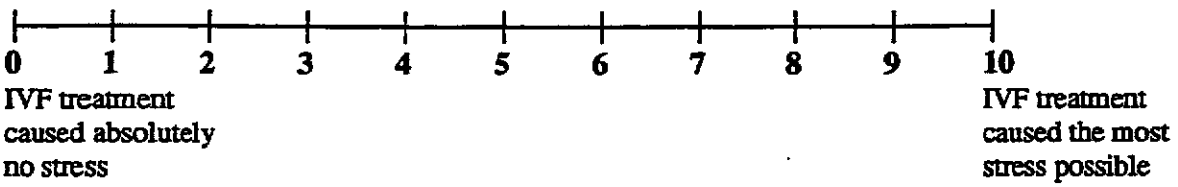
2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:



4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐₀ → go to Qn 6

   yes ☐₁ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐₀ → go to Qn 7

   yes ☐₁ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription or non-prescription ('over the counter') medication** today, other than prescribed by PIVET?

   no ☐0 → go to Qn 8

   yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

   no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

   no ☐0 → go to Qn 9

   yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

   no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

   yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

   no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

    no ☐0

11. Did you eat any vegetables today?

    yes ☐1 → How many servings (see back page)_____

    no ☐0

12. Did you visit PIVET today?

    yes ☐1 → What time did you arrive at PIVET_____am/pm

    no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any **tea** beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐0 → go to Qn 20

yes ☐1 → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐0

yes ☐1 → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐1 → How many?_____

no ☐0

22. Did you smoke any other tobacco products today?

no ☐0 → Please go to **the end of the page**

yes ☐1 → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of today's diary, THANK YOU

DIARY - DAY 10

Work Commitments

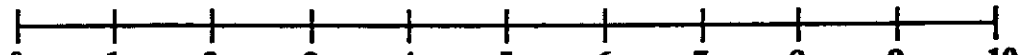
Date ___/___/___

1. How many hours of **paid** work did you do today? _____**hours**

2. How many hours of **unpaid** work did you do today?_____**hours**
   (For example, voluntary work, caregiving or house and garden duties)

Stress & Exercise

3. Indicate how you felt about your day in terms of **stress due to daily living, in general,** by placing a cross (X) on the stress scale below:

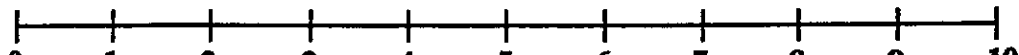


0 1 2 3 4 5 6 7 8 9 10

0 absolutely no stress

10 the most stress possible

4. Indicate how you felt about your day in terms of **stress due to your IVF treatment** by placing a cross (X) on the stress scale below:



0 1 2 3 4 5 6 7 8 9 10

0 IVF treatment caused absolutely no stress

10 IVF treatment caused the most stress possible

5. Did you engage in any activity today for the specific purpose of reducing stress?

   no ☐$_0$ → go to Qn 6

   yes ☐$_1$ → Please complete the table below:

Type of Activity eg. jogging, yoga or counselling	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M), light (L) or N/A

6. Did you do any exercise today (other than what you may have listed above)?

   no ☐$_0$ → go to Qn 7

   yes ☐$_1$ → Please complete the table below:

Type of Exercise eg. tennis, gym workout or swimming	**Duration** eg. 10 mins or 1½ hours	**Intensity** vigorous (V), moderate (M) or light (L)

Medication

7. Did you take any **prescription** or **non-prescription** ('over the counter') **medication** today, other than prescribed by PIVET?

no ☐0 → go to Qn 8

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 8

no ☐1 → Please complete the table below:

Brand Name eg. Aspirin	**Ingredients** eg. paracetamol	**Quantity** eg. 2 tablets x 500mg

8. Did you take any vitamin or mineral supplements today?

no ☐0 → go to Qn 9

yes ☐1 →Was it the same as yesterday? yes ☐0 → go to Qn 9

no ☐1 → Please complete the table below:

Brand Name	**Type of Supplement** eg. multi-vitamin, vitamin B, zinc, folate + iron	**Type** eg. tablet or powder	**Quantity** eg. 2 tablets or 5mg

Diet

9. Did you eat breakfast today?

yes ☐1 → Did your breakfast include breakfast cereals? yes ☐1

no ☐0 no ☐0

10. Did you eat any fruit today? yes ☐1 → How many servings (see back page)_____

no ☐0

11. Did you eat any vegetables today?

yes ☐1 → How many servings (see back page)_____

no ☐0

12. Did you visit PIVET today?

yes ☐1 → What time did you arrive at PIVET_____am/pm

no ☐0

Caffeine Consumption

13. Did you consume any coffee beverages today? no ☐0 → go to Qn 16
yes ☐1

14. Did you consume any of your **usual coffee beverages** today?
yes ☐1 → How many?_____
no ☐0

15. Did you consume any other coffee beverages today?
no ☐0 → go to Qn 16
yes ☐1 → Please complete the table below:

Type or Brewing Method eg. cappuccino or plunge	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

16. Did you consume any tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1

17. Did you consume any of your **usual tea beverages** today?
yes ☐1 → How many?_____
no ☐0

18. Did you consume any other tea beverages today including **herbal** or **iced** tea?
no ☐0 → go to Qn 19
yes ☐1 → Please complete the table below:

Name or Brewing Method eg. brewed tea or Liptonice	Regular (R) or Decaffeinated (D)	Quantity (mls) or Cup (C) or Mug (M)	No. of Drinks

19. Did you consume any **cola drinks** or **iced coffee drinks** today?

no ☐$_0$ → go to Qn 20

yes ☐$_1$ → Please complete the table below:

Brand Name eg. Diet Pepsi or Brownes Iced Milk	Regular (R) or decaffeinated (D)	Quantity (mls) eg. 375ml can	No. of Drinks

Alcohol Consumption

20. Did you consume any alcoholic drinks today?

no ☐$_0$

yes ☐$_1$ → How many **standard** drinks of alcohol (see back page) did you consume today?_____

If you do not smoke, this is the end of today's diary, THANK YOU.

Tobacco Consumption

21. Did you smoke any cigarettes today? yes ☐$_1$ → How many?_____

no ☐$_0$

22. Did you smoke any other tobacco products today?

no ☐$_0$ → Please go to **the end of the page**

yes ☐$_1$ → Please complete the table below:

Description eg. 'rollie' cigarette	**Brand** eg. Drum tobacco	**Quantity** eg. 2 cigarettes

This is the end of the diary, THANK YOU

We appreciate that it is sometimes difficult to know your exact consumption of alcohol, fruit and vegetable consumption, therefore, we request you provide your best approximation based on the following.

Standard Alcohol Drinks

Any drink containing 10 grams of alcohol is a standard drink. These are standard drinks:

You can also check for standard drink information on bottles and cans.

Fruit

(fresh, frozen, canned)

1 serve =

- 1 medium piece of fruit
- 1 cup of diced pieces
- 3 pieces of smaller sized fruit (apricots, plums, kiwi-fruit, figs)
- ¼ medium sized melon (rockmelon, honeydew)
- 4-6 pieces of dried fruit
- 2tbs sultanas and/or raisins
- 1 cup berries
- 20 grapes
- 10 strawberries

Vegetables

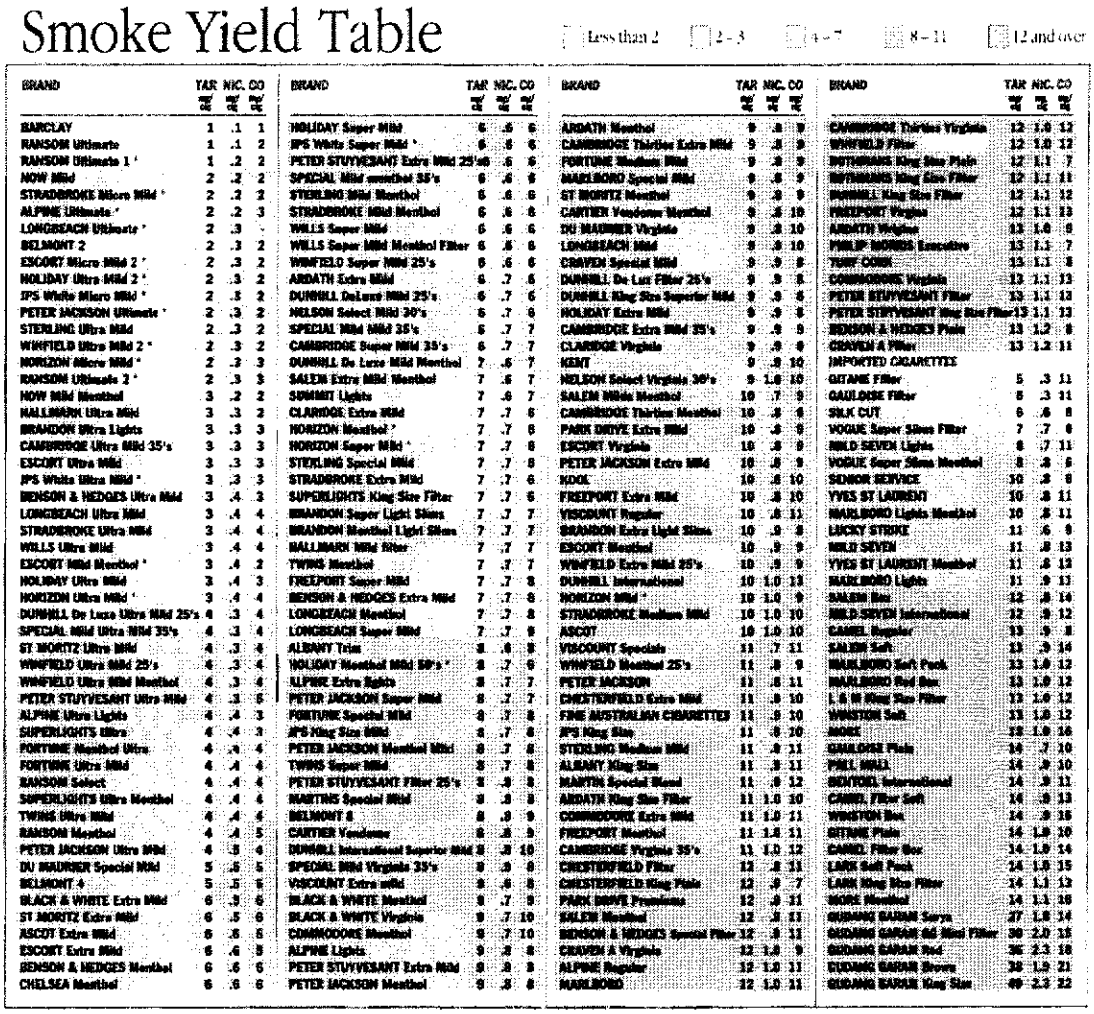
(fresh, frozen, canned)

1 serve =

- 1 small to medium potato
- ½ cup of cooked vegetables
- ½ to 1 cup of salad

Appendix E

Smoke Yield Table



Smoke Yield Table

Less than 2 | 2 - 3 | 4 - 7 | 8 - 11 | 12 and over

BRAND	TAR mg	NIC. mg	CO mg
BARCLAY	1	.1	1
RANSOM Ultimate	1	.1	2
RANSOM Ultimate 1 *	1	.2	2
NOW Mild	2	.2	2
STRADBROKE Micro Mild *	2	.2	2
ALPINE Ultimate *	2	.2	3
LONGBEACH Ultimate *	2	.3	-
BELMONT 2	2	.3	2
ESCORT Micro Mild 2 *	2	.3	2
HOLIDAY Ultra Mild 2 *	2	.3	2
JPS White Micro Mild *	2	.3	2
PETER JACKSON Ultimate *	2	.3	2
STERLING Ultra Mild	2	.3	2
WINFIELD Ultra Mild 2 *	2	.3	2
HORIZON Micro Mild *	2	.3	3
RANSOM Ultimate 2 *	2	.3	3
NOW Mild Menthol	3	.2	2
HALLMARK Ultra Mild	3	.3	2
BRANDON Ultra Lights	3	.3	3
CAMBRIDGE Ultra Mild 35's	3	.3	3
ESCORT Ultra Mild	3	.3	3
JPS White Ultra Mild *	3	.3	3
BENSON & HEDGES Ultra Mild	3	.4	3
LONGBEACH Ultra Mild	3	.4	4
STRADBROKE Ultra Mild	3	.4	4
WILLS Ultra Mild	3	.4	4
ESCORT Mild Menthol *	3	.4	2
HOLIDAY Ultra Mild	3	.4	3
HORIZON Ultra Mild *	3	.4	4
DUNHILL De Luxe Ultra Mild 25's	4	.3	4
SPECIAL Mild Ultra Mild 35's	4	.3	4
ST MORITZ Ultra Mild	4	.3	4
WINFIELD Ultra Mild 25's	4	.3	4
WINFIELD Ultra Mild Menthol	4	.3	4
PETER STUYVESANT Ultra Mild	4	.3	5
ALPINE Ultra Lights	4	.4	3
SUPERLIGHTS Ultra	4	.4	3
FORTUNE Menthol Ultra	4	.4	4
FORTUNE Ultra Mild	4	.4	4
RANSOM Select	4	.4	4
SUPERLIGHTS Ultra Menthol	4	.4	4
TWINS Ultra Mild	4	.4	4
RANSOM Menthol	4	.4	5
PETER JACKSON Ultra Mild	4	.5	4
DU MAURIER Special Mild	5	.5	5
BELMONT 4	5	.5	6
BLACK & WHITE Extra Mild	6	.5	6
ST MORITZ Extra Mild	6	.5	6
ASCOT Extra Mild	6	.6	5
ESCORT Extra Mild	6	.6	5
BENSON & HEDGES Menthol	6	.6	6
CHELSEA Menthol	6	.6	6
HOLIDAY Super Mild	6	.6	6
JPS White Super Mild *	6	.6	6
PETER STUYVESANT Extra Mild 25's	6	.6	6
SPECIAL Mild menthol 35's	6	.6	8
STERLING Mild Menthol	6	.6	6
STRADBROKE Mild Menthol	6	.6	6
WILLS Super Mild	6	.6	6
WILLS Super Mild Menthol Filter	6	.6	6
WINFIELD Super Mild 25's	6	.6	6
ARDATH Extra Mild	6	.7	6
DUNHILL DeLuxe Mild 25's	6	.7	6
NELSON Select Mild 30's	6	.7	6
SPECIAL Mild Mild 35's	6	.7	7
CAMBRIDGE Super Mild 35's	6	.7	7
DUNHILL De Luxe Mild Menthol	7	.6	7
SALEM Extra Mild Menthol	7	.6	7
SUMMIT Lights	7	.6	7
CLARIDGE Extra Mild	7	.7	6
HORIZON Menthol *	7	.7	6
HORIZON Super Mild *	7	.7	6
STERLING Special Mild	7	.7	6
STRADBROKE Extra Mild	7	.7	6
SUPERLIGHTS King Size Filter	7	.7	6
BRANDON Super Light Skims	7	.7	7
BRANDON Menthol Light Skims	7	.7	7
HALLMARK Mild Filter	7	.7	7
TWINS Menthol	7	.7	7
FREEPORT Super Mild	7	.7	8
BENSON & HEDGES Extra Mild	7	.7	8
LONGBEACH Menthol	7	.7	8
LONGBEACH Super Mild	7	.7	8
ALBANY Trim	8	.6	8
HOLIDAY Menthol Mild 50's *	8	.7	9
ALPINE Extra Lights	8	.7	7
PETER JACKSON Super Mild	8	.7	7
FORTUNE Special Mild	8	.7	8
JPS King Size Mild	8	.7	8
PETER JACKSON Menthol Mild	8	.7	8
TWINS Super Mild	8	.7	8
PETER STUYVESANT Filter 25's	8	.8	8
MARTINS Special Mild	8	.8	8
BELMONT 8	8	.8	9
CARTIER Vendome	8	.8	9
DUNHILL International Superior Mild	8	.8	10
SPECIAL Mild Virginia 35's	8	.8	8
VISCOUNT Extra mild	8	.8	8
BLACK & WHITE Menthol	8	.7	9
BLACK & WHITE Virginia	9	.7	10
COMMODORE Menthol	9	.7	10
DUNHILL International Mild	9	.8	8
ALPINE Lights	9	.8	8
PETER STUYVESANT Extra Mild	9	.8	8
PETER JACKSON Menthol	9	.8	8
ARDATH Menthol	9	.8	9
CAMBRIDGE Thirties Extra Mild	9	.8	9
FORTUNE Medium Mild	9	.8	9
MARLBORO Special Mild	9	.8	9
ST MORITZ Menthol	9	.8	9
CARTIER Vendome Menthol	9	.8	10
DU MAURIER Virginia	9	.8	10
LONGBEACH Mild	9	.8	10
CRAVEN Special Mild	9	.9	8
DUNHILL De Luxe Filter 25's	9	.9	8
DUNHILL King Size Superior Mild	9	.9	8
HOLIDAY Extra Mild	9	.9	8
CAMBRIDGE Extra Mild 35's	9	.9	9
CLARIDGE Virginia	9	.9	9
KENT	9	.9	10
NELSON Select Virginia 30's	9	1.0	10
SALEM Milds Menthol	10	.7	9
CAMBRIDGE Thirties Menthol	10	.8	8
PARK DRIVE Extra Mild	10	.8	9
ESCORT Virginia	10	.8	9
PETER JACKSON Extra Mild	10	.8	9
KOOL	10	.8	10
FREEPORT Extra Mild	10	.8	10
VISCOUNT Regular	10	.8	11
BRANDON Extra Light Skims	10	.9	8
ESCORT Menthol	10	.9	9
WINFIELD Extra Mild 25's	10	.9	9
DUNHILL International	10	1.0	13
HORIZON Mild *	10	1.0	9
STRADBROKE Medium Mild	10	1.0	10
ASCOT	10	1.0	10
VISCOUNT Specials	11	.7	11
WINFIELD Menthol 25's	11	.8	9
PETER JACKSON	11	.8	11
CHESTERFIELD Extra Mild	11	.8	10
FINE AUSTRALIAN CIGARETTES	11	.8	10
JPS King Size	11	.8	10
STERLING Medium Mild	11	.8	11
ALBANY King Size	11	.8	11
MARTIN Special Blend	11	.8	12
ARDATH King Size Filter	11	1.0	10
COMMODORE Extra Mild	11	1.0	11
FREEPORT Menthol	11	1.0	11
CAMBRIDGE Virginia 35's	11	1.0	12
CHESTERFIELD Filter	12	.8	11
CHESTERFIELD King Plain	12	.9	7
PARK DRIVE Premium	12	.9	11
SALEM Menthol	12	.9	11
BENSON & HEDGES Special Filter	12	.9	11
CRAVEN A Virginia	12	1.0	9
ALPINE Regular	12	1.0	11
MARLBORO	12	1.0	11
CAMBRIDGE Thirties Virginia	12	1.0	12
WINFIELD Filter	12	1.0	12
BENSON & HEDGES King Size Plain	12	1.1	7
DUNHILL King Size Filter	12	1.1	11
FREEPORT Virginia	12	1.1	13
ARDATH Virginia	13	1.0	9
PHILIP MORRIS Executive	13	1.1	7
TURF CORK	13	1.1	8
COMMODORE Virginia	13	1.1	11
PETER STUYVESANT Filter	13	1.1	13
PETER STUYVESANT King Size Filter	13	1.1	13
BENSON & HEDGES Plain	13	1.2	8
CRAVEN A Filter	13	1.2	11
IMPORTED CIGARETTES			
GITANE Filter	5	.3	11
GAULOISE Filter	6	.3	11
SILK CUT	6	.6	8
VOGUE Super Slims Filter	7	.7	8
MILD SEVEN Lights	8	.7	11
VOGUE Super Slims Menthol	8	.8	8
SENIOR SERVICE	10	.8	8
YVES ST LAURENT	10	.8	11
MARLBORO Lights Menthol	10	.8	11
LUCKY STRIKE	11	.6	9
MILD SEVEN	11	.8	13
YVES ST LAURENT Menthol	11	.8	13
MARLBORO Lights	11	.9	13
SALEM Box	12	.8	14
MILD SEVEN International	12	.9	12
CAMEL Regular	13	.9	8
SALEM Soft	13	.9	14
MARLBORO Soft Pack	13	1.0	13
MARLBORO Red Box	13	1.0	12
L & M King Size Filter	13	1.0	12
WINSTON Soft	13	1.0	12
MORE	13	1.0	16
GAULOISE Plain	14	.7	10
PALL MALL	14	.9	10
BENSON International	14	.9	11
CAMEL Filter Soft	14	.9	13
WINSTON Box	14	.9	16
GITANE Plain	14	1.0	10
CAMEL Filter Box	14	1.0	14
LARK Soft Pack	14	1.0	15
LARK King Size Filter	14	1.1	13
MORE Menthol	14	1.1	16
SUDANI SARAH Surya	27	1.8	14
SUDANI SARAH 66 Mini Filter	30	2.0	15
SUDANI SARAH Red	36	2.3	18
SUDANI SARAH Brown	38	1.9	21
SUDANI SARAH King Size	40	2.3	22

ABOUT THIS TABLE

This table shows the tar, nicotine (nic.) and carbon monoxide (CO) present in cigarette smoke and is measured in milligrams per cigarette. It is presented in ascending order of tar yield. The higher the smoke yield the more dangerous the cigarette is for you.

Tar - condensed smoke containing many chemicals, including some that cause cancer.

Nicotine - a poisonous and addictive drug.

Carbon Monoxide - a deadly gas which reduces the ability of blood to carry oxygen.

Tests were performed in 1991 by the Australia Government Analytical Laboratories (AGAL). * Manufacturers result only. Not yet tested by AGAL.

THE DRUG OFFENSIVE
A Federal and State Initiative.

Appendix F

Supplementary Data Analysis:

Male Smoking and Sperm Quality

Table F: Spermatozoa Classification by Smoking Status

	smokers % (n)	non-smokers % (n)	total % (n)	*P*-value
oligozoospermia[a]	8.6 (3)	6.8 (8)	7.2 (11)	0.728
asthenozoospermia[b]	20.0 (7)	21.4 (25)	21.1 (32)	0.862
teratozoospermia[c]	14.3 (5)	17.1 (20)	16.4 (25)	0.694
total	32	120	152	-

[a] χ^2=0.121, 1df

[b] χ^2=0.030, 1df

[c] χ^2=0.155, 1df