

Optimising Human Sperm Use in Subfertility

A thesis submitted for the degree of MD by
Madhavi Gudipati MBBS, MD (India), MRCOG
Newcastle Fertility Centre at Life
Institute of Genetic Medicine
Newcastle University

Abstract

Introduction: There is a nationwide shortage of sperm donors and over the last few years this has been evident at Newcastle Fertility Centre (NFC). As the most common cause for rejection of sperm donors is suboptimal semen quality, external factors that may influence semen quality (i.e. season and vitamin D) were studied to investigate their impact (if any) to improve donor recruitment.

Methods: A retrospective review of donor sperm treatments at NFC between Jan 2000 and Dec 2010 was performed to investigate sperm donor shortage. A retrospective review (Dec 2006 to Nov 2009) and a longitudinal study (32 sperm donors) of the semen analyses were conducted to investigate seasonal variation in semen parameters. We performed a retrospective review of donor insemination treatments over 6 years to investigate seasonal variation in donor conceptions. The correlation between semen parameters and serum vitamin D was investigated in a cross sectional study (125 participants) and a cohort study, to examine the change in semen parameters with a rise in serum vitamin D level (with vitamin D supplementation and seasonal rise).

Results: A significant reduction in the number of sperm donors recruited and a smaller pool of available donors was seen, which lead to fewer patients receiving treatment and a longer wait for treatment. Seasonal variation with improved semen parameters in winter / spring was noted, but was more prevalent in sperm donors than in patients attending NFC. However, there were no variation donor conceptions by the season of original sperm production. In the cross sectional association study there was no significant difference in the semen parameters between men with different serum vitamin D levels, however in the cohort study, semen parameters deteriorated significantly with increased serum vitamin D levels secondary to vitamin D supplementation and also seasonal rise.

Conclusions: A significant local problem of sperm donor shortage is confirmed. Despite significant seasonal differences in donor semen parameters (but not donor conceptions), we do not recommend restricting recruitment of sperm donors to winter /

spring. A negative association between vitamin D and semen parameters is noted; therefore vitamin D supplementation should not be recommended to improve to semen parameters.

Acknowledgements

I owe my sincere thanks to all who mentored, supported, helped and cheered me through completion of this dissertation.

I am truly indebted and grateful to my principal supervisor Mr. Kevin McEleny for his intellectual and emotional support, patience, and encouragement throughout the project. I thank him for guiding me through several international / national presentations and interview with Newspaper '*Daily Telegraph*' staff too! I truly believe that the completion of this project would have been impossible without his help.

I owe my sincere and earnest thanks to my supervisor Dr. Jane Stewart for contriving the project and advising me through the data analysis. I thank her immensely for her encouragement and helping me set realistic time scale of achieving targets (for a full time working mum!)

I am grateful to my supervisor Professor Simon Pearce for providing me valuable information sources and guiding me through the management of my research participants.

I would like to show my gratitude to statistician Kim Pearce for her time, expertise and involvement.

I am grateful to embryologists Victoria Hemingway and Lynne Nowak for training me in semen analysis even when that meant staying out of hours and David Cullen for his help with data collection and IT expertise.

It is a great pleasure to thank my colleagues Dr. Meenakshi Choudhary for sharing her enthusiasm and knowledge in research field; Dr. Mohar Goswami for inspiring me with her focus and determination during her MD.

I cannot thank my husband Sreedhar Gudipati enough for his understanding, unwavering support and for his countless months of 'single parenting'. My son Varun's cheerful face kept me going and I could not have let down my daughter Neha who

whenever heard the word finish would ask ‘Momma have you finished your thesis?’ I would like to thank my parents Bachu Chandraiah and Bachu Rajamani for their moral support and my brother Umesh Kumar Bachu for helping me visualize a broader perspective and a brighter future.

Finally, I am grateful to all my research participants without whom the project would have not been possible.

Dedication

I wish to dedicate this thesis to my husband Sreedhar Gudipati for his unwavering support and our gorgeous kids Neha and Varun.

Declaration

This is to confirm that the work done in this thesis project is original and all material which is not my own work has been identified. I certify that no material is included for which a degree has previously been conferred upon me.

Madhavi Gudipati.

Table of contents

Abstract	ii
Acknowledgements	iv
Dedication	vi
Declaration	vii
Table of contents	viii
Table of Tables.....	xiv
Table of figures	xvi
Abbreviations	xvii
Chapter 1 Introduction	1
1.1 Is there a problem with the sperm donor recruitment currently at Newcastle Fertility Centre?	1
1.1.1 Removal of sperm donor anonymity.....	2
1.1.2 Payment to the donors	6
1.1.3 Updated UK guidelines for the screening of sperm donors	11
1.1.4 A comprehensive Andrology service	11
1.1.5 Sperm donor shortage	12
1.2 Factors effecting semen quality.....	12
1.3 Season and semen parameters	13
1.4 Seasonal variation in donor conceptions	21
1.5 Association of vitamin D and semen parameters	23
1.5.1 Background	23
1.5.2 Sources	25
1.5.3 Physiology.....	30
1.5.4 Mode of Action	34
1.5.5 Epidemiology	39
1.5.6 Risk Factors for vitamin D deficiency	39

1.5.7	Effects of vitamin D	41
1.5.8	Assessment of vitamin D status	44
1.5.9	Classification of vitamin D status	45
1.5.10	Recommended daily intake of vitamin D.....	46
1.5.11	Management of vitamin D deficiency	47
1.5.12	Vitamin D and Reproduction	49
1.6	Summary of introduction.....	62
Chapter 2 Aims of the project		66
Chapter 3 Is there a problem with sperm donor recruitment currently at NFC?.....		68
3.1	Introduction	68
3.2	Aim.....	69
3.3	Objectives	69
3.4	Outcome Measures	69
3.5	Subjects and Methods.....	69
3.5.1	Subjects	69
3.5.2	Recruitment of sperm donors at the Centre.....	69
3.5.3	Donor programme	70
3.5.4	Methodology	71
3.6	Statistical analysis	72
3.7	Results	72
3.7.1	Recruitment of donors.....	73
3.7.2	Donor sperm treatments	75
3.7.3	Sperm export and imports	79
3.7.4	Waiting list issues	79
3.8	Discussion	82
3.8.1	Recruitment of donors.....	82
3.8.2	Donor sperm treatments	85
3.8.3	Sperm export and imports	87

3.8.4	Waiting list issues	87
3.9	Strategies to cope with the sperm donor shortage	89
3.10	Limitations of the study	93
3.11	Conclusion	93
Chapter 4	Seasonal variation in semen parameters	94
4.1	Introduction	94
4.2	Aim	95
4.3	Objectives	95
4.4	Outcome measures	95
4.5	Subjects and Methods	95
4.5.1	Subjects	95
4.5.2	Methods	95
4.5.3	Study size	95
4.5.4	Semen Analysis	96
4.5.5	Quality assurance	98
4.5.6	Semen Parameters	98
4.5.7	Seasons	98
4.5.8	Subsets	98
4.6	Statistical Analysis	99
4.7	Results	99
4.8	Discussion	104
4.9	Limitations of the study	108
4.10	Conclusion	110
Chapter 5	Seasonal variation in donor conceptions	111
5.1	Introduction	111
5.2	Aim	111
5.3	Objectives	111
5.4	Outcome measures	111

5.5	Subjects and Methods.....	111
5.5.1	Subjects	111
5.5.2	Donor insemination treatment (DI).....	112
5.5.3	Laboratory processing of the semen from recruited sperm donors.....	113
5.5.4	Methodology	114
5.5.5	Study size	115
5.6	Statistical Analysis	115
5.7	Results	115
5.7.1	Is the pregnancy rate following DI treatment higher when sperm produced in a particular season was used?	116
5.7.2	Is the pregnancy rate following DI treatment higher when treatment occurs in a particular season?	118
5.7.3	Is the outcome of DI treatment dependent on the number of inseminations?.....	119
5.7.4	Donor semen parameters.....	120
5.7.5	Is the cryopreservation of sperm more effective in any particular season? 125	
5.8	Discussion	126
5.9	Limitations of the study.....	129
5.10	Conclusion	130
Chapter 6 Association of vitamin D and semen parameters		131
6.1	Introduction	131
6.2	Aim.....	131
6.3	Objectives	131
6.4	Outcome measures	132
6.5	Subjects and Methods.....	132
6.5.1	Subjects	132
6.5.2	Study design / size.....	132
6.5.3	Recruitment.....	132

6.5.4	Eligibility	135
6.5.5	Exclusion Criteria.....	135
6.5.6	Procedure.....	135
6.5.7	Follow up clinics	136
6.5.8	Laboratory Procedures	138
6.5.9	Data Handling	147
6.5.10	Handling of results	147
6.5.11	Vitamin D deficiency	147
6.5.12	Vitamin D compliance	147
6.6	Statistical Analysis	148
6.7	Results	149
6.7.1	Recruitment.....	149
6.7.2	Prospective observational association study	152
6.7.3	Follow up	163
6.7.4	Cohort study	165
6.7.5	Annual seasonal variation of vitamin D.....	171
6.7.6	Is there a threshold for Vitamin D to be achieved to influence semen parameters?	173
6.7.7	Is high vitamin D (>125nmol/l) associated with a negative impact on semen parameters?	175
6.8	Discussion	177
6.9	Limitations of the study.....	185
6.10	Conclusion	186
Chapter 7 Vitamin D and male fertility.....		188
7.1	Introduction	188
7.2	Aim.....	190
7.3	Objectives	190
7.4	Outcome measures	190

7.5	Subjects and Methods.....	190
7.5.1	Subjects	190
7.5.2	Study design	191
7.5.3	Investigations	191
7.5.4	Study method	191
7.6	Statistical analysis	195
7.7	Results	195
7.7.1	Semen quality in four vitamin D groups	195
7.7.2	Semen quality with vitamin D supplementation	197
7.7.3	Semen quality with seasonal rise in vitamin D	200
7.7.4	Vitamin D and IVF/ICSI outcome	203
7.8	Discussion	207
7.9	Limitations of the study.....	208
7.10	Conclusion	208
	Chapter 8 Discussion	210
	Academic Achievements so far from this project	224
	Appendix A: Patient information sheet.....	226
	Appendix B: Questionnaire.....	230
	Appendix C: Consent form	232
	Appendix D: Invitation letter to the participants.....	233
	Appendix E: NEQAS calculations and results interpretation	235
	Appendix F: Training check list.....	237
	Appendix G: NEQAS: My accreditation paper work	239
	Appendix H: Letter to participants with vitamin D level deficiency	251
	Appendix I: Letter to Participant's GP.....	252
	Appendix J: Vitamin D information leaflet	253
	Appendix K: Embryo grading.....	254
	References	255

Table of Tables

Table 1: Dietary, Supplemental, and Pharmaceutical Sources of Vitamins D2 and D3	28
Table 2: Vitamin D supplements available in UK	29
Table 3: Chromosomal location of vitamin D receptor and metabolising enzymes	31
Table 4: Deficiency [25(OH)D <25 nmol/l) – Treatment for Adults	48
Table 5: Maintenance treatment for adults following treatment of Deficiency [25(OH)D <25 nmol/l)	48
Table 6: Sperm donor enquiries, recruitment, sperm export / import, and waiting times for donor sperm treatment at the Newcastle Fertility Centre from 2000 to 2010	74
Table 7: Rising proportion of Donor IVF in donor sperm treatment cycles	77
Table 8: Patients added to the waiting list for donor sperm treatment – with their indications	80
Table 9: Patients on the waiting list- Outcome	81
Table 10: Characteristics of the semen samples by seasons in the total study group (N= 4067 samples)	100
Table 11: Characteristics of the semen samples by seasons in samples with sperm concentration ≥ 1 million / ml (N= 3905 samples)	101
Table 12: Characteristics of the semen samples by seasons in samples with sperm concentration ≥ 5 million / ml (N=3748 samples)	102
Table 13: Characteristics of the semen samples by seasons in samples with sperm concentration ≥ 10 million / ml (N= 3600 samples)	103
Table 14: Donor insemination treatment outcome by the season of semen sample production	117
Table 15: Treatment outcome by the season of donor insemination	118
Table 16: Association between number of inseminations and pregnancy outcome of DI treatment	119
Table 17: Details of the donor semen samples	120
Table 18: Pre freeze semen parameters of donor samples by season of sample production (N= 1108 samples)	122
Table 19: Post thaw semen parameters of cryopreserved donor samples by season of sample production (N= 906 samples)	124
Table 20: Association between season of sample production and cryopreservation	125
Table 21: Follow up clinics	137
Table 22: Calculation of dilution for sperm concentration	143

Table 23: Determination of squares to count by number of sperm seen.....	143
Table 24: Conversion factor to obtain sperm concentration in millions per millilitre..	143
Table 25: Training in semen analysis.....	146
Table 26: Male factor subfertility	151
Table 27: Indicators of vitamin D status in the various groups	155
Table 28: Characteristics of 124 men by serum vitamin D groups.....	157
Table 29: Risk factors in the participants.....	159
Table 30: Semen parameters of 124 men divided by the vitamin D groups	161
Table 31: Participants failing to return for follow up	164
Table 33: Vitamin D and Semen parameters in the 36 vitamin D supplemented men .	168
Table 34: Semen parameters with seasonal rise in vitamin D levels	170
Table 35: Seasonal variation of vitamin D levels – Annual pattern	172
Table 36: Semen parameters in men with vitamin D deficiency that improved to optimal levels with supplementation	174
Table 37: Vitamin D and Semen parameters in the 30 vitamin D supplemented men .	176
Table 38: WHO 2010 criteria for semen parameters	188
Table 39: Semen quality by vitamin D group	196
Table 40: Semen quality with vitamin D supplementation	197
Table 41: Serum vitamin D levels and semen parameters before and after supplementation in 6 participants with change in semen quality from Optimal to suboptimal.....	199
Table 42: Semen quality with seasonal rise in vitamin D.....	200
Table 43: Serum vitamin D levels and semen parameters in a participant with change in semen quality (suboptimal to optimal) from Winter/Spring to Summer/ Autumn	201
Table 44: Serum vitamin D levels and semen parameters in two participants with change in semen quality (optimal to suboptimal) from Winter/Spring to Summer/Autumn	202
Table 45: Comparison of fertilisation and embryo quality between Winter/Spring and Summer/Autumn	205
Table 46: Comparison of pregnancy and embryo freezing rates between Winter/Spring and Summer/Autumn	206
Table 47: Embryo grading Day 2 / day 3	254
Table 48: Embryo grading	254

Table of figures

Figure 1: Vitamin D3 (Colecalciferol).....	24
Figure 2: Schematic illustration of the Solar Zenith Angle (SZA) and Viewing Zenith Angle (VZA).....	26
Figure 3: Metabolic pathway of vitamin D.....	32
Figure 4: Regulation of vitamin D.....	33
Figure 5: Diagrammatic representation of the known molecular events in the regulation of gene expression by the vitamin D hormone, 1,25(OH) ₂ D ₃ acting through its receptor, VDR.	36
Figure 6: Number of patients starting treatment with donor sperm per year.....	75
Figure 7: Number of treatment cycles with donor sperm per year.....	76
Figure 8: Indications for use of donor sperm.....	78
Figure 9: Sequence of events in IVF.....	134
Figure 10: Participant's recruitment and follow up.....	150
Figure 11: Serum vitamin D levels achieved in vitamin D deficiency participants in various treatment groups.....	166
Figure 12: Monthly variation of serum vitamin D levels.....	172
Figure 13: Participant's flow chart for Vitamin D and "Semen quality" study.....	193
Figure 14: Participant's flow chart for vitamin D (VD) and IVF/ICSI study.....	203

Abbreviations

25(OH)D- 25-hydroxyvitamin D

1,25(OH)₂D- 1,25-dihydroxyvitamin D

ABCA1- ATP- binding cassette transporter

AMH- Anti mullerian hormone

ALTM- All laboratory trimmed mean

BAS- British Andrology society

BFS- British Fertility Society

BMD- Bone mineral density

BMI – Body mass index

CASA- Computer assisted semen analysis

CLIA- Chemiluminescent immunoassay

CV- Co-efficient of variation

CYP- Cytochrome P 450

CYP27A1- Vitamin D-25hydroxylase

CYP2R1- Vitamin D-25hydroxylase

CYP27B1-25-hydroxyvitamin D-1 α -hydroxylase

CYP24A1- 25-hydroxyvitamin D-24hydroxylase

DBP- Vitamin D binding protein

D-IVF-Donor IVF

EIA- enzyme immunoassay

ERC- Excess residual cytoplasm

ESHRE- European Society of Human Reproduction and Embryology

FSH- Follicle stimulating hormone

GnRH- Gonadotropin releasing hormone

GP- General Practitioner

HCG- Human Chorionic Gonadotrophin

HFEA- Human Fertilisation and Embryo Authority

HPLC- high-performance liquid chromatography

HPT axis-Hypothalamo pituitary axis

HTLV- Human T cell Lymphotropic virus

ICSI- Intra cytoplasmic sperm injection

IL1 –Interleukin 1

IL2- Interleukin 2

IOM- Institute of Medicine

IU- International Units

IUI- Intra uterine insemination

IVF- In vitro fertilisation

kDa- Kilo Daltons

LC-MS/MS - liquid chromatography tandem mass spectrometry

LH- Luteinising hormone

MAP kinase - Mitogen Activated Protein Kinase

Micro TESE- Micro surgical testicular sperm extraction

MG- Madhavi Gudipati

MM- Method Mean

NFC- Newcastle Fertility Centre

NGDT – National Gamete Donation Trust

NICE- National Institute of Clinical Excellence

PC- Personal computer

PCOS- Polycystic ovarian syndrome

PESA- Percutaneous epididymal sperm aspiration

PKA- Protein Kinase A

PKC- Protein Kinase C

PTH- Parathyroid hormone

RCOG –Royal College of Obstetrics and Gynaecology

RCT-Randomised controlled trial

RDA – Recommended daily allowance

RIA- Radioimmunoassay

RVI- Royal Victoria Infirmary

SD- Standard deviation

SEED review- Sperm, egg and embryo donation review

SEM- Standard error of mean

SHBG- Sex hormone binding globulin

SNP- Single nucleotide polymorphism

TTP - Time to pregnancy

UKFSA- United Kingdom Food standards agency

UVB –Ultra violet radiation

VD- Vitamin D (represents vitamin D3 and D2)

VDR – Vitamin D receptor

VDRE – Vitamin D response element

Chapter 1 Introduction

1.1 Is there a problem with the sperm donor recruitment currently at Newcastle Fertility Centre?

Assisted conception treatment with donor sperm is a vital treatment option for couples with severe male factor infertility, same sex couples and single women. Between 1992 and 2009, 26,638 children were born in the UK from 226,662 cycles of treatment with donor sperm (HFEA, 2011). The need for donor sperm treatment continues despite the wide availability of ICSI, which since 2000 has become a well-established treatment for male factor infertility (Hamilton *et al.*, 2008a; Pacey, 2010). It is estimated that approximately 4000 patients need donor sperm treatment every year (Hamilton *et al.*, 2008a) in the UK.

Over recent years, at the Newcastle Fertility Centre (NFC), a long waiting time for donor sperm treatments (about 18 months) has developed with the perception of an increase in the amount of sperm imported to the centre from overseas commercial sperm banks. These issues were non-existent in the early part of the 21st century, but significant national and local changes since then have influenced the sperm donor programme at the centre and various strategies have been introduced to cope with the changes.

The significant national changes were:

1. Removal of gamete donor anonymity in 2005.
2. Changes to the sperm donor payment in 2006.
3. Guidelines for the screening of sperm (egg and embryo) donors updated in 2008.

The local changes are:

1. Introduction of a comprehensive integrated Andrology service in 2009.

1.1.1 Removal of sperm donor anonymity

Assisted conception treatment with donor sperm was legalized in the UK from 1992, (HMSO, 1990) although it had been practiced from the 1930s in secrecy (Frith, 2001). Mary Barton published on the use of donor sperm to treat what was described as incurable male infertility and for genetic reasons (Barton, 1945). This was condemned across the world and specifically by religious leaders.

Over the following few decades, committees were set up to consider various aspects of artificial insemination with donor sperm. Namely the Feversham Committee (Earl of Feversham's report 1960), which held the view that donor insemination should not be allowed and the Peel committee (Peel report 1973), which accepted that donor insemination treatment should be carried out in the NHS, available as a clinical service from 1968. During these years donor insemination treatments were carried out with anonymous sperm donation and without any regulation or maintenance of records.

The UK government in 1982 appointed a Committee of Inquiry into Human Fertilisation and Embryology under the guidance of Mary Warnock to ensure that the new technology of IVF was not abused. The Warnock Report published in 1984, included recommendations on the issues of the creation of embryos *in vitro*, the storage of gametes / embryos and on research involving embryos. The Warnock Report, essentially supported the practice of treatment with donor sperm but suggested that it should remain anonymous to prevent any disturbance in the family dynamics (Spallone, 1986).

With anonymous sperm donation treatments, the woman's husband was registered as the birth father to keep the birth origin a secret. In reality this was an offence against the law until the 1987 Family Law Reform Act, which gave the right to the women's husband to register as the father legally (McWhinnie, 2001).

The Human Fertilisation Embryology Act 1990 (which was implemented from August 1991) legitimised donor sperm treatment. The act supported anonymous sperm donation and also gave protection to the sperm donor with no legal liability for any off spring. The act also led to the establishment of the Human Fertilisation Embryo Authority (HFEA) as an independent regulator of all treatments and research involving gametes and embryos, which is answerable to Parliament.

Only HFEA licensed centres can offer treatment with donor sperm and the HFEA maintains a central register for all donors and the information on donor-conceived children. The information about the donor was held only to allow tracking if the child was diagnosed with any heritable disorder. In exceptional circumstances a donor offspring upon reaching 18 years can sue the donor if born with a disability which the donor should have disclosed (Van den Akker, 2006). The Act also put in place a legal safeguard at the time that should the practice of anonymity be considered to be removed, it would need to be done by legislation (Frith, 2001).

The first country to remove donor anonymity was Sweden in 1984 (Frith, 2001). Since then approximately 35 countries, mainly economically developed countries like Austria, Germany, Finland, Norway, Holland, Switzerland and also Australia and New Zealand have moved to non-anonymous sperm donation (Janssens *et al.*, 2011), where the child born from donor sperm treatment has a right to know the identifying details of the genetic father when they reach majority, which varies from 16 to 18 years in different countries.

Removal of donor anonymity has been an issue of public debate in the UK from about the 1990s (Cooke, 1993; Haines, 1993; Snowden, 1993; HFEA, 1998). The debates on this topic were published in various journals such as articles published in the *Journal of assisted Reproduction and Genetics* in 1997 entitled 'Controversies in assisted reproduction and genetics -Privacy versus disclosure' and in 2001 in *Human Reproduction* titled 'Gamete donation and anonymity'. Some of these and others' arguments are presented in the following paragraphs.

The fundamental issue raised in support of removing anonymity was that it is a child's right to know their parents identity as per the 1989 United Nations Convention on the Rights of the Child (McWhinnie, 2001), although this was written for children in general and not taking children born by gamete donation specifically into account (Frith, 2001). It has been suggested that they have a right to know the truth (Daniels and Taylor, 1993) and the importance of genetic knowledge for health information was emphasized (Van den Akker, 2006).

A frequent theme emerging from the life experiences of donor offspring is that the system in place deceived them and was not justified (McWhinnie, 2001). As the HFEA

Code of Practice emphasizes that women are offered treatment only after accounting for the 'welfare of the child' to be born as a result of treatment, (Shenfield, 1994; Daniels, 1997) secrecy may not be considered in their best interest (Van den Akker, 2006). The qualitative study by Turner and Coyle of 16 donor-conceived adults between the ages of 26 and 55 has raised issues in donor conceived children such as a lack of trust in the family, a struggle to cope with their new identity and frustration because of opposition in the quest for their biological parent (Turner and Coyle, 2000).

Although there were concerns about the psychological wellbeing of children due to the damaging effect of secrecy and deception, (Daniels and Taylor, 1993) generally it was shown that children conceived using donor sperm develop normally with good psychosocial and emotional wellbeing and good parent-child relationships between parents and donor offspring (Golombok, 1997; McWhinnie, 2001; Van den Akker, 2006). However, it is also acknowledged that as the majority of children born by sperm donation treatments are unaware of the truth (Janssens *et al.*, 2006; Van den Akker, 2006), it is difficult to conduct large enough studies to know with certainty the impact of disclosure and anonymity (Frith, 2001; McWhinnie, 2001).

The concerns about removing anonymity included the parental worries about the rejection of the child by relatives, also the possibility of the child having to face isolation and bullying by their peers. Moreover the practice of anonymity helped the couple keep the male infertility problem a secret (Daniels and Taylor, 1993; Frith, 2001). Parents were worried that revealing the truth may cause psychological problems and rifts in the family, particularly between a father and his son (Frith, 2001).

While most of the damaging effects on donor offspring by denying them the knowledge of their birth origins is inferred from the literature on adopted children (Golombok, 1997), it was argued that the two groups are in fact different, as unlike adopted children, donor offspring are not disowned by their biological parents and also have direct genetic links with their mother (Frith, 2001). Despite the fact that clinicians and regulators in Reproductive Medicine worried about a potential fall in donor numbers, associated with the removal of donor anonymity (Cooke, 1993; Daniels and Taylor, 1993; Snowden, 1993; Hunt and Fleming, 2002; Dyer, 2004; Daniels *et al.*, 2005; Van den Akker, 2006), it was maintained that it did not justify denying the donor offspring the truth of their birth origin (Gollancz, 2001).

Donor conceived children campaigned for a change in the law to remove donor anonymity (Gollancz, 2001; Hamilton, 2010; Pacey, 2010). A public consultation was held in 2001 by the Department of Health to consider the issue (Department of Health, 2001). Although the British Fertility Society in general supported the move towards openness from anonymity, it felt that it was not the time for a radical change as both donors and recipients needed to be prepared for this with information, support and counselling. The concern of possible decline in the number of sperm donors coming forward was discussed. However, the idea of a twin track system with both anonymous and identifiable donors was not favoured to avoid discrimination and also to avoid potential conflicts in families where anonymous donors were used (Hunt and Fleming, 2002).

Following the feedback from public consultation alongside a background of public debates and campaigns from donor conceived individuals, the HFEA Disclosure of donor information Regulations 2004 (HMSO, 2004) was approved in the UK Parliament, which meant that prospectively donor conceived children would be allowed to know identifying information about their donor when they reached the age of 18. Only donors who agreed to be identified to the children born of their donation could be registered with the HFEA after 1st April 2005, which means that donor conceived children from 2005 will be able to contact their genetic parent (if they were aware that they were conceived using a donor gamete and wished to make contact with their biological parent). The amended Act came into force from 1st April 2005 and was not applied retrospectively. Licensed centres were allowed a transition period of one year during which they could continue to offer the service using previously recruited anonymous donors whilst recruiting identifiable donors. Therefore the Act was implemented in full with identifiable donors only from April 2006. Thereafter anonymous sperm donations from prior to 2005 could only be used for siblings of children conceived with the same donors.

After the removal of anonymity, a shortage of donor sperm was reported through workshops and surveys (Pike and Pacey, 2005; BBC survey, 2006). The experience from other countries that moved from anonymous to open donations prior to the UK (Austria, Australia and The Netherlands) has shown that the change did not negatively affect donor recruitment although the evidence was limited at the time of regulatory

change in the UK (Hunt and Fleming, 2002; Van den Akker, 2006; Blyth and Frith, 2008).

Sweden appeared to recover from the shortage (Daniels and Lalos, 1995; Gollancz, 2001; Dyer, 2004; Van den Akker, 2006), but recent reports indicate a shortage of sperm donors, long waiting time for donor sperm treatments and more than 250 Swedish sperm recipients travelling abroad every year for treatment, suggesting that the donor supply does not meet their needs (Ernst *et al.*, 2007; Ekerhovd and Faurskov, 2008).

In the Netherlands where gamete donor anonymity was abolished in 2004, during the 15 years of debate that preceded the legislative change, the number of donors decreased by more than 70% and the number of sperm banks by 50% (Janssens *et al.*, 2006). A questionnaire study on Danish sperm donors' views suggested that lifting anonymity may lead to shortage of sperm donors (Ernst *et al.*, 2007). In Denmark anonymous sperm donation is legal and Denmark also has (for unknown reasons) a higher number of sperm donors than other European countries (Pennings, 2010).

However a sperm donor shortage is seen globally and is not restricted to countries where donor anonymity has been removed. A decline in sperm donors has been reported in France, where donor anonymity is protected (Blyth and Frith, 2008). In the United States where there is no legislation regarding gamete donation (both identifiable and anonymous donors are accepted), an internet search of the 31 centres offering donor insemination which also recruit their own donors, showed that the number of open identity programmes were increasing and more importantly the longer a centre had offered an open identity donor recruitment programme, the higher the ratio of identifiable to anonymous donors (Scheib and Cushing, 2007) suggesting a shift towards being identifiable.

1.1.2 Payment to the donors

Prior to the HFEA, the payment to sperm donors for their donation was decided by local policies and these payments varied considerably from minimal to large amounts. When the HFEA was established it was decided that all clinics who were paying donors prior to 1991 could continue to do so with an upper limit set at £15 in addition to 'reasonable expenses', whilst clinics paying less than £15 were not allowed to increase to £15 and

new clinics were not allowed to make any payments at all until the authority had considered the issue in greater depth in the future (HFEA, 1996; Johnson, 1997).

The intention of the HFEA was to eventually phase out donor payment, and although it was unsure over what period the transition should take place research was commissioned to obtain information on the clinics' views on semen donation and the attitudes and motivations of semen donors.

A cross sectional survey of 144 sperm donors from 14 clinics revealed that 81% of donors were young, single students mainly motivated by payment and a survey of 81 fertility centres in the UK reported that the centres estimated to lose more than 80% of their donors, if the donors were not paid (Golombok and Cook, 1994; Cook and Golombok, 1995). The working group set up by HFEA reported in 1998, that gamete donation should be a gift voluntarily given with informed consent. The donation should be financially neutral; therefore the donors were to be reimbursed for the expenses incurred as a result of the donation but not for any loss of earnings. However recognizing that the removal of payments would further jeopardize the recruitment of donors, the HFEA decided to allow the centers to continue with payments of up to £15 for each gamete donation. So overall the sperm donors received £15 and a reimbursement of the expenses for donation (HFEA, 1998).

Sperm donors donate on an average 45 times to allow enough semen samples to create 10 families (Paul *et al.*, 2006). As the payment was for each donation, the sperm donors eventually received a higher total payment compared to egg donors because of the multiple ejaculates donated (Gazvani *et al.*, 1997; Pacey, 2010).

The discussion on payments to gamete donors has been ongoing since the commencement of HFEA in 1991. Some of the arguments are outlined below.

1.1.2.1 Arguments in favor of nonpayment of donors

The term 'donation' means an act of generosity whilst receiving payment alters the perception to one that views human gametes as a commodity. (Johnson, 1997; Pennings, 1997). Offering payment for the donation will promote a market for buyers and sellers of human body parts; the social implications of this being that the donor-conceived child could be perceived as a commodity from a commercial transaction and the welfare

of the child might therefore be compromised (Johnson, 1997; Yee, 2009; Hamilton, 2010). Furthermore payment may encourage both clinics and patients to relegate the role of a sperm donor in donor insemination treatment (Johnson, 1997).

Payment can financially induce donors by attracting vulnerable groups such as students or unemployed people. Financially motivated donors may not fully consider the implications of their donation. They may fail to recognize the potential emotional or psychological impact of their donation, the potential to generate problems in their own families in future and may even regret their decision years later (Johnson, 1997; Pennings, 1997).

The applicants could equally falsify their medical history and conceal important medical information that would otherwise make them unsuitable to become a donor (Guerin, 1998). However it is important to acknowledge that there is no evidence to suggest that paid donors were at higher risk for the transmission of any genetic or infectious diseases when compared to altruistic donors (Yee, 2009). On the contrary, altruistic donors with well-established careers may find payment demeaning which may in turn deter them from donation (Yee, 2009).

1.1.2.2 Arguments in favor of payment of donors

Studies have shown that removal of payments will further reduce the number of donors (Golombok and Cook, 1994; Gazvani *et al.*, 1997). The arguments in favor of payment to the donors are that the process of donation involves time, effort and also expense to the donor. It is not acceptable and amounts to exploitation if donors lose financially by donating gametes. Donors should be allowed fair payment for their time, effort, inconvenience and loss of earnings during the course of the donation, although authorities have difficulty in calculating the appropriate compensation for inconvenience that does not involve risk (Johnson, 1997; HFEA, 2011f).

The idea that sperm donation should be a gift without any background financial motivation, in circumstances where the patients are paying up to £8000, in a private setting for their fertility treatment and where private fertility specialists in the so called 'fertility industry' make good money by treating infertility, is argued to be inconsistent and irrational (Blackburn-Starza, 2009; Hamilton, 2010). It was widely argued that UK sperm donors were not adequately compensated and yet clinics were using imported

sperm from overseas commercial sperm banks, where the donors may have been paid (Heng, 2007; Hamilton, 2010; Tomlinson *et al.*, 2010).

Removal of payment to donors has the potential to attract different groups of men who may consider donating for the wrong reasons such as to prove their own fertility; however it can be argued that they are likely to come even if paid. Paid donors are likely to be more emotionally and psychologically detached from the outcome of their donation and therefore may be preferred to altruistic donors (Pennings, 1997; Yee, 2009).

Previously the majority of sperm donors were young single students. Many studies showed that they were motivated by the payment unlike married men with children who were keen on altruistic donation. Only studies with students showed that payment is the reason to donate and that they would stop donating if not paid (Cook and Golombok, 1995; Daniels *et al.*, 2006). The HFEA SEED review in 2004-5, revealed that the commonest age group for sperm donors was between 36 and 40. 69% of the donors were aged above 30 and 41.5% of the donors already had children. This is in contrast to 1994-1995 when the commonest age for sperm donors was between 18- 24 and only 21% of them had children of their own at the time of donation (SEED Report, 2005). This suggests a move towards more altruistic donors (Hamilton, 2010). Also a review of the 22 studies between 1980 and 2003 on sperm donors revealed that men who are willing to be identified shared their demographics (such as age and parental status) with men keen on altruistic donation (Daniels *et al.*, 2006).

The regulations on payment were further considered in the Sperm, Egg and Embryo Donation Review (SEED), which was published in January 2006 and implemented from 1st April 2006. The SEED Review steering group considered the evidence from a survey of UK clinics and reviewed the scientific evidence supporting HFEA policies followed by public consultation to produce the report.

At the time, the UK also adopted the EU Tissues and Cells Directive which led to a requirement to comply with new standards of quality and safety. Article 12(1) of the EU Tissues and Cells Directive provides that *'Donors may receive compensation which is strictly limited to making good the expenses and inconveniences related to the*

donation'. (Directive 2004/23/EC, 2004). This essentially supported voluntary and unpaid donations.

The SEED review concluded

- *'Donors may be reimbursed all demonstrable out-of-pocket expenses incurred within the UK in connection with gamete or embryo donation'*.
- *In addition to reimbursement of out-of-pocket expenses, donors may be compensated for loss of earnings (but not for other costs or inconveniences) up to a daily maximum commensurate with jury service but with an overall limit of £250 (or the equivalent in local currency) for each 'course' of sperm donation or each cycle of egg donation.*
- *Gamete donors may receive benefits in kind in return for supplying gametes for the treatment of others but these benefits should be limited to discounted treatment services (SEED Report 2005).*

However a “course” of donation was interpreted variously, even though the HFEA has defined it as the period commencing from the first consultation to the release of sample for treatment. It could be applied to a single sample or to all the samples donated by a single donor (Tomlinson *et al.*, 2010). Sperm donors donate about 45 times on average (Paul *et al.*, 2006) and an amount of 250 pounds for the entire course of donation is unlikely to cover all the costs incurred by the donor during the process of donation. In 2004 in Canada, the implementation of the Assisted Human Reproduction Act, led to the replacement of donor payment with reimbursement limited to reasonable expenses. In a study by Del Valle *et al.* (2008), of the 246 donor applicants who responded to the survey, 37.5% were willing to participate without reimbursement, whilst 44.4% declined and 18.1% were unsure, revealing the difficulty in recruitment without financial incentive. However, the willingness to participate without reimbursement was similar in all age groups, irrespective of occupation, marital status or whether the potential donor had children or not, contrary to previous findings.

It is noteworthy that in France, semen donation is entirely altruistic and has been offered for over 30 years (the donors are men living together with a woman, who have at least one child and are anonymous). Although a decline in donors has been reported, it reveals that donor recruitment is possible without payment (Guerin, 1998; Daniels *et al.*, 2006; Blyth and Frith, 2008).

In early 2011 the HFEA launched a 3 month public consultation to review some policies with a view to improve sperm and egg donation services in UK. The outcome of the consultation raised the possibility that donors might be losing money due to the expense incurred and inconvenience of donation. Compensation had to be decided at a level that would not deter men interested in donation, but at the same time would not financially induce donors. Following the consultation the new policy allows a fixed sum of £35 per visit including expenses for sperm donors. Gamete donors may continue to receive benefits in kind such as discounted treatment services or moving up the waiting list for treatment (HFEA, 2011a). The new policy is outside the period examined in this study and its impact will only be revealed over the next few years.

1.1.3 Updated UK guidelines for the screening of sperm donors

The guidelines in 2008 (Association of Biomedical Andrologists *et al.*, 2008), formulated by a multi-disciplinary group including BFS (British Fertility Society), BAS (British Andrology Society) and RCOG (Royal college of Obstetrics and Gynaecology) updated the 1999 British Andrology Society (BAS) guidelines (British Andrology Society, 1999) for the screening of sperm donors. The major changes to the previous guidelines included screening for Human T cell Lymphotropic viruses (HTLV) 1 and 2 and to exclude men with considerable risk of transmissible spongiform encephalopathies from donating sperm. The introduction of new guidelines did not lead to increased rejection of donors and therefore does not appear to have had any significant impact on the donor recruitment.

1.1.4 A comprehensive Andrology service

Locally, changes include the introduction of a comprehensive and integrated Andrology service in 2009 led by a Consultant Uro-Andrologist. Prior to 2009, the centre offered surgical sperm retrievals in the form of PESA (percutaneous epididymal sperm aspiration) and patients were referred elsewhere if they needed sperm retrievals for non-obstructive azoospermia. Since 2009 the centre has offered advanced surgical sperm retrieval techniques including micro TESE (micro surgical testicular sperm extraction) which has made own gamete treatment accessible to many more couples. The treatments usually involve the female partner going through controlled ovarian hyper stimulation concurrently. If sperm retrieval is unsuccessful those couples who have already been counseled and accepted donor back up, continue with donor IVF (D-IVF) treatment.

1.1.5 Sperm donor shortage

The British Fertility Society Working Party Report acknowledged a problem of donor sperm shortage which continued to affect the majority of UK fertility centres, leading to the cessation of donor sperm treatments in some centres. With the estimate that at least 500 sperm donors are needed per year to meet the UK's demand for treatment, (Hamilton *et al.*, 2008a) and with what is generally a low recruitment rate of less than 5% of applicants ultimately becoming sperm donors (Paul *et al.*, 2006), a large numbers of applicants are needed to come forward to meet the demand for sperm donation.

Many fertility centres have in the meantime introduced strategies to improve the efficiency of local donor recruitment. Despite the absence of published evidence, it is felt that centres have coped variably with the problem of sperm donor shortage. While the majority of UK fertility centres are struggling to recruit donors (Wardle, 2008), some centres are coping (Adams *et al.*, 2006; Ahuja, 2008; Tomlinson *et al.*, 2010). This problem of a national sperm donor shortage was recognised by the Department of Health and in consultation with the BFS in 2010, a project was funded to explore and improve national sperm donation although the findings have not yet been made public (Hamilton, 2010). Following a further public consultation in 2011 the HFEA introduced policies attempting to improve sperm donation services in the UK (HFEA, 2011f).

Overall briefly, in view of the current long waiting time for donor sperm treatments of about 18 months and a perception of an increase in sperm imports to NFC over recent years, we have discussed the various changes nationally and locally in the early years of the 21st century which may have had an influence on the centre's sperm donor programme. The described study aims to analyse the changes or trends in various aspects of the sperm donor programme at the NFC over 11 years from 2000 to 2010 to investigate if there is a problem with sperm donor recruitment.

1.2 Factors effecting semen quality

If a problem in donor recruitment is present then a more efficient use of existing resources may be of benefit. Previous work done at the centre revealed that only 3.63% of applicants were eventually released as sperm donors; 65% of applicants were rejected and the most common reason for this was suboptimal semen quality (Paul *et al.*, 2006) which is similar to the experience of several other UK fertility centres (Hamilton *et al.*, 2008a).

Suboptimal semen quality is a common problem and is poorly understood. Biological variation in semen quality is well known and is linked to accessory gland secretions, testicular size, sexual activity, length of abstinence and completeness of the sample collection at analysis. The intra-individual variations range widely and are associated with largely uncontrollable factors (World Health Organisation, 2010). Some external factors that may influence semen quality are reviewed here. Influences of season and vitamin D on semen parameters have been considered in the following sections as these two factors have been widely studied. A better understanding of their impact on semen parameters (if any) may be of value in improving the efficiency of a sperm donor programme and also donor recruitment.

1.3 Season and semen parameters

Seasonal variation in birth rate is reported in the majority of human populations, but the patterns have been variable (James, 1990; Rojansky *et al.*, 1992; Bronson, 1995; Ombelet *et al.*, 1996; Wellings *et al.*, 1999). For instance there was a deficit of spring births in United States, but in Canada and Europe the births are at their highest in late winter and spring (Bronson, 1995; Levine, 1999). However, studies also reported on changing patterns over time and recently on decline or loss of seasonality in births in western countries (James, 1990; Russell *et al.*, 1993; Cancho-Candela *et al.*, 2007).

Seasonal variation in birth is mostly due to corresponding changes in conception which in turn could be due to variations in the frequency of intercourse, female factors, (such as egg quality / ovulation / endometrial receptivity), semen quality or the loss of embryos at an early stage (James, 1990; Rojansky *et al.*, 1992; Bronson, 1995). Levine *et al.* (1990) suggested that the link may be stronger with a seasonal variation in semen parameters, as the female reproductive axis is protected from environmental changes by homeostatic mechanisms, as opposed to the restricted thermo regulatory capacity of the scrotum. Furthermore it was shown that the fluctuations in sperm parameters paralleled fluctuations in natural and IVF conceptions (Fisch *et al.*, 1997; Ossenbühn, 1998).

Seasonal variation in testicular function and spermatogenesis is seen in several animal species (Fraile *et al.*, 1989; Gosch and Fischer, 1989; Chemineau *et al.*, 2008). In humans also seasonal variation in semen parameters has been reported widely in the literature; however the findings are variable and inconsistent. Studies have been

conducted in various regions globally, but mostly in the Northern hemisphere (Levine *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Krause and Krause, 2002; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005; Detti *et al.*, 2006; Calonge *et al.*, 2009) than Southern (Sobreiro *et al.*, 2005) and in places with temperate (Levine *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Detti *et al.*, 2006; Calonge *et al.*, 2009), rather than tropical climates (Chia *et al.*, 2001; Sobreiro *et al.*, 2005). Studies from the UK are sparse.

As it is difficult to recruit men for longitudinal semen quality studies (Mortimer *et al.*, 1983) which may necessitate financial incentives to improve recruitment (Levine *et al.*, 1990; Levine *et al.*, 1992; Malm *et al.*, 2004). The majority of studies are retrospective observational studies, but some with large population size (Tjoa *et al.*, 1982; Levine *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Centola and Eberly, 1999; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Chia *et al.*, 2001; Chen *et al.*, 2003; Moskovtsev and Mullen, 2005; Detti *et al.*, 2006; Calonge *et al.*, 2009). Poster presentations in scientific conferences have also contributed to our knowledge (Moskovtsev and Mullen, 2005; Detti *et al.*, 2006). Few studies are prospective (Levine *et al.*, 1990; Levine *et al.*, 1992; Jørgensen *et al.*, 2001; Carlsen *et al.*, 2004; Sobreiro *et al.*, 2005).

A retrospective study involving 1159 samples from 903 men in New Orleans by Levine *et al.* (1988) revealed a significant deterioration in all semen parameters (count, motility and morphology) except semen volume in summer. The findings were confirmed in a subset of 61 men with paired samples (i.e. one in summer and the other in a season other than summer). The authors established the findings again in a prospective longitudinal study on 131 outdoor workers in San Antonio (Texas). There was significant deterioration in sperm count, total count and motile concentration in summer compared to winter by 34%, 24% and 28% respectively, but there was no seasonal difference in the percentage of motile sperm, furthermore sperm morphology was not assessed (Levine *et al.*, 1990).

Similar findings were seen in the large retrospective study by Gyllenborg *et al.* (1999) involving 1927 donors over a period from 1977 to 1995 conducted in Copenhagen, where there was a significant deterioration in sperm count and total concentration in

summer / autumn compared to winter / spring with no seasonal variation in semen volume or percent motility. Convincingly, the pattern of seasonal variation described was seen every year from 1977 to 1995 with few exceptions. Furthermore, a subset analysis of 32 donors with paired samples (summer / autumn and winter / spring) also confirmed the seasonal trend as described above although this did not reach statistical significance.

Sobreiro *et al.* (2005) study involving 500 men coming in for vasectomy from 1999 to 2002 at Sao Paulo (Brazil) in southern hemisphere where seasons are opposite to northern hemisphere (i.e. June to August is summer in northern hemisphere whilst it is winter in southern hemisphere), revealed a significant decline in all the semen parameters in summer compared to winter similar to the seasonal trends seen in many regions in the northern hemisphere (Levine, 1999).

Overall the majority of studies, (largely retrospective cross-sectional association studies) reported a significant seasonal variation in semen parameters whilst few studies disputed this (Mortimer *et al.*, 1982; Mallidis *et al.*, 1991; Ombelet *et al.*, 1996; Chia *et al.*, 2001; Carlsen *et al.*, 2004). Of those studies that showed a seasonal variation in semen parameters, a majority of the studies reported a deterioration in summer and or autumn or an improvement in winter and or spring (Tjoa *et al.*, 1982; Levine *et al.*, 1988; Reinberg *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Jørgensen *et al.*, 2001; Chen *et al.*, 2003; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005; Calonge *et al.*, 2009; Levitas *et al.*) whilst other studies reported varied patterns, for instance that sperm counts were lower in winter but higher in spring and autumn (Menge and Beitner, 1989); or the percent of abnormal sperm was higher in spring (Swatowski *et al.*, 1994), or progressive sperm motility was lower in summer and also spring (Centola and Eberly, 1999) and also that morphology was better in summer and winter (Detti *et al.*, 2006).

As mentioned some studies revealed a lack of seasonal variation. Ombelet *et al.* (1996) in their study, that analysed 340 semen samples from 107 men, did not reveal any seasonal variation in semen parameters (count, motility or morphology). The authors suggested that the lack of seasonal variation could be explained by large intra-individual variation in semen parameters. The effect of intra-individual variation in semen

parameters was reduced by considering the difference of each result from the mean (of at least 3 samples) for that individual.

It was pointed out that generally longitudinal studies, for no clear reason, revealed a lack of seasonal variation (Mallidis *et al.*, 1991; Ombelet *et al.*, 1996; Carlsen *et al.*, 2004) although this is not always seen (Levine *et al.*, 1990; Levine *et al.*, 1992). The longitudinal studies apart from allowing for intra-individual variation in semen parameters also avoid selection bias by seasonal recruitment (Carlsen *et al.*, 2004).

In the study by Krause *et al.*, although a significant trend for sperm count was seen in sub groups of men arranged by birth cohorts, with the peak counts being in different months (i.e. November, October and August), when the total group was analysed there was no seasonal variation identified (Krause and Krause, 2002).

The majority of studies investigated the seasonal variation in all semen parameters (i.e. volume, concentration, motility and morphology) whilst some parameters were omitted in a few studies particularly sperm morphology and sometimes motility (Tjoa *et al.*, 1982; Reinberg *et al.*, 1988; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Gyllenberg *et al.*, 1999; Chia *et al.*, 2001).

In studies showing seasonal variation, whilst some studies revealed a deterioration in all semen parameters (except volume) (Levine *et al.*, 1988; Andolz *et al.*, 2001; Sobreiro *et al.*, 2005); other studies revealed significant variation only in some parameters, mostly in sperm count or concentration parameters (i.e. total concentration, motile concentration etc) (Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Gyllenberg *et al.*, 1999; Krause and Krause, 2002; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005).

In the meta-analysis by Levine (1999) including 11 studies, although a pattern of low summer sperm counts was confirmed, there was no consistent seasonal variation in the rest of the parameters i.e. semen volume, sperm motility or morphology. In the study by Chen *et al.* (2004), where the sperm counts were significantly higher in spring compared to other three seasons, sperm motility and morphology also showed a similar trend, but were not statistically significant. In the study by Saint Pol *et al.* (1989), although there was higher sperm count in winter / spring in 4196 semen donors, there was no seasonal change seen in sperm motility.

Seasonal changes in semen volume have not been reported in majority of studies (Levine *et al.*, 1988; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Centola and Eberly, 1999; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Chen *et al.*, 2003; Moskovtsev and Mullen, 2005; Sobreiro *et al.*, 2005), however Reinberg *et al.* (1988) reported an increased semen volume in spring in men pre-vasectomy.

A prospective study conducted in four European cities (Denmark, Paris, Edinburgh and Turku i.e. temperate climate) revealed that despite regional differences in semen parameters, likely to be related to lifestyle and environmental exposure, there was a general seasonal variation with higher sperm counts in winter compared to summer (Jørgensen *et al.*, 2001).

Seasonal variation appeared to be more noticeable in temperate climates or non-equatorial countries than in equatorial / tropical climates, but this was not always the case. Despite being conducted in temperate climates, a prospective study of 27 healthy men over 16 months in Copenhagen (Carlsen *et al.*, 2004) and a large retrospective study with more than 1500 samples over 5 years in Edinburgh (Mortimer *et al.*, 1983), did not show any significant seasonal variation in semen parameters. However, in both studies there was a tendency for improved sperm counts in winter and or spring with no differences in other semen parameters.

In tropical climates, whilst a study by Chia *et al* conducted in Singapore revealed the absence of seasonal variation (Chia *et al.*, 2001), another study from Sao Paulo (Brazil) showed seasonal variation in sperm count, motility and morphology but not semen volume with parameters being significantly better in winter compared to summer (Sobreiro *et al.*, 2005).

Interestingly some studies considered four seasons whilst others noted the variation between two seasons or compared between summer and seasons other than summer. The majority of the studies defined winter as December to February, spring as March to May, summer as June to August and autumn as September to November (Centola and Eberly, 1999; Chen *et al.*, 2003; Carlsen *et al.*, 2004; Chen *et al.*, 2004; Levitas *et al.*, 2013), however the definitions of seasons varied (Moskovtsev and Mullen, 2005). Notably even in studies concurring with the seasonal variation, the semen parameters were at their peak in different months (Ossenbühn, 1998), for instance peak sperm

counts were seen in February / March in the Tjoa *et al* (1982) study whilst in April / May in the Reinberg *et al* (1988) study.

Almost all studies considered the season in which the semen sample was produced, however a recent study by Levitas *et al.* (2013), in addition, analysed the data by the season 70 days prior to the sample production i.e. season at spermatogenesis. The trend of seasonal variation by the season of sample production in semen parameters (better sperm concentration, rapid motility and sperm morphology in winter) persisted when the data was reanalysed by the season of spermatogenesis.

Overall there appears to be some evidence for a seasonal variation in semen parameters, at least in temperate climates, although the exact cause is unknown. A strong inverse association between the rising global temperatures and declining birth rates has been reported (Fisch *et al.*, 2003) and although the relationship between sperm count and male fertility is crude, a strong correlation between sperm counts and birth rates was shown in a population based study (Fisch *et al.*, 1997).

It was suggested that summer heat may suppress spermatogenesis and or testicular steroidogenesis or have a damaging effect on epididymal spermatozoa (Levine *et al.*, 1988; Bronson, 1995). In semen samples sperm viability and motility decrease at a significantly higher rate at 37 degrees Celsius compared to 20 degrees Celsius (Appell *et al.*, 1977). A temperature of 2-3°C lower than core temperature is needed for spermatogenesis (Snyder, 1990). A study by Mieusset and Bujan (1994) to investigate the potential of mild testicular heating as a contraceptive method for men revealed that one to two degree Celsius increase in testicular temperature decreased the sperm count and motility significantly. Men exposed to excessive heat at work such as 'plant and machine operators' were at higher risk of oligospermia (Chia *et al.*, 1994).

However it was argued (Snyder, 1990; Gyllenborg *et al.*, 1999) that summer heat may not be a major reason, as studies conducted in cooler climates i.e. where summer temperatures are moderate such as Basel in Switzerland, Lille in France and Edinburgh in Scotland also showed a considerable deterioration in sperm counts in summer compared to winter and spring (Mortimer *et al.*, 1982; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989).

Moreover, Levine *et al.* (1992) in their further study revealed that the deterioration in semen parameters in summer was not much different between outdoor workers and indoor workers (who worked in air conditioned environments), this did not support their former hypothesis of detrimental summer heat (Levine *et al.*, 1988; Levine *et al.*, 1990) and so the possible effect of photoperiod on endogenous circannual rhythm was suggested.

There was no seasonal variation in semen parameters (semen volume and sperm count) noted in men from Singapore i.e. a tropical region where the temperatures and photoperiod are generally stable throughout the year (Chia *et al.*, 2001). Similarly the lack of seasonal variation in Ombelet *et al.*'s study conducted in Belgium was also suggested secondary to minimal changes in the temperatures / photoperiod in summer and winter. However the authors mainly attributed this to the large intra-individual variation of semen parameters (Ombelet *et al.*, 1996).

Photoperiod (ratio of hours of day length to hours of darkness) may influence gametogenesis and steroidogenesis by a neuroendocrine pathway. The main components include melatonin (N-acetyl-5-methoxytryptamine) a neurohormone secreted by pineal gland. In response to the stimuli received by the retinohypothalamic tract, the suprachiasmatic nucleus in the hypothalamus generates a signal of day length, which is passed on to the pineal gland via the paraventricular nucleus (Bronson, 2004). Increased melatonin secretion leads to suppression of gonadotropin releasing hormone (GnRH), thereby suppressing follicle stimulating hormone (FSH) and luteinising hormone (LH) concentrations that control the changes in the testis. In animal studies the mode of action of the photoperiod was inferred as a signal that may either start or stop a breeding season by triggering gonadal development or regression; or which can synchronize an endogenously generated circannual rhythm of reproduction with seasonal changes in energy conditions (Bronson, 1995).

In humans, melatonin is secreted in a circadian pattern and mostly at night. As the secretion is dependent on the duration of darkness, a longer duration is expected in winter compared to summer, however studies on seasonal variation of human melatonin secretion have been conflicting and inconclusive (Bronson, 2004). Photo responsive mammals sense the seasons by the circulating levels of melatonin and in many farm animal species (sheep, goats, horses) artificial photoperiodic treatments such as indoor

lighting during short days or melatonin during long days are widely used to alter the breeding season or overcome the seasonal variation in semen parameters in artificial insemination centres (Chemineau *et al.*, 2008).

The neuroendocrine literature however, refutes that there is any form of reproductive photo responsiveness in humans (Bronson, 1995). In humans it has been shown that there is no difference in the duration of nocturnal melatonin secretion in summer and winter secondary to modern artificial lighting (Wehr *et al.*, 1995). Furthermore, some suggest that this phenomenon may be an evolutionary remnant in humans rather than a physiological functional mechanism in the current environmental conditions i.e. post industrial revolution (Gyllenborg *et al.*, 1999; Wehr, 2001) whilst others believe that there is large individual variation in responsiveness to photoperiod which may be responsible for the inconsistencies in studies in the literature (Bronson, 2004).

Malm *et al.* (2004) conducted a longitudinal semen quality study to investigate the effect of varying lengths of photoperiod involving 2 regions, north (69°N) and south (60°N) of the Arctic Circle (67°N). There is two months of total darkness during winter and 24 hour daylight during summer at the Arctic Circle. Men produced 2 semen samples one in summer and the other in winter, however there was no seasonal difference in the semen parameters (volume, count, motility) despite the fact that during the 11 week period prior to semen sample production (i.e. period of spermatogenesis), there was large variation in the daylight period (at least 19 hours longer in summer compared to winter at 69°N), suggesting that photoperiod does not affect semen parameters significantly.

Publication bias regarding the seasonal variation in semen parameters was suggested (Malm *et al.*, 2004) as studies investigating temporal trends, coming across significant seasonal variation as a chance finding, may have published although this was not the premise of the study originally.

The United Kingdom lies at a latitude of 50 to 58 degrees north and has a temperate climate, i.e. the weather is mild with temperatures generally not too far below 0°C in winter and not much higher than 32°C in summer. NFC's catchment area is most of the North East region in England. As the study undertaken nearest to our centre geographically, in Edinburgh, revealed a pattern of improved sperm counts in winter

and spring (though not statistically significant) (Mortimer *et al.*, 1983), we wondered if a similar pattern in semen parameters would emerge in men attending our centre. Evaluation of seasonal variation in semen parameters can be of clinical importance, as it could potentially have an impact on donor recruitment. This could then be positively utilised by timing the assessment of potential sperm donors for semen quality during the months of improved quality, to decrease the rejection rates related to suboptimal semen samples and also aiming for collection of semen donations, thus improving the efficiency of the sperm donor recruitment and donor programme.

1.4 Seasonal variation in donor conceptions

If a seasonal variation in semen parameters is confirmed, it is also worthwhile investigating the influence of this on conception rates to not only further understand the impact of season but also to strengthen the proposal of seasonal recruitment of donors. Semen quality is a surrogate measure of male fertility. In a population based study by Fisch *et al.* (1997) a significant correlation between sperm counts and birthrates was shown. Significantly improved semen parameters in winter were associated with peak of deliveries in autumn (Levitas *et al.*, 2013) similar to the poor parameters in summer associated with a deficit of spring births in USA (Levine, 1999). Furthermore in Ossenbühn's retrospective study, it was shown that in IVF patients, the fluctuations in the semen parameters were congruent with fluctuations in the IVF conception rates and this also coincided with fluctuations in natural conceptions in the normal population (Ossenbühn, 1998). Although there are several factors influencing the chance of conception, the studies mentioned reveal a possible role for variation in semen parameters to influence this.

Bonde *et al.* (1998) have shown that an increase in sperm count (but only up to a count of 40 million/ml) improved the chances of conception. Higher sperm concentrations did not improve the likelihood of conception. The contradicting reports in the literature on the association between sperm parameters and natural or assisted conception may be because of the lack of adjustment for confounding factors (such as female factors). In a review by Tomlinson *et al.* (2013), progressive motile sperm concentration was the most predictive semen parameter in both natural and artificial insemination for likelihood of conception. However the association between sperm morphology and conception was unclear owing to the variety of assessment methodologies, criteria for classification used and changing reference values.

Despite the fact that only men with optimal semen quality are recruited as donors, fecundity of sperm donors is variable and not always apparent from traditional semen parameters (Paraskevaides *et al.*, 1991; Thyer *et al.*, 1999; Navarrete *et al.*, 2000). Could this be related to the season of sperm production?

To evaluate the effect of seasonality of sperm on conception, we selected women undergoing donor insemination. The primary reason for this is that both the season in which sperm used for treatment was produced and the season of conception as well as the outcome (conceived or not) is known with certainty in this scenario. As this group of women are ovulating and are at low risk for tubal disease, they constitute a reasonably uniform group and variables such as frequency of intercourse and regularity of ovulation which affect the chances of conception can be excluded in this setting. To reduce the bias of other factors which can influence the chance of conception, we chose only unstimulated (natural cycles without ovulation induction drugs) and the intracervical insemination of unprepared sperm. Intrauterine inseminations which involve sperm preparation were excluded.

A study by Paraskevaides *et al.* (1988), showed that donor conceptions in the artificial insemination programme of their clinic were highest between October and March. Similarly in another study by Ronnberg (1989) in Northern Finland, i.e. Sub-arctic region, increased conceptions by donor insemination from October to March were seen. In contrast, in a large French epidemiological survey of an artificial insemination programme over six years, where frozen sperm was used, there was no seasonal variation in the donor conceptions (Mayaux and Spira, 1989).

As generally sperm used in donor insemination treatments is cryopreserved for quarantine purposes (to avoid transmission of sexually transmitted infections), it is usually considered as a constant factor unlikely to influence the seasonal variation in the donor conceptions. It is well known that freezing and thawing is generally associated with reduced motility, morphology, viability and sperm function (Mahadevan and Trounson, 1984; Yogev *et al.*, 2004), however interestingly seasonal variations are also reported in post thaw donor semen parameters with post thaw progressive motile density being considerably higher in winter /spring (Yogev *et al.*, 2004; Zhang and Yao, 2010).

Furthermore, post-thaw motility and morphology are positively correlated with pregnancy rates in donor insemination treatments (Mahadevan and Trounson, 1984; Johnston *et al.*, 1994). There is also some evidence to support that a prolonged duration of freezing does not adversely affect progressive motile concentration (Yogev *et al.*, 2010) and that fertilizing capacity is still comparable to that of fresh sperm (Marcus-Braun *et al.*, 2004). Therefore we decided for a novel approach of investigating the outcome of donor insemination treatments by the season of sperm production.

I aimed to analyse the distribution of conceptions achieved by donor insemination (DI) treatment, with a view to finding out if there was any variation in relation to the seasonal timing of the original sperm donation. I also aimed to investigate the seasonal variation in the semen parameters of donors used for the above insemination programme to get a comprehensive picture of the influence of season on semen parameters and pregnancy rates.

1.5 Association of vitamin D and semen parameters

1.5.1 Background

Vitamin D has been mainly recognised for its role in calcium homeostasis and bone integrity. The association of lack of exposure to sunlight and rickets was first revealed by Sniadecki in 1822 and only 100 years later it was demonstrated by Huldshinsky (1919) and Chick *et al.* (1923) independently that exposure to sunlight or artificially produced ultraviolet radiation B (UVB) cured rickets (DeLuca, 2004; Holick and Chen, 2008). However research in the past couple of decades has revealed the diverse role of vitamin D in human physiology. As vitamins are substances essential for health but not endogenously produced in required quantities, vitamin D is not a true vitamin owing to its significant cutaneous synthesis (Bosomworth, 2011). Furthermore vitamin D exhibits properties of hormones such as activation, release into the circulation and exertion of biological activity in various target tissues via receptors (DeLuca, 2004; Reichranth *et al.*, 2007; Bosomworth, 2011) and has been referred as a pluripotent steroid hormone (Verstuyf *et al.*, 2010).

The term vitamin D (VD) (calciferol) generally includes vitamin D2 (ergocalciferol) and vitamin D3 (colecalciferol) [Figure 1]. Vitamin D3 is formed by photosynthesis in the skin while vitamin D2 is derived by irradiation of ergosterol present in some yeast and fungi (mushrooms) (DeLuca, 2004). The A, B, C, and D ring structure is derived

from the cyclopentanoperhydrophenanthrene ring structure of steroids. As one of the rings is broken (the 9, 10 carbon-carbon bond of ring B for vitamin D₃) they are classified as seco-steroids.

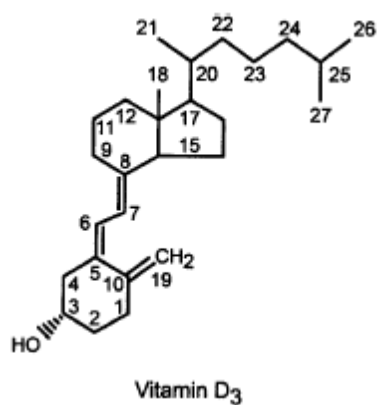


Figure 1: Vitamin D₃ (Colecalciferol)

1.5.2 Sources

The sources of vitamin D include endogenous cutaneous photosynthesis and dietary sources.

1.5.2.1 Cutaneous photosynthesis

The main source of vitamin D₃, (about 90% for most of the individuals) is synthesis in the skin upon exposure to ultraviolet solar irradiation (Webb *et al.*, 1988; Holick and Chen, 2008; Yuen and Jablonski, 2010). The initial step in the production of vitamin D₃ is the conversion of 7-dehydrocholesterol present in the dermal fibroblasts and epidermal keratinocytes of the skin to previtamin D₃ upon exposure to UV-B solar radiation at wave lengths between 290 -315nm. *In vitro* experiments have shown that there is absent cutaneous photosynthesis above this wave range (Webb *et al.*, 1988). This wave band (290 -315nm) is at the shortest wave length range of the solar radiation that reaches the earth and changes in magnitude with changes at the zenith angle. Solar zenith angle is the angle between a point on the ground (local zenith) and the line of sight from that point to the sun (Figure 2). Solar zenith angle alters with earth's revolution (season) and rotation (day and night).

It is well known that season is the major determinant of vitamin D₃ levels (Ashwell *et al.*, 2010). Apart from season, latitude affects the quantity and quality of solar radiation, in particular UVB radiation reaching the earth. Therefore cutaneous photosynthesis of vitamin D₃ is dependent on latitude, season of the year and time of the day. In winter months and in the early morning / late afternoon, the solar zenith angle is increased and the solar radiation is at a more oblique angle and whilst filtering through the ozone layer, leads to a decrease in the UVB radiation (290 -315nm) reaching the earth's surface (Webb *et al.*, 1988; Holick, 2007; Holick and Chen, 2008) and therefore reducing vitamin D₃ synthesis. Furthermore, clouds and aerosols can also considerably decrease the vitamin D₃ synthesis by affecting the UVB reaching the earth (Engelsen *et al.*, 2005).

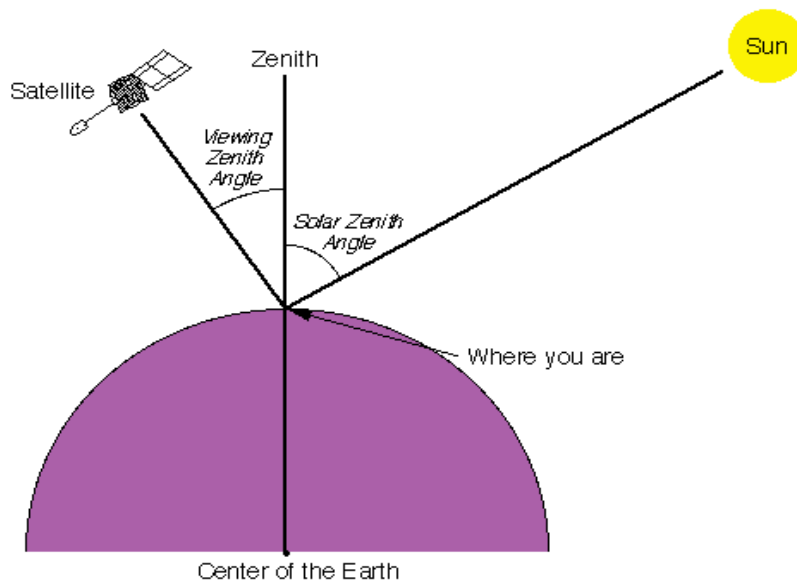


Figure 2: Schematic illustration of the Solar Zenith Angle (SZA) and Viewing Zenith Angle (VZA)

(<http://sacs.aeronomie.be/info/sza.php>)

In Boston (42.2 °N) there is very little UVB in the range (290 -315nm) detected between November to February and there is no cutaneous photosynthesis. Moving further in the northern direction to Edmonton (52 °N), there is no cutaneous photosynthesis between October to March whilst it is seen even in January as we move south towards the equator to Puerto Rico (18 °N) (Webb *et al.*, 1988). Unsurprisingly, as the United Kingdom lies at latitude of 50 to 58 °N, there is hardly any cutaneous photosynthesis between October and April (Pearce and Cheetam, 2010).

Importantly skin also contains a range of substances that absorb UVB, such as melanin, which affect cutaneous photosynthesis (Holick and Chen, 2008). It is estimated that two to three (sub-erythematous) sunlight exposures of thirty minutes duration per week, to the face and forearms at midday (when UVB intensity is at its peak) between April to October are sufficient to achieve healthy vitamin D levels in fair skinned individuals of the majority of UK (Department of Health, 1998; Ashwell *et al.*, 2010); while the frequency and duration needs to be increased two to ten fold in dark skinned individuals to achieve similar levels (Pearce and Cheetam, 2010).

1.5.2.2 Dietary sources

Dietary sources only play a minor role (Holick and Chen, 2008). The research by UK food standards agency (UKFSA) confirmed that the typical dietary intake of vitamin D (3.5-5.5µg) contributed less than exposure to sunlight to the average year round vitamin D levels, however it did acknowledge the major contribution from the dietary sources during the winter months (Ashwell *et al.*, 2010).

Dietary sources include: oily fish (salmon, trout, mackerel, herring, sardines, anchovies, pilchards and fresh tuna), egg yolk, cod liver oil, mushrooms and red meat. Margarine (7-8.8µg/ 100g), some breakfast cereals (17-33% of the RDA of vitamin D per serving) and infant formula milk (40-100IU per 100kcal) are the only foods fortified with vitamin D in the UK (Department of Health, 2000).

In 1930s fortified milk (100IU vitamin D₂ per 8 ounces of milk) helped eradicate rickets in the United States and Europe. An outbreak of hyper-calcaemia in the UK in 1950s, despite an absence of good evidence was attributed to the vitamin D fortification of milk and lead to the abandonment of the fortification of dairy products in Europe (Holick and Chen, 2008).

Vitamin D supplements are available in multivitamin preparations or by themselves in various strengths such as 400IU and 1000IU. In Canada the pharmaceutical form is vitamin D3 whilst in United States and Australia it is vitamin D2 (Holick and Chen, 2008; Roth *et al.*, 2008). Some of the food sources with vitamin D content are shown in Table 1 and vitamin D preparations available in the UK are shown in Table 2.

Source Type	Source	Vitamin D Content
Natural Sources	Salmon	
	Fresh, wild (3.5 oz) / Canned (3.5 oz)	About 600–1000IU of vitamin D3
	Fresh, farmed (3.5 oz)	About 100–250 IU of vitamin D3or D2
	Canned (3.5 oz)	About 300–600IU of vitamin D3
	Sardines, canned (3.5 oz)	About 300IU of vitamin D3
	Mackerel, canned (3.5 oz)	About 250IU of vitamin D3
	Tuna, canned (3.6 oz)	About 230IU of vitamin D3
	Cod liver oil (1 tsp)	About 400–1000IU of vitamin D3
	Shiitake mushrooms Fresh (3.5 oz)	About 100IU of vitamin D2
	Egg yolk	About 20IU of vitamin D3 or D2
Fortified foods	Fortified milk	About 100IU/8 oz, usually vitamin D3
	Fortified orange juice	About 100IU/8 oz vitamin D3
	Infant formulas	About 100IU/8 oz vitamin D3
	Fortified yogurts	About 100IU/8 oz, usually vitamin D3
	Fortified butter	About 50IU/3.5 oz, usually vitamin D3
	Fortified margarine	About 430IU/3.5 oz, usually vitamin D3
	Fortified cheeses	About 100IU/3 oz, usually vitamin D3
	Fortified breakfast cereals	About 100IU/serving, usually vitamin D3

Table 1: Dietary, Supplemental, and Pharmaceutical Sources of Vitamins D2 and D3

** IU denotes international unit, which equals 25 ng. To convert values from ounces (oz) to grams, multiply by 28.3. To convert values from ounces to millilitres, multiply by 29.6. ‡ When the term used on the product label is vitamin D or calciferol, the product usually contains vitamin D2; colecalciferol or vitamin D3 indicates that the product contains vitamin D3. [Adapted from (Holick, 2007)]*

Product	Vitamin D content
Colevit D3 (colecalciferol) solution	20,000IU/ml of vitamin D3
ProD3 (colecalciferol)	10,000IU capsules of vitamin D3
Dekristol	20,000IU capsules of vitamin D3
Sterogyl liquid	20,000IU/MI of vitamin D2
Adcal D3	400IU of vitamin D3 +600mg calcium
Calcichew D3 Forte	400IU of vitamin D3 +500mg calcium
Over the counter	
Calciferol tablets	400IU, 500IU, 1000IU

Table 2: Vitamin D supplements available in UK

1.5.3 Physiology

The natural vitamin D₃ is absorbed from the diet or more commonly produced in the skin upon ultraviolet irradiation of the cholesterol derivative, 7-dehydrocholesterol (provitamin D₃). The reaction in the skin appears to be an enzyme independent photochemical process. Ultraviolet light causes the electro cyclic rupture of the 9, 10 bond to produce previtamin D₃. Previtamin D₃ is biologically inert and isomerizes spontaneously to vitamin D₃. This is a temperature dependent process and at body temperature takes about 2-3 days (Webb *et al.*, 1988). The vitamin D₂ and D₃ from dietary sources are integrated in the chylomicrons and transported by the lymphatics to the circulation.

Vitamin D (include D₂ and D₃ from here onwards unless specified) is transported to the liver by vitamin D binding protein (DBP), a serum glycoprotein produced by the liver. Several cytochrome P450 enzymes (CYP) located in endoplasmic reticulum or mitochondria are involved in vitamin D metabolism (Table 3). Vitamin D is converted by mitochondrial vitamin D-25-hydroxylase (CYP2R1 supported by CYP27A1) to 25-hydroxyvitamin D [25(OH)D], which is the major circulating form of vitamin D. This is further metabolised by 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) to 1,25-dihydroxyvitamin D₃ (calcitriol) [1,25(OH)₂D], a biologically active form of vitamin D produced primarily in kidney but also in many tissues throughout the body (extra renal or local production) when sufficient substrate is available (Figure 3).

CYP27B1 is regulated by parathyroid hormone (PTH), calcium, phosphate and vitamin D metabolites, in particular by 1,25(OH)₂D (Figure 4) via feedback mechanisms to maintain optimal concentrations of 1,25(OH)₂D both in the circulation and also target cells (Prosser and Jones, 2004).

Gene name	Vitamin D metabolism	Protein function	Chromosomal location in human
CYP2R1	Activation	25-hydroxylase	11p15.2
CYP27A1	Activation	25-hydroxylase	2q33-qter
CYP27B1	Activation	1 α -hydroxylase	12q13.1-q13.3
VDR	Mediator	Receptor	12q13.11
CYP24A1	Inactivation	24-hydroxylase	20q13

Table 3: Chromosomal location of vitamin D receptor and metabolising enzymes
Adapted from (Blomberg Jensen, 2012)

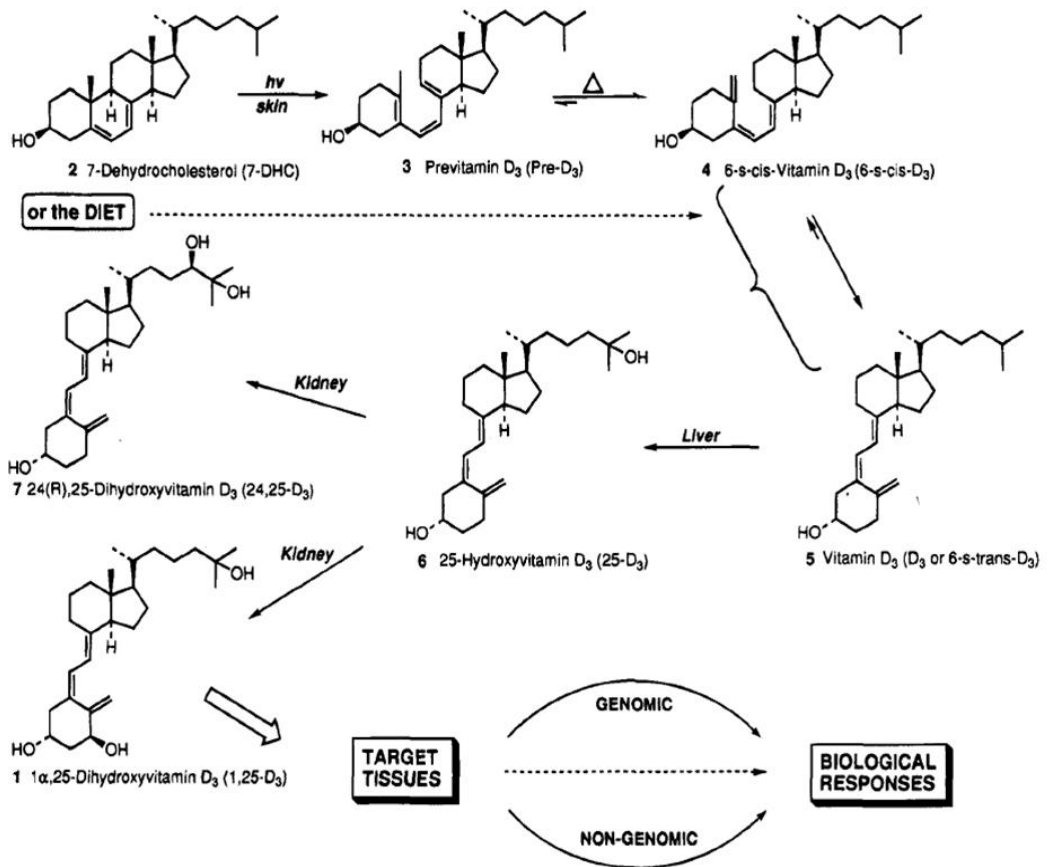


Figure 3: Metabolic pathway of vitamin D
Adapted from (Zhu and Okamura, 1995)

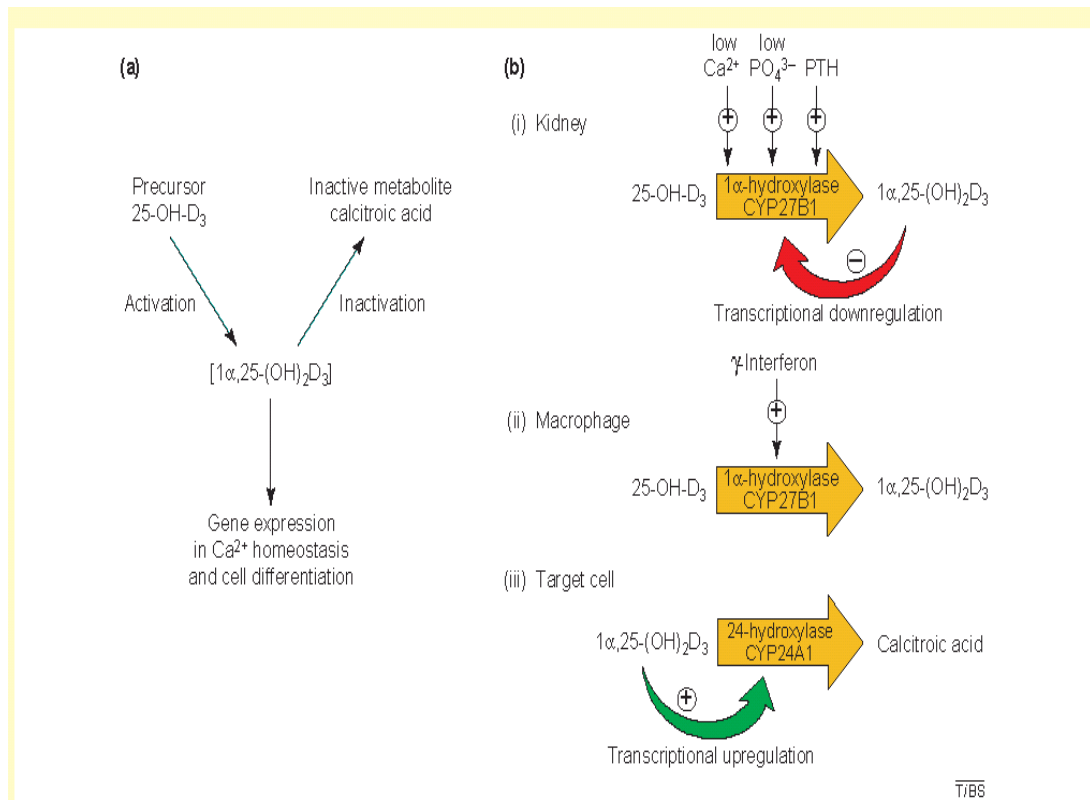


Figure 4: Regulation of vitamin D

(a) Regulation of the $1\alpha, 25\text{-(OH)}_2\text{D}_3$ concentration in vitamin-D-dependent gene expression. (b) Regulation of the expression of vitamin D hydroxylases: (i) renal 25-OH-D_3 - 1α -hydroxylase (CYP27B1); (ii) extrarenal 25-OH-D_3 - 1α -hydroxylase (CYP27B1); (iii) target cell $1\alpha, 25\text{-(OH)}_2\text{D}_3$ - 24 -hydroxylase (CYP24A1). Adapted from (Prosser and Jones, 2004)

25(OH)D has a half-life of 2-3 weeks whilst it is shorter for 1,25(OH)₂D (4-6 hours). When optimal levels of 1,25(OH)₂D are reached, 25-hydroxyvitamin D3-24-hydroxylase (CYP24A1) catabolises 25(OH)D and 1,25(OH)₂D in the kidney to 24,25-dihydroxyvitamin D [24,25(OH)₂D], which is further catabolised to water soluble inactive calcitroic acid and excreted in bile (Zhu and Okamura, 1995; Prosser and Jones, 2004; Lips, 2006). CYP24A1 is induced by 1,25(OH)₂D (feedback mechanisms to avoid toxicity) and CYP24A1 remains silent in vitamin D deficiency. Therefore CYP24A1 plays an important role in the determining the levels of 1,25(OH)₂D (Figure 4). Approximately 33 vitamin D metabolites, intermediates in the degradation of this molecule, have been identified (DeLuca, 2004).

Vitamin D and its metabolites including 25(OH)D, 1,25(OH)₂D and 24,25(OH)₂D are carried in the circulation bound to DBP (Zanatta *et al.*, 2011).

Cellular vitamin D responsiveness is complex and not as tightly regulated as systemic vitamin D metabolism. It is dependent on VDR expression, uptake of the substrate by the cells and metabolism of the circulating forms of vitamin D (Blomberg Jensen, 2012).

1.5.4 Mode of Action

1,25(OH)₂D is transported to the various target tissues by DBP where it binds to specific receptors leading to genomic and non-genomic responses.

1.5.4.1 Vitamin D receptors (VDR)

VDR is a DNA binding protein (427 amino acid peptide) with a molecular weight of about 50kDa and the gene encoding VDR is located on chromosome 12 (DeLuca, 2004).

The VDRs are not only localized in the classic target organs of 1,25(OH)₂D i.e. intestine, bone and kidney where they facilitate calcium homeostasis (Lips 2006), but also in over 38 different tissues such as the immune system, the reproductive system, and the endocrine system (DeLuca, 2004; Aquila *et al.*, 2008; Haussler *et al.*, 2011; Zanatta *et al.*, 2011).

A variety of tissues like bone (osteoblasts), colon, brain, breast, placenta, pancreas, prostate, lymph nodes, macrophages, skin and testis can produce 1,25(OH)₂D locally, by CYP27B1 under the influence of cytokines and this does not contribute to the circulatory 1,25(OH)₂D (Somjen *et al.*, 2007; Holick and Chen, 2008; Zanatta *et al.*, 2011). The presence of VDR and CYP27B1 in various tissues is suggestive of the autocrine / paracrine role of vitamin D.

1.5.4.2 Genomic response

The receptor for 1,25(OH)₂D belongs to the nuclear receptor superfamily which includes receptors for estrogen, progesterone, testosterone and cortisone. When 1,25(OH)₂D binds to the VDR, the receptor dimerizes with retinoid receptor and thereafter binds to the specific nucleotide sequences, vitamin D responsive elements (VDRE) located in the promoter regions of the target genes, recruiting either co-activators or co-repressors leading to either activation or suppression of the target genes (Figure 5).

This binding of 1,25(OH)₂D to VDR induces phosphorylation, conformational changes in the VDR and the release of co-repressors to allow gene expression. Following transcription and translation, a protein is formed (eg: osteocalcin), which elicits a physiological response (Lips, 2006; Haussler *et al.*, 2011; Zanatta *et al.*, 2011). The genomic pathway takes hours to days to produce the changes in the gene transcription (Lerchbaum and Obermayer-Pietsch, 2012). Vitamin D has been shown to regulate the expression of more than 1000 genes, reflecting its widespread biological activities (Bouillon *et al.*, 2008; Haussler *et al.*, 2011; Zanatta *et al.*, 2011) whereas the traditional genomic action of vitamin D included intestinal calcium and phosphate absorption, calcium reabsorption in the kidney and calcium mobilisation in the bone (DeLuca, 2004; Haussler *et al.*, 2011).

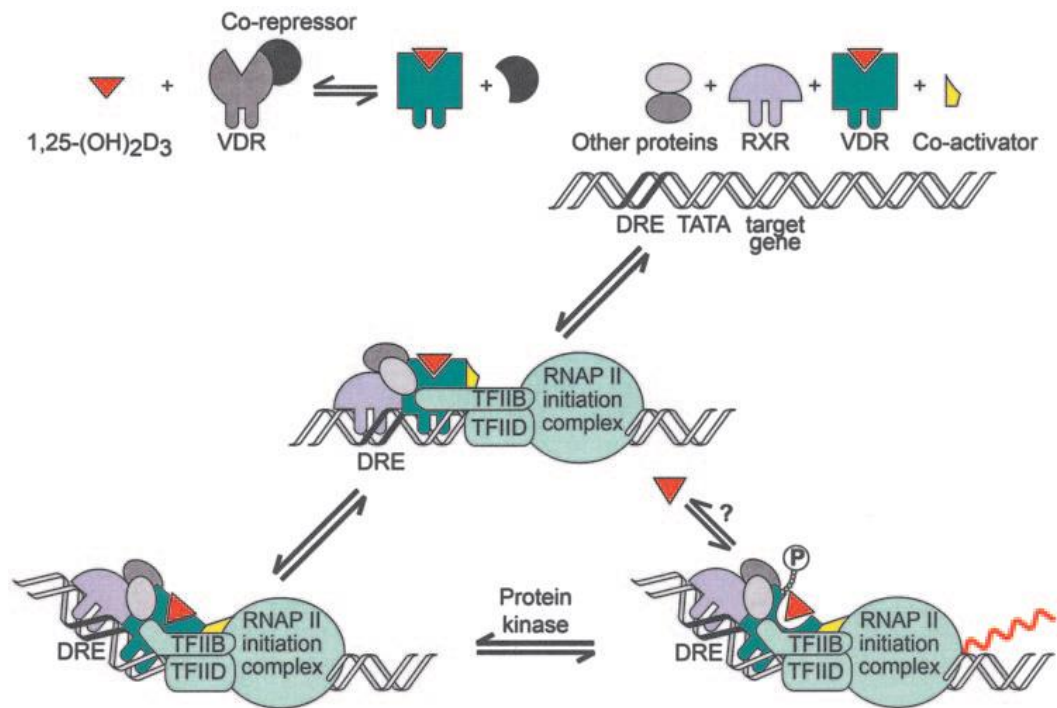


Figure 5: Diagrammatic representation of the known molecular events in the regulation of gene expression by the vitamin D hormone, 1,25(OH)₂D₃ acting through its receptor, VDR.

The result of regulation may be either suppression or activation. RXR, Retinoid X receptor; VDRE, Vitamin D response elements; TFIIB, transcription factor IIB; TFIID, transcription factor IID; RNAP, RNA polymerase. Adopted from (DeLuca, 2004).

1.5.4.3 Non-genomic response

The non-genomic actions of 1,25(OH)₂D are rapid (taking seconds to minutes) and act through a putative plasma membrane associated receptor. The membrane VDR primarily acts as a regulator of cytosolic second messengers such as cyclic Adenosine monophosphate (cyclic AMP) which in turn modulates the activity of various kinases [such as Protein Kinase A (PKA), Protein Kinase (PKC), Mitogen Activated Protein Kinase (MAP kinase)] and phosphatases. These enzymes are involved in the opening of calcium and chloride ionic channels leading to a biological response (Zanatta *et al.*, 2011).

This mode of action is seen in transcaltachia (the Rapid Hormonal Stimulation of Intestinal Calcium Transport), insulin secretion by pancreatic beta cells, in monocytes and vascular smooth muscle (Lips, 2006). In Sertoli cells in testis, 1,25(OH)₂D can modulate the kinase activities and influx of ions (such as chloride and calcium) at the plasma membrane, thus regulating the secretory process in these cells (Zanatta *et al.*, 2011).

Contrary to the description of receptors of vitamin D as above (nuclear and membrane), it has been argued that there is only one receptor for vitamin D mediating all its actions (DeLuca, 2004; Haussler *et al.*, 2011; Zanatta *et al.*, 2011). Structure function studies have revealed that VDR mediated rapid responses use a different ligand (6-s-cis) of the VDR, whilst the nuclear actions are generated when 6-s-trans ligand is occupied. In other words, VDR contains two ligand binding sites, a genomic pocket (VDR-GP) and an alternative pocket (VDR-AP), which when occupied by conformationally flexible vitamin D metabolites, lead to genomic and non-genomic responses respectively (Haussler *et al.*, 2011; Zanatta *et al.*, 2011). There is also some evidence to suggest that some of the genomic actions of vitamin D are initially triggered by the non-genomic pathway (Zanatta *et al.*, 2011).

1.5.4.4 Vitamin D receptor gene polymorphism

VDR gene polymorphism is the variation in the VDR gene DNA structure. VDR gene comprises 8 protein coding exons 2-9, 6 untranslated exons 1a-1f and several promoter regions (Nejentsev *et al.*, 2004). There are more than 470 single nucleotide polymorphisms (SNP) identified in the VDR (Toner *et al.*, 2010). Some of the commonly studied VDR polymorphisms are *FokI*, *BsmI*, *TaqI*, *Cdx-2* and *ApaI*. The

polymorphisms are generally named after the restriction enzymes initially used for genotyping. The effects of these polymorphisms on VDR activity are still not clear.

Although not seen in all studies, an increase in bone mineral density (BMD) with vitamin D supplementation that is dependent on the VDR genotype has been demonstrated (Graafmans *et al.*, 1997). This was also dependent on environmental factors such as calcium and vitamin D intake, with low calcium intake showing a positive association between specific VDR genotype and BMD, whilst increasing calcium intake showed no association. This can be explained by the fact that VDR dependent calcium absorption only occurs in calcium restrictive environments as opposed to when calcium is replete and VDR is not essential for calcium absorption (Pols *et al.*, 1998). VDR polymorphisms (*BsmI*, *FokI* and *Cdx2*) have been linked to low BMD and fracture risk but the results are inconsistent (Casado-Diaz *et al.*, 2013).

Furthermore in the recent past there are several studies in the literature to suggest an association of specific VDR polymorphisms with cancer (such as breast, colon and skin), atopic dermatitis, diabetes and immune mediated disorders (Toner *et al.*, 2010; Engel *et al.*, 2012; Gündüz *et al.*, 2012; Monticielo *et al.*, 2012; Sahin *et al.*, 2012; Heine *et al.*, 2013) but again the results are not always consistent. However the studies suggest that VDR gene polymorphism may alter the relationship between vitamin D status and various health conditions and increasing knowledge of these polymorphisms may explain some of the inconsistencies in the literature and also identify at risk groups for certain diseases.

1.5.4.4.1 Vitamin D receptor gene polymorphism and reproduction

VDR polymorphisms have been demonstrated in some gynaecological conditions such as endometriosis and PCOS.

Endometriosis: In a Brazilian study that recruited 132 women with endometriosis-related infertility, 62 women with unexplained infertility and 133 controls, similar VDR polymorphisms (*ApaI*, *TaqI*, *FokI*, and *BmsI*) were seen in the different groups. There was no difference in VDR polymorphisms in infertile women with and without endometriosis (Vilarino *et al.*, 2011).

Polycystic ovarian syndrome (PCOS): In a study by Wehr *et al.* (2011), including 545 PCOS women and 145 control women, genotyping of VDR (Cdx2, Bsm-I, Fok-I, Apa-I, and Taq-I), was performed. In PCOS women with the Cdx2 genotype, lower levels of fasting insulin, lower insulin resistance and higher insulin sensitivity was noted.

1.5.5 Epidemiology

Vitamin D deficiency is now identified as a worldwide problem because of its prevalence in the United States, Europe, the Middle East, Asia and Australia, affecting both adults and children (Holick and Chen, 2008; Bosomworth, 2011). Season mainly influences vitamin D levels in Northern latitudes by affecting cutaneous photosynthesis of vitamin D₃ (Webb *et al.*, 1988). From October to March, at latitudes north of 52° N, such as Scandinavia, the majority of Western Europe including 90% of the UK and 50% of North America, lack exposure to the UV-B necessary for cutaneous photosynthesis (Webb *et al.*, 1988; Pearce and Cheetam, 2010).

A nationwide survey of middle-aged British adults revealed that 16% had severe deficiency during winter and spring and the prevalence of hypovitaminosis D increased on moving northward in the UK, with the highest rates seen in Scotland, northern England and Northern Ireland (Hyppönen and Power, 2007). Similar findings were seen in other United Kingdom Food standards agency (UKFSA) funded studies (Ashwell *et al.*, 2010).

In the past vitamin D deficiency has been considered as a health problem predominantly affecting ethnic minority population in the UK, but the national survey revealed that it is a problem among the indigenous British population as well and is more common than previously thought.

However more recently, it was argued that the “epidemic” of vitamin D deficiency in North America was based on overestimation of adequacy and therefore population screening was not recommended (Aloia, 2011).

1.5.6 Risk Factors for vitamin D deficiency

One of the main risk factor for vitamin D deficiency is a lack of exposure to the sun. Increased time spent indoors, traditional clothing in some cultures (where most of the skin is covered) and pigmented skin limits the absorption of UV-B leading to reduced

synthesis (Glerup *et al.*, 2000; Lips, 2006; McKinney *et al.*, 2008; Greene-Finestone *et al.*, 2011). Furthermore melanin in the skin also reduces the synthesis of 7-dehydrocholesterol, thereby decreasing the availability of the substrate for vitamin D₃ synthesis (Aloia, 2011), making dark skinned individuals a high risk group. The use of sunscreen with sun protection factor 15 or more can block 99% of cutaneous synthesis of vitamin D₃ (Pearce and Cheetam, 2010). Advancing age is recognised as a risk factor as the skin synthesis of vitamin D₃ is reduced due to a decrease in precursor (7-dehydrocholesterol) concentrations in the skin as well as from a potential decrease in sun exposure (Lips, 2006; Holick and Chen, 2008).

There is a decreased to absent cutaneous synthesis of vitamin D₃ in winter months in northern latitudes (Webb *et al.*, 1988). One study however revealed higher vitamin D levels in northern European countries compared to southern, contrary to expectation. This difference could possibly be explained by dietary habits (consumption of fatty fish, a rich source of vitamin D in Northern European countries) and lifestyle with decreased sun exposure in residents of southern European countries (Lips *et al.*, 2001).

Vegetarians or people consuming a diet free of oily fish, pregnant and breast feeding women, infants and young children under 5 years and immigrants (specifically people with pigmented skin moving towards polar areas) are also at an increased risk (Holvik *et al.*, 2004; Andersen *et al.*, 2007). Obesity (as vitamin D gets sequestered in fat), chronic liver and renal disease, mal-absorption syndromes, use of some medications like anticonvulsants and glucocorticoids are other risk factors (Holvik *et al.*, 2004; Lips, 2006; McKinney *et al.*, 2008; Gagnon *et al.*, 2010; Aloia, 2011; Bosomworth, 2011; Greene-Finestone *et al.*, 2011).

A recent genome wide association study involving 33,996 participants of European origin from 15 cohorts (5 discovery cohorts, 5 replication cohorts and 5 de-novo replication cohorts), revealed that in individuals with genetic variation at the loci involved in cholesterol synthesis (DHCR7), vitamin D hydroxylation (CYP2R1) and vitamin D transport (GC) were at significantly increased risk of vitamin D insufficiency (Wang *et al.*, 2010).

1.5.7 Effects of vitamin D

The effects of vitamin D can be classified into skeletal and non-skeletal effects.

1.5.7.1 Skeletal effects

Vitamin D plays a vital role in calcium and phosphorous homeostasis. The classic effect of 1,25(OH)₂D is enabling active calcium absorption in the intestine by synthesizing calcium binding protein by its genomic action (Lips, 2006) and also promoting reabsorption of calcium in kidneys (DeLuca, 2004). Vitamin D promotes mineralization of the skeleton and the typical manifestation of vitamin D deficiency is rickets in children and osteomalacia in adults where mineralization of osteoid is affected.

Vitamin D deficiency leads to rise in serum parathyroid hormone levels (secondary hyperparathyroidism) causing bone resorption and osteoporosis (reduction in bone mineral density) thus increasing the risk of fractures (Lips, 2006). There is some evidence to suggest that vitamin D supplementation reduces the risk of falls and fractures (Yuen and Jablonski, 2010; Bosomworth, 2011). However it was pointed out that most of the studies included calcium supplements which makes it difficult to interpret the results (Aloia, 2011). In contrast a recent randomised controlled trial (RCT) where 500,000IU of vitamin D was given once yearly for 3 years revealed a 25% higher risk of fracture and a higher incidence of falls (Sanders *et al.*, 2010).

1.5.7.2 Non-skeletal effects

The presence of VDR in the majority of the tissues in the human body along with increasing observational data showing a link between vitamin D and various non-skeletal conditions such as autoimmune diseases, infections, cardiovascular diseases and cancers has triggered a plethora of studies to investigate this further. Studies also investigated the therapeutic role of vitamin D in these non-skeletal conditions. Reviews on the non-skeletal effects of vitamin D have been published (Toner *et al.*, 2010; Aloia, 2011; Rosen *et al.*, 2012). Here we briefly describe the biological plausibility of the association and a summary of the evidence.

1.5.7.2.1 Vitamin D and infections

Observational studies have shown increased an incidence of infections (such as influenza A, common cold and Respiratory Syncytial Virus infections) in winter months, which led to the hypothesis that low vitamin D may have a role in causation.

Further studies revealed an association between low serum vitamin D levels and an increased risk of viral and bacterial infections including tuberculosis and there is also some evidence to suggest vitamin D supplementation decreased this risk (Yuen and Jablonski, 2010; Bosomworth, 2011). Historically it was noted that increased exposure to sunlight was beneficial in the treatment of tuberculosis.

There is data to suggest that macrophages infected with mycobacterium tuberculosis increased the production of 1,25(OH)₂D locally (paracrine pathway) and increased the expression of VDR. This combination then leads to up-regulation of the gene expressing the bactericidal protein cathelicidin, which attacks Mycobacterium tuberculosis or other infective agents and also triggers cells' autophagy pathways (Holick and Chen, 2008; Rosen *et al.*, 2012). However, the data is still inadequate to suggest vitamin D supplementation for treatment or prevention of infections.

1.5.7.2.2 Vitamin D and cancer

The potential role of vitamin D in cancer can be explained by *in vitro* studies, which apart from revealing VDR expression in most tissues including tumors also suggest the possibility of local production of 1,25(OH)₂D in a variety of tissues. Furthermore *in vitro* studies suggest 1,25(OH)₂D promotes cell differentiation, inhibits cell growth (by regulating genes responsible for cell proliferation) in a variety of cell types and also exhibits pro-apoptotic and anti-angiogenic properties (Lips, 2006; Fleet, 2008; Toner *et al.*, 2010; Verstuyf *et al.*, 2010; Bremmer *et al.*, 2012; Rosen *et al.*, 2012).

Several observational studies have shown the link between living at high altitude or low exposure to sunlight or low serum 25(OH)D levels, to an increased risk of breast, colon, prostate and other cancers (Holick and Chen, 2008; Verstuyf *et al.*, 2010; Yuen and Jablonski, 2010). As higher levels of vitamin D were associated with lower estradiol and progesterone levels in healthy young women, it was hypothesized that this may be a potential mechanism in the reduction of breast cancer risk (Knight *et al.*, 2010). However there are studies in post-menopausal women which did not show an inverse relationship between vitamin D and breast cancer (Freedman *et al.*, 2008). Furthermore the studies investigating the association of vitamin D and risk of prostate cancer revealed inconsistent results (Toner *et al.*, 2010). Studies including a meta-analysis suggested that increasing intake of vitamin D was associated with a decreased risk of colon, breast and other cancers. Whilst the evidence is promising for colorectal

cancer it is inconsistent for breast cancer (Toner *et al.*, 2010). In the WHI (Women's Health initiative) trial involving 36,000 women, the 7 year intervention of vitamin D3 400IU and 1000mg of calcium per day did not reduce the incidence of total cancer or cancer mortality (Wactawski-Wende *et al.*, 2006; Chlebowski *et al.*, 2008). A large scale study found no evidence of a protective association between higher serum 25(OH)D and cancer outcome in 7 less common cancers including endometrial and gastric cancers. Conversely an increased risk of pancreatic cancer was noted with serum 25(OH)D levels above 100nmol/l (Helzlsouer and Vdpp Steering Committee, 2010). Furthermore there is some evidence of increased risk of esophageal and prostate cancers with higher levels of vitamin D levels in subgroups of the general population (Toner *et al.*, 2010).

1.5.7.2.3 Vitamin D and cardiovascular disease

Epidemiological studies revealed higher rates of hypertension and cardiovascular diseases with increasing distance from the equator, raising the possibility for a role of vitamin D. The potential for a role in cardiovascular diseases is explained by the expression of VDR in cardiac muscles and vascular smooth muscle. Vitamin D deficient mice or VDR knockout mice develop hypertension and cardiac hypertrophy (Verstuyf *et al.*, 2010). Pilz *et al.* (2008) revealed that low levels of vitamin D were associated with an increased risk of heart failure and sudden cardiac death. However there are only a few observational studies investigating the association between serum 25(OH)D and cardiovascular disease and hypertension and the results are inconsistent (Rosen *et al.*, 2012) although a 'U' shaped association as described later has also been suggested (Ross *et al.*, 2011).

1.5.7.2.4 Vitamin D and autoimmune disease

The role of vitamin D as an immune modulator can be explained by the presence of VDR in most cells of the immune system (monocytes, macrophages and lymphocytes) and many cells can synthesize the active form of vitamin D locally. It is involved in cell mediated immunity by down regulating inflammatory markers such as interleukins and tumour necrosis factor (IL1, IL2, IL-6 and TNF α) (Lips, 2006; Verstuyf *et al.*, 2010; Grundmann and von Versen-Hoyneck, 2011). The localization of VDRE in the human insulin receptor gene promoter (Maestro *et al.*, 2003) makes it biologically plausible to hypothesize a role for vitamin D in the pathogenesis of diabetes. Furthermore in

diabetic rats, vitamin D supplementation improved the insulin response to glucose transport in adipocytes (Calle *et al.*, 2008).

Epidemiological studies have shown the association between low vitamin D levels and auto immune diseases such as Type I diabetes, multiple sclerosis and inflammatory bowel disease (DeLuca, 2004). The data is inadequate however, to support vitamin D supplementation for the treatment or prevention of autoimmune disorders.

Overall, following a comprehensive review of the available evidence regarding vitamin D and extra skeletal outcomes, it was concluded that the associations are inconsistent and the evidence regarding causation was insufficient and inconclusive (Ross *et al.*, 2011; Rosen *et al.*, 2012). Furthermore there is some evidence suggestive of a reverse 'J' or 'U' shaped association i.e. increased risk at high vitamin D levels of some non-skeletal conditions including cardiovascular disease, pancreatic cancer, oesophageal cancer and overall mortality (Ross *et al.*, 2011; Rosen *et al.*, 2012). Clinicians are warned of the potential adverse effects of levels above 125nmol/l (50ng/ml) (Aloia, 2011). It has to be acknowledged however, that the data on the extra-skeletal benefits of vitamin D are convincing enough to have initiated large scale randomised controlled studies.

1.5.8 Assessment of vitamin D status

Serum 25(OH)D is considered as the best measure of estimating vitamin D status, (Roth *et al.*, 2008; Pearce and Cheetam, 2010; Aloia, 2011), as it has a long circulating half-life of 2 to 3 weeks (Lips, 2001). This also enables the achievement of consistent results (Bosomworth, 2011).

Methods

There are several methods of measuring serum 25(OH)D and the variability in measurements due to utilized assay technology is well known (Carter *et al.*, 2004; Roth *et al.*, 2008; Thienpont *et al.*, 2012). The two common methods used are immunological (antibody-type) methods and liquid chromatography based methods. The assay methods essentially involve the process of releasing the 25(OH)D from its binding protein followed by quantification of the molecule. The gold standard is liquid chromatography tandem mass spectrometry (LC-MS/MS) (Holick and Chen, 2008; Roth *et al.*, 2008; Aloia, 2011). This involves chemical release of hydroxyvitamin D,

followed by a protein precipitation (sample purification) and then separation of the 25(OH)D by chromatography and finally quantification by mass spectrometry.

Some of the other routinely available methods are high-performance liquid chromatography (HPLC), the Immunodiagnostic System's radioimmunoassay (IDS-RIA) and enzyme immunoassay (IDS-EIA) and DiaSorin's automated immunoassays (Liaison 1 and Liaison 2) and automated immunoassay (Elecsys). With increasing demand for vitamin D assays many laboratories have moved onto automated immune assays from the traditional manual techniques. As there are method-related differences in 25(OH)D results, standardization of methods against a reference method would help to reduce the inter-method variability (Roth *et al.*, 2008).

The assays also differ in 25-hydroxyvitamin D3 and D2 metabolite recognition. Assays estimating 25-hydroxyvitamin D3 and D2 incorporate cutaneous synthesis and dietary intake (such as foods, fortified products and supplements), although it is noteworthy that 25-hydroxyvitamin D2 levels are low to undetectable in those who have not received vitamin D supplementation. Moreover in the majority of countries vitamin D supplementation is by Colecalciferol, however in United States and Australia it is by Ergocalciferol (Roth *et al.*, 2008).

The majority of the concerns about imprecise serum 25(OH)D assays are overcome by advances in methodology, calibration of equipment and regular participation in quality control assessments (Ross *et al.*, 2011). The Vitamin D external Quality Assessment scheme (DEQAS) is an international on-going multicentre trial of measuring various vitamin D assays for their specificity and accuracy apart from offering an opportunity for the participating centres to maintain their standards (Carter *et al.*, 2004). DEQAS is discussed in further detail in Chapter 6 (Section 6.5.8.1.1).

1.5.9 Classification of vitamin D status

The classification of vitamin D status including the optimal levels of vitamin D is ongoing and debated extensively (Grundmann and von Versen-Hoynck, 2011) although many appear to accept serum concentrations of 50nmol/l or ≥ 75 nmol/l as optimal. The higher levels were suggested based on the evidence of suppression of parathyroid hormone and some evidence on maximal intestinal calcium absorption above this level whilst levels of 50nmol/l were recommended based on the evidence on optimal skeletal

health (Dawson-Hughes *et al.*, 2005; Lips, 2006; Hyppönen and Power, 2007; Holick and Chen, 2008; Aloia, 2011). Serum concentrations of vitamin D < 25nmol/l are considered as deficiency as this can cause softening of bone and present as rickets in children and osteomalacia in adults (Ashwell *et al.*, 2010; Bosomworth, 2011), therefore levels above this (i.e. >25nmol/l) are internationally recognised as the most conservative threshold of adequacy.

A widely used classification of vitamin D status in relation to serum concentration is presented here (Pearce and Cheetam, 2010; Blomberg Jensen *et al.*, 2011) whilst recognising the modified classifications published in literature (Aloia, 2011; Bosomworth, 2011)

- < 25nmol/l - Deficiency
- 25-50nmol/l –Insufficiency
- 50-75nmol/l – Adequate
- >75nmol/l – Optimal

The Institute of Medicine (IOM) has further added:

- >125nmol/l – Risk of excess (Aloia, 2011)

The common variations noted are:

- < 40nmol/l –insufficiency or inadequacy.

The concentrations of vitamin D are given in molar units (nmol/l); to convert to mass units ng/ml divide the molar units by 2.496; to convert to mass units µg/l, multiply the molar units by 0.4.

1.5.10 Recommended daily intake of vitamin D

In the UK, the recommended daily intake of vitamin D for an adult is 400IU (Department of Health, 1998), however it has been argued that this intake would provide adequate vitamin D levels to only prevent osteomalacia (Pearce and Cheetam, 2010). The institute of medicine (IOM) recommends a daily intake of 600IU of vitamin D for ages 1-70 years and 800IU for above 70 years old, with optimal bone health being the target. This advised intake is also based on the assumption of a lack of adequate sun exposure and aims for 50nmol/l of serum vitamin D. The recommendations by Endocrine Society of North America however, for patients at risk for vitamin D deficiency (acknowledging the fact that vitamin D deficiency is very common)

suggested a higher intake and aim for sufficiency of serum vitamin D i.e. 75nmol/l. (Holick *et al.*, 2011).

Despite a high therapeutic index of vitamin D and toxicity seen at levels of 500nmol/l, the upper limits for intake in adults suggested are 4000IU / day (corresponding to serum vitamin D level of 125nmol/l) as the risk of harm such as hypercalcemia, hypercalciuria, soft tissue calcification, and nephrolithiasis increases above this level (Aloia, 2011; Ross *et al.*, 2011). Pharmaceutical preparations with vitamin D2 are as effective as vitamin D3 in maintaining serum 25(OH)D levels (Holick and Chen, 2008).

1.5.11 Management of vitamin D deficiency

Although there are several treatment regimens, the principle of treatment remains to replenish the stores with high dose vitamin D for a short period of 8-12 weeks, followed by low dose maintenance therapy. It has been demonstrated that serum vitamin D increases by approximately 2.5 nmol/l (1 ng/ml) for every 100IU of oral vitamin D taken each day (Heaney, 2008). The oral route is better tolerated and is more effective because of improved absorption than parenteral forms (Grundmann and von Versen-Hoynck, 2011).

As vitamin D is stored in fat and gradually released, it does not matter whether vitamin D is given weekly, monthly, 6 monthly or annually (Lips, 2001; Bosomworth, 2011). Hydroxylated and potent forms like Calcidiol and Calcitriol are not recommended as they are not only ineffective but can cause hypercalcemia, which can be dangerous. Life style changes such as maximal use of sunlight and dietary modification also improve vitamin D. Some suggested medical regimes are shown in Table 4 and Table 5.

Calciferol dose	Frequency	Route	Length of course
10,000 IU (0.25mg)	daily	Oral (capsules)	8-12 weeks
60,000 IU (1.5mg)	Once Weekly	Oral	8-12 weeks
300,000 IU (7.5mg) Ergocalciferol	Twice	Oral / IM	Two stat doses, one month apart

Table 4: Deficiency [25(OH)D <25 nmol/l) – Treatment for Adults

	Frequency	Route	Length of course
Colecalciferol or Ergocalciferol tablets 1000-2000 IU	Daily	Oral	Indefinite
Colecalciferol capsules 10,000 IU.	Weekly or twice monthly	Oral	Indefinite

Table 5: Maintenance treatment for adults following treatment of Deficiency [25(OH)D <25 nmol/l)

Tables adapted from (Pearce and Cheetam, 2010)

1.5.12 Vitamin D and Reproduction

The diverse roles of vitamin D in human physiology also include a potential role in reproductive physiology. The role of vitamin D in reproduction both in males and females has been shown in animal experiments for decades, however there is increasing evidence in the recent literature to suggest a potential role of vitamin D in human reproduction also.

1.5.12.1 Vitamin D and Female Fertility

The expression of VDR in female reproductive tract followed by studies investigating the role of vitamin D in female reproduction and pregnancy are described in this section.

1.5.12.1.1 Expression of VDR and vitamin D metabolising enzymes in female reproductive system

In female rats, immunohistochemistry studies using polyclonal antibodies against VDR have localized VDR in ovary (granulosa cells, theca cells, stroma, corpus luteum and germinal epithelium), Fallopian tubes and uterus (Johnson *et al.*, 1996). In humans VDR and the metabolising enzymes are expressed in the endometrium and ovaries (Agic *et al.*, 2007; Parikh *et al.*, 2010; Grundmann and von Versen-Hoynck, 2011). Furthermore VDR expression in the human pituitary gland has been shown suggesting a role of vitamin D in hormone secretion (Pérez-Fernandez *et al.*, 1996).

1.5.12.1.2 Studies on female reproductive function

Animal studies have shown that vitamin D deficiency affects female reproductive function, reducing overall fertility by 75% and litter sizes by 30% (Halloran and DeLuca 1980). VDR null mutant mice showed impaired folliculogenesis, infrequent conception, uterine hypoplasia, decreased aromatase expression, hypergonadotrophic hypogonadism, fewer viable fetuses in utero and also more offspring with low birth weight (Yoshizawa *et al.*, 1997; Kinuta *et al.*, 2000; Kovacs *et al.*, 2005) than wild type controls.

Similarly the transgenic 25-hydroxyvitamin D-1 α -hydroxylase female mice, were infertile and showed decreased estrogen and progesterone levels, elevated FSH and LH, adverse effects on folliculogenesis, uterine hypoplasia and decreased ovarian expression

of angiogenic factors such as VEGF and angiopoetin (Panda *et al.*, 2001; Sun *et al.*, 2010).

Vitamin D deficient female rats maintained on diets varying in calcium concentrations including normal calcium levels, when mated with vitamin D replete males, showed reduced litter sizes and fertility compared to vitamin D replete female rats. Furthermore the effect was reversed on supplementing vitamin D to deficient female rats suggesting that vitamin D has a direct effect on female reproductive function (Kwiecek *et al.*, 1989b). Similarly Kinuta *et al.* also showed supplementation of calcium only partially corrected the hypogonadism in VDR null mice and vitamin D directly regulated aromatase gene expression (necessary for estrogen synthesis) and was essential for full gonadal function.

However there have been studies undertaken on VDR null mice or transgenic 25-hydroxyvitamin D-1 α -hydroxylase mice where impaired reproductive function was restored by calcium, or calcium and phosphorous supplementation, suggesting an indirect action of vitamin D (Johnson and DeLuca, 2001; Sun *et al.*, 2010).

A possible mechanism of action of vitamin D in female fertility is through its role in steroidogenesis. In a study on cultured human ovarian cells, vitamin D stimulated steroidogenesis (progesterone production by 13%, estradiol production by 9% and estrone production by 21%) and insulin-like growth factor binding protein-1 (IGFBP-1) production in the ovary (Parikh *et al.*, 2010). In female rats injection of 1,25(OH)₂D induced a decidual reaction and increased uterine weight (Halhali *et al.*, 1991). In mice, peripubertal vitamin D deficiency delayed puberty and disturbed the estrous cycle suggesting vitamin D has a role as a regulator of the hypothalamo-pituitary axis, as an adequate vitamin D level appears to play an important role in the pubertal transition (Dicken *et al.*, 2012).

Malloy *et al.* (2009) identified a sequence in the anti-mullerian hormone (AMH) promoter gene that is almost identical to VDRE. In a HeLa (cervical cancer cell line) transfected with VDR, calcitriol stimulated the AMH promoter activity. As AMH is produced by the granulosa cells of the ovarian follicles and regulates follicular recruitment and selection, it was hypothesized that vitamin D may play a role in ovarian physiology by its regulatory role on AMH expression (Luk *et al.*, 2012)

Conversely in the study by Horii *et al.* (1992), vitamin D (with associated hypercalcemia) caused disturbances of the estrous cycle, adverse functional changes in the corpus luteum, uterus, endometrium and decreased progesterone but these changes were reversible on discontinuing vitamin D. Also in 101 healthy young women aged between 18 and 22 years, it was shown that higher levels of vitamin D are associated with decreased estradiol and progesterone levels (Knight *et al.*, 2010).

Studies have been undertaken to investigate the association of vitamin D and gynaecological conditions causing infertility (such as endometriosis and PCOS) and also in the IVF setting.

1.5.12.1.2.1 Endometriosis

An increased expression of the VDR and metabolising enzymes is shown in endometriosis, whilst the serum concentrations of 25(OH)D were comparable in women with and without endometriosis, suggesting vitamin D by its potential autocrine / paracrine role may influence local immune cells and cytokines involved in the pathogenesis of endometriosis (Agic *et al.*, 2007). In a prospective cohort study however, involving 87 women with endometriosis and 53 controls, higher serum levels of vitamin D were associated with increased risk of endometriosis. Moreover a positive gradient was recorded between serum vitamin D and the severity of the endometriosis (Somigliana *et al.*, 2007). Serum and peritoneal concentrations DBP are comparable in women with and without endometriosis (Borkowski *et al.*, 2008), however in a study by Faserl *et al.* (2011), DBP polymorphism (GC-2) was more prevalent in women with endometriosis compared to controls.

1.5.12.1.2.2 PCOS (Polycystic ovarian syndrome)

As both vitamin D deficiency and PCOS are associated with features of the metabolic syndrome, (particularly insulin resistance) several studies have investigated the role of vitamin D in the pathogenesis of PCOS (Luk *et al.*, 2012). Women with PCOS are at a higher risk of vitamin D insufficiency and deficiency compared to women without PCOS (Wehr *et al.*, 2009; Selimoglu *et al.*, 2010; Li *et al.*, 2011). In PCOS women, vitamin D levels show a significant inverse relation with BMI, free androgen index and insulin resistance (generally measured by the Homeostasis model assessment of insulin resistance –HOMA IR) and a positive correlation with insulin sensitivity (generally

measured by quantitative insulin sensitivity check index QUICKI) (Wehr *et al.*, 2009; Li *et al.*, 2011; Wehr *et al.*, 2011).

PCOS women with metabolic syndrome have lower levels of serum vitamin D levels compared to PCOS without metabolic syndrome (Wehr *et al.*, 2009). Similarly in the study by Yildizhan *et al.* (2009) serum vitamin D levels were lower in obese PCOS participants compared to non-obese PCOS and an association of low vitamin D with increased insulin resistance, BMI, testosterone and DHEAS was noted in obese PCOS participants.

It has been argued that a major limitation in most of the studies investigating the association of low vitamin D levels and PCOS is the confounding factor obesity and therefore whether vitamin D deficiency independently contributes to pathogenesis of PCOS is still debatable (Grundmann and von Versen-Hoynck, 2011; Lerchbaum and Obermayer-Pietsch, 2012; Luk *et al.*, 2012). Also it has to be acknowledged that the relationship between hypovitaminosis D and obesity is complex and interestingly there are studies to suggest low vitamin D to be an independent risk factor for obesity (Kamycheva *et al.*, 2003; Foss, 2009).

Studies on vitamin D supplementation in PCOS women show inconsistent results. In a study by Selimoglu, eleven obese insulin-resistant women with PCOS were supplemented with vitamin D (single dose of 300,000 units of vitamin D3 orally) and the HOMA-IR decreased significantly, suggesting a potential beneficial role of vitamin D replacement therapy in obese PCOS women (Selimoglu *et al.*, 2010). However a more recent randomized, placebo-controlled, double-blinded trial with 50 PCOS women failed to demonstrate a beneficial effect of vitamin D supplementation on insulin sensitivity and insulin resistance (Ardabili *et al.*, 2012).

1.5.12.1.2.3 IVF

There are few studies of vitamin D and IVF and they provide conflicting results. Whilst a study by Ozkan *et al* revealed that a higher vitamin D level in the serum and follicular fluid in IVF was significantly more likely to achieve pregnancy (Ozkan *et al.*, 2010), Anifandis *et al* found excess serum and follicular fluid vitamin D levels to be detrimental to the IVF outcome (Anifandis *et al.*, 2010).

1.5.12.2 Vitamin D and Pregnancy

CYP27B1 is localized in human placenta (Barrera *et al.*, 2008) and the placenta synthesizes 1,25(OH)₂D (Stephanou *et al.*, 1994). Furthermore the localization of VDR in the placenta (Tanamura *et al.*, 1995) suggests a possible role for vitamin D in placental function.

1,25(OH)₂D regulates the secretion of human chorionic gonadotrophin (HCG) through VDR and a cAMP / PKA pathway (Barrera *et al.*, 2008), stimulates estradiol and progesterone production in trophoblasts (Barrera *et al.*, 2007), stimulates synthesis and release of human placental lactogen (genomic pathway) (Stephanou *et al.*, 1994) and up regulates the HOXA10 expression necessary for embryo implantation in endometrial cells (genomic pathway) (Du *et al.*, 2005).

The phases in life of rapid growth like foetal life, infancy, early childhood, puberty, pregnancy and lactation, where demand can exceed supply, are the vulnerable times for vitamin D deficiency (Bosomworth, 2011). The National Institute of Clinical Excellence (NICE) 'Antenatal care' guideline was updated in 2008 (NICE, 2008) and now includes a statement that; *'All women should be informed at the booking appointment about the importance for their own and their baby's health of maintaining adequate vitamin D stores during pregnancy and whilst breastfeeding. In order to achieve this, women may choose to take 10 micrograms of vitamin D per day'*.

At the present time the advice is focussed on women with deficiency or those in at-risk groups only. Although the safety aspects of vitamin D supplementation in pregnancy have been proven in many studies, (Hollis *et al.*, 2011) further research is warranted to investigate the effectiveness of routine vitamin D supplementation to improve pregnancy outcomes for healthy pregnant women at low risk of vitamin D deficiency (NICE, 2008). More recently however, UK health departments are recommending that all pregnant and breastfeeding women take a vitamin D supplement (10µg/ day) to meet maternal requirements and build adequate fetal stores (Department of Health, 2012).

There are observational studies linking vitamin D deficiency to an increased risk of pre-eclampsia, gestational diabetes, preterm delivery, bacterial vaginosis and an increased risk of caesarean section, but so far the evidence is equivocal and large scale clinical

trials confirming these associations are lacking (Barrett and McElduff, 2010; Grundmann and von Versen-Hoynck, 2011).

The vitamin D status of the new-born is dependent on the vitamin D status of the mother (Thomas *et al.*, 2011). Studies have shown that children of mothers with inadequate vitamin D levels are at risk of osteoporotic fractures (Javaid *et al.*, 2006) and are more susceptible to rickets. A study by Camargo *et al* showed a strong inverse association between maternal vitamin D supplementation during pregnancy and the risk of recurrent wheeze in children at three years of age (Camargo *et al.*, 2007). It is well known that exclusively breastfed babies have lower levels of vitamin D compared to formula milk fed babies as breast milk is a poor source of vitamin D and the latter is fortified with vitamin D. Therefore breastfed babies are more prone to rickets (Jain *et al.*, 2011).

1.5.12.3 Vitamin D and Male fertility

The expression of VDR in the male reproductive tract followed by studies (animal / human lab based / clinical) investigating the role of vitamin D in male reproduction are described in this section.

1.5.12.3.1 Expression of VDR and Vitamin D metabolising enzymes

In animal studies, the VDR has been identified in the testis (seminiferous tubules, epididymis, Sertoli cells), elsewhere in the male reproductive tract (seminal vesicles and prostate) and throughout various stages of spermatogenesis (spermatogonia, spermatocytes but only sparsely in sperm) (Merke *et al.*, 1985; Johnson *et al.*, 1996).

Similarly in humans, the VDR is expressed widely in testis (Sertoli, Leydig cells, epididymis), the male reproductive tract (seminal vesicles and prostate) and various stages of spermatogenesis (spermatogonia, spermatid and sperm) (Nangia *et al.*, 2007; Hirai *et al.*, 2009; Blomberg Jensen *et al.*, 2010; Zanatta *et al.*, 2011) . A variation in the expression of VDR in human sperm is noted. Studies have revealed that VDRs are localized mostly in the sperm nucleus and neck (Corbett *et al.*, 2006; Aquila *et al.*, 2008), whilst more recently it was revealed that other predominant areas of VDR expression in sperm were the post-acrosomal region of the head and mid-piece (Blomberg Jensen *et al.*, 2010). The sperm nucleus is comprised of transcriptionally silent and highly condensed chromatin (Aquila *et al.*, 2008), but the VDR expressed in

the post-acrosomal region of the head, neck and mid-piece evoke responses via non-genomic pathways (Blomberg Jensen and Dissing, 2012) .

Moreover, vitamin D metabolising enzymes are expressed in the male genital tract (Leydig cells, epididymis, seminal vesicles, prostate, sperm) suggesting that 1,25 (OH)₂D is locally produced, increasing the possibility for a role for vitamin D in human male reproduction (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2010; Foresta *et al.*, 2011).

In sperm, vitamin D metabolising enzymes are expressed (Blomberg Jensen *et al.*, 2012) as detailed below:

- CYP2R1, CYP27A1 – neck, tail
- CYP27B1 - neck, post acrosomal region of the head
- CYP24A1 - neck, annulus

Interestingly, infertile men have lower expression of VDR and vitamin D metabolising enzymes in testis and sperm compared to normal men (Aquila *et al.*, 2009; Foresta *et al.*, 2011; Blomberg Jensen *et al.*, 2012).

1.5.12.3.2 Animal studies

Several animal studies have been conducted to investigate the effect of vitamin D deficiency on male fertility. The proportion of vitamin D deficient male rats inseminating female rats (as determined by sperm positive vaginal smears) was significantly lower (73%) when compared to vitamin D replete rats (90%). Furthermore there was a significant reduction in the size of litters from vitamin D deficient male rat inseminations compared to inseminations by vitamin D replete rats (Kwiecinski *et al.*, 1989a). Vitamin D deficient rats revealed adverse histological changes in the testis such as reduced tubule diameter in the seminiferous tubules and marked disruption of spermatogenesis (Osmundsen *et al.*, 1989).

Mechanism(s) of action suggested from animal studies

Various studies have suggested the possible mechanism(s) by which vitamin D affects male reproductive function.

The impaired fertility of vitamin D deficient rats was restored by treating with either vitamin D or calcium suggesting that the probable mode of action of vitamin D is by regulating calcium levels in reproductive tissues (Uhland *et al.*, 1992). This fits in with

the classic role of vitamin D i.e. calcium homeostasis, particularly with the role of calcium having been demonstrated during spermatogenesis, sperm motility, sperm capacitation and the acrosome reaction (Benoff *et al.*, 1994; Breitbart, 2002; Yoshida *et al.*, 2008; Mendoza *et al.*, 2012). Furthermore as calcium increases VDR expression, it is speculated that calcium may be an important co-factor in VDR expression (Nangia *et al.*, 2007).

It has also been shown however that calcium supplementation only partially corrects hypogonadism in VDR null mutant mice (Kinuta *et al.*, 2000) as described later and also a direct positive effect of vitamin D action on semen quality has been shown (Blomberg Jensen *et al.*, 2011; Blomberg Jensen, 2012).

In vitamin D deficient rats, there is a significant reduction in testicular and epididymal sperm count, testicular glutamyl transpeptidase activity (an index of Sertoli cell function as glutamyl transpeptidase is involved in the synthesis of specific proteins secreted by sertoli cells) and Leydig cell count along with degenerative changes in germinal epithelium, but there is no difference in testicular lactate dehydrogenase activity (an index of germ cell function) compared to rats with vitamin D replete diets (Sood *et al.*, 1992). These results suggested that vitamin D deficiency retards spermatogenesis by interfering with the function of Sertoli and Leydig cells.

In another study by the same authors, Sood *et al.* (1995) showed that the above changes in testicular function were reversed by supplementation with an optimal dose of vitamin D. However they also showed that high dose of vitamin D caused deterioration in testicular function.

As $1,25(\text{OH})_2\text{D}$ via its non-genomic pathway leads to chloride channel activation and exocytosis in a mouse sertoli cell line, it has been suggested that vitamin D may be involved in the secretory activity of the Sertoli cells, essential for spermatogenesis (Menegaz *et al.*, 2010).

Some studies suggest that vitamin D affects fertility by the regulation of certain genes. VDR null mutant mice show reduced gene expression of aromatase (essential for estrogen synthesis), decreased aromatase activity and hypergonadotropic hypogonadism; and also demonstrate histological abnormalities in testes (dilated

seminiferous tubules, thinner layer of epithelial cells and decreased spermatogenesis). Calcium supplementation only partially corrects the hypogonadism (increased aromatase gene expression and activity without a change in FSH and LH levels), suggesting a direct vitamin D action. Supplementation of estradiol normalizes the histological abnormalities in the testes suggesting that vitamin D is an important factor in estrogen biosynthesis in male gonads by maintaining calcium homeostasis and also by regulation of aromatase gene expression (Kinuta *et al.*, 2000), thus explaining a possible regulatory role for vitamin D in gonadal function.

Furthermore as estrogen receptor alpha and aromatase null mice had a similar reproductive and gonadal phenotype to VDR knockout mice, it was hypothesized that the impaired spermatogenesis in the VDR knockout mice may be mediated via deficiencies in estrogen signalling (Mahato *et al.*, 2000; Couse *et al.*, 2001; Luk *et al.*, 2012).

A study by Hirai *et al.* (2009) noted that 19 out of 2483 testis-specific genes showed up-regulation by vitamin D treatment. Of these genes, the regulator of cellular cholesterol homeostasis, ABCA1 (ATP- binding cassette transporter) was expressed mainly in Sertoli cells and the study concluded that vitamin D contributes to spermatogenesis by up regulating certain specific genes in Sertoli cells.

ABCA1 causes the outflow of cellular cholesterol and phospholipid, which is the rate-limiting step in the synthesis of high-density lipoprotein, the main source of cholesterol in steroidogenic tissues (such as for testosterone synthesis by Leydig cells). It has been shown that ABCA1 knockout mice had an accumulation of lipids in Sertoli cells and significant reduction in intratesticular testosterone levels and sperm counts (Selva *et al.*, 2004).

Calbindin, (vitamin D dependent calcium binding protein) was localized in developing and growing chick testes. A comparison of the time course of the appearance and increase in the calbindin content in spermatogonia and spermatocytes in chickens suggests that calbindin may be involved in the mitotic process in spermatogenesis (Inpanbutr and Taylor, 1992).

1,25(OH)₂D stimulated amino acid accumulation in 11 day old rat testis which was blocked by cycloheximide and verapamil /apamine, providing evidence for a dual action of vitamin D, by both genomic (triggered by PKA) and rapid non genomic pathways (involving Ca⁺² dependent K⁺ channels on the plasma membrane) (Menegaz *et al.*, 2009).

1.5.12.3.3 Human lab based Studies

The lab studies investigating the association between vitamin D and sperm and also vitamin D and androgens are described in this section.

1.5.12.3.3.1 Vitamin D and sperm

In vitro human studies have shown that vitamin D enables sperm capacitation by contributing to involved processes such as increasing cholesterol efflux, protein phosphorylation and also by improving sperm survival (Aquila *et al.*, 2008).

1,25(OH)₂D increases intracellular calcium levels in sperm by 5-10 fold from baseline via a non-genomic pathway. Furthermore vitamin D in *in vitro* studies at levels corresponding closely to the physiologic levels in serum improved sperm motility and also increased the acrosome reacted sperm numbers significantly (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011). As the association between vitamin D and sperm motility was not affected by an adjustment for calcium levels, it was suggested that the improvement in sperm motility may be due to a direct action of vitamin D, rather than being secondary to increasing calcium levels.

It was shown in sperm that 1,25(OH)₂D (via VDR) decreased triglyceride content with concurrent increase in lipase activity. The authors suggested that the energy demands of the capacitation process may be met by this vitamin D triggered energy expenditure (Aquila *et al.*, 2009).

This association between vitamin D and sperm function (acrosin activity or sperm motility) in the *in vitro* studies appears to be dose dependent with higher concentrations either being ineffective or inhibitory (Aquila *et al.*, 2008; Blomberg Jensen *et al.*, 2011), suggesting the possibility of receptor down-regulation with higher levels of hormones (Aquila *et al.*, 2009).

Interestingly further *in vitro* experiments by Blomberg Jensen *et al.* (2012) on 22 young and 18 sub-fertile men revealed 1,25(OH)₂D increased sperm motility in young men, but not in sub-fertile men.

1.5.12.3.3.2 Vitamin D and androgens

Concomitant expression of receptors and metabolising enzymes in Leydig cells suggested the possible role of vitamin D in hormone production (Blomberg Jensen *et al.*, 2010). The interaction between vitamin D and androgens appears to involve complex pathways (Lerchbaum and Obermayer-Pietsch, 2012). Testosterone down regulated VDR expression in the testis, whilst increasing concentrations of vitamin D increased VDR expression (Nangia *et al.*, 2007). However in cultured human osteoblasts, dihydrotestosterone increased CYP27B1 activity and 1,25(OH)₂D levels (Somjen *et al.*, 2007).

In the LPB-Tag [large probasin promoter (LPB) linked to the large T antigen (Tag)] transgenic mouse model of prostate cancer, tumour progression was significantly more rapid in VDR knockout mice compared to wild type mice. However following testosterone supplementation, the differences in the tumour progression were abolished, suggesting considerable cross interaction between androgens and vitamin D (Mordan-McCombs *et al.*, 2010).

1.5.12.3.4 Clinical studies

Several clinical studies have investigated the role of vitamin D in human male reproduction. To ease classification, I have divided the studies into those investigating the association between:

- Vitamin D and androgens
- Vitamin D and semen parameters
- Vitamin D and testicular function

1.5.12.3.4.1 Vitamin D and androgens

A positive association including a concordant seasonal variation has been shown between serum vitamin D levels and androgens (testosterone and free androgen index) (Wehr *et al.*, 2010b). Nimptsch *et al.* (2012) confirmed this positive association in their association study but denied any seasonal variation in serum testosterone levels.

However, other studies showed either no link between serum vitamin D levels and androgens or indicators of spermatogenesis (Blomberg Jensen *et al.*, 2011; Hammoud *et al.*, 2012; Yang *et al.*, 2012) or a negative association as men with high serum vitamin D had a lower free androgen index (Ramlau-Hansen *et al.*, 2011).

To explain these study findings in further detail, Blomberg Jensen *et al.* (2011) revealed that there is no association between serum vitamin D levels and FSH / inhibin B. In a study by Hammoud *et al.* (2012) there was no significant difference between the mean levels of total testosterone, sex hormone-binding globulin (SHBG), FSH and LH in the various vitamin D groups. In a cross sectional association study by Ramlau-Hansen *et al.* (2011), men with higher vitamin D levels (94- 227nmol/l) had 16% higher levels of SHBG and 11% lower levels of free androgen index compared to men with vitamin D levels between 8 and 62nmol/l. There was no association between serum vitamin D levels and estradiol, testosterone, FSH, LH and inhibin B levels. Yang *et al.* (2012) showed there was no correlation between serum testosterone and serum vitamin D levels in either fertile or infertile men.

In a large cross sectional population study (European male ageing study-EMAS) involving 3369 participants from 8 European centres, there was no association between serum vitamin D levels and hormones of the hypothalamo-pituitary (HPT) axis. Unlike Wehr *et al.* (2010a) study, there was no seasonal variation noted in reproductive hormones. Vitamin D deficiency defined in their study as less than 50nmol/l was associated with compensated (normal testosterone 10.5nmol/l and high LH >9.4IU/l), and secondary (low testosterone <10.5nmol/l and normal LH 9.4IU/l) hypogonadism, which are strongly linked to obesity and limited mobility respectively. Based on this significant association with hypogonadism and serum vitamin D levels, the authors speculated that low vitamin D could potentially adversely affect the HPT axis at multiple levels (Lee *et al.*, 2012). However the clinical significance of this association is not understood and needs further investigation.

Interestingly, a placebo controlled trial involving 54 men participating in a weight reduction programme revealed that men who were supplemented with vitamin D (3332IU daily for 1 year) had higher levels of total and free testosterone compared to the placebo group (Pilz *et al.*, 2011).

Following a review of 13 studies investigating the association between vitamin D levels and androgens, Blomberg Jensen (2012) reported that a positive association is generally seen in men above 40 years and with co-morbidities whilst in young healthy men either there was a negative association or no association noted. Caution was advised in interpreting these studies as the interaction between bone metabolism, gonadal and pituitary function and calcium homeostasis is complex, particularly as various compensatory mechanisms can affect the association between vitamin D and serum testosterone.

1.5.12.3.4.2 Vitamin D and semen parameters

Increased serum vitamin D has been associated with improved sperm motility but not sperm counts or morphology (Blomberg Jensen *et al.*, 2011). Conversely a study by Ramlau Hansen *et al* showed no correlation between serum vitamin D and semen parameters (Ramlau-Hansen *et al.*, 2011). Interestingly in an association study conducted in Salt Lake, Utah, sperm motility and morphology were lower in men with low (<50nmol/l) as well as high (above 125nmol/l) concentrations of vitamin D (Hammoud *et al.*, 2012). All the above association studies were conducted in men from the general population.

Studies recruiting sub-fertile men include a further small cross sectional association study by Blomberg Jensen *et al.* (2012) of 70 men [25 men (partners of infertile couples) and 45 young unselected men], which revealed progressive sperm motility was significantly better in the vitamin D non-deficient group (>25nmol/l) compared to the vitamin D deficiency group. There was no statistical difference in other semen parameters (i.e. sperm concentration, total motility and morphology) between the groups.

In a large recent cross sectional association study by Yang *et al.* (2012) (314 infertile and 195 fertile men), a significant positive association between serum vitamin D and sperm motility and sperm morphology but not sperm concentration was seen in the infertile male group. The positive association between vitamin D and sperm motility and morphology was only of borderline significance in fertile men and there was no association between sperm concentration and vitamin D in the fertile male group. However in another small study recruiting 90 men attending an infertility clinic, there was no correlation between vitamin D and semen parameters (poster presentation at

American Urological Association) (Knopf *et al.*, 2011). Recently CYP24A1 expression in sperm has been suggested as a marker of semen quality owing to its positive association with semen parameters and ability to distinguish infertile and fertile men with high positive predictive value (Blomberg Jensen *et al.*, 2012).

Overall, despite some studies showing no link between serum vitamin D and semen parameters, a significant positive association (mainly on sperm motility and morphology, but not on semen volume or sperm count) has been reported in fertile and infertile men with some evidence of a negative association at higher vitamin D levels.

1.5.12.3.4.3 Vitamin D and testicular function

In a study by Foresta *et al.* (2011), 57 infertile men (azoospermia secondary to sertoli cell only syndrome and oligoazoospermia) were compared with 41 men with normal testicular function. Men with normal testicular function had significant higher expression of CYP2R1 gene expression compared to men with testicular pathology. Men in the testicular pathology group had significantly lower levels of serum vitamin D, higher levels of PTH and higher levels of bone specific alkaline phosphatase. Moreover these men were more likely to have osteopenia and osteoporosis despite normal testosterone and estrogen levels compared to men with normal testicular function, suggesting that testis was involved in systemic activation of vitamin D.

The presence of VDR and metabolising enzyme expression in the male genital tract increases the possibility of a role for vitamin D in human male reproduction, however the importance of these receptors and the role of vitamin D in spermatogenesis and the male reproductive tract is still not completely understood and therefore the biological mechanisms are speculative.

Further studies are needed to elucidate the precise role of vitamin D in human spermatogenesis and reproduction. In this study I aimed to investigate the hypothesis that vitamin D has an important role in male fertility by investigating if there was a correlation between serum levels of vitamin D and semen parameters.

1.6 Summary of introduction

To summarize, over recent years at NFC the waiting time for donor sperm treatments, was not only over a year but also perceived to be increasing. There have been some

significant changes nationally and locally during the first decade of the millennium, including the regulations regarding the removal of donor anonymity, which may have influenced the sperm donor programme at the centre. The Disclosure of Donor Information Regulations introduced by the Human Fertilisation and Embryology Authority (HFEA) were approved in the UK Parliament in 2004 (HMSO, 2004). From 1st April 2005, only donors who have agreed to reveal their identity to the children born by their donation are allowed to donate.

The British Fertility Society (BFS) Working Party report in 2008 recognised the problem of donor sperm shortage to affect most of fertility centres in UK (Hamilton *et al.*, 2008a). Despite the absence of published evidence, it appears that centres have coped variably with the majority of the UK fertility centres struggling to recruit donors (Wardle, 2008), whilst some centres are successful (Adams *et al.*, 2006; Ahuja, 2008; Tomlinson *et al.*, 2010). We aimed to analyse the changes or trends in various aspects of the sperm donor programme at the NFC over 11 years from 2000 to 2010 to investigate if there is a problem with sperm donor recruitment.

The most common reason for rejection of potential sperm donors is suboptimal semen quality (Paul *et al.*, 2006; Hamilton *et al.*, 2008a). Suboptimal semen quality is a common problem but is poorly understood. I have reviewed a couple of external factors, widely studied in literature (i.e. season and vitamin D) that may influence semen parameters. A better understanding of their impact on semen quality (if any) may be of value in improving the efficiency of a sperm donor programme and also donor recruitment.

The findings on seasonal variation in semen parameters, although extensively described in literature, have been variable and inconsistent. The majority of studies reveal a significant seasonal variation with improved parameters in Winter / Spring (or worsening parameters in Summer / Autumn) (Tjoa *et al.*, 1982; Levine *et al.*, 1988; Reinberg *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Jørgensen *et al.*, 2001; Chen *et al.*, 2003; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005; Calonge *et al.*, 2009) whilst some have revealed an absence of seasonal variation (Mortimer *et al.*, 1982; Mallidis *et al.*, 1991; Ombelet *et al.*, 1996; Chia *et al.*, 2001; Carlsen *et al.*, 2004).

Most of the data is accrued from retrospective cross sectional observation studies as there are few longitudinal studies. Although not always, seasonal variation is more commonly seen in temperate than tropical climates (Chia *et al.*, 2001). Seasonal variation has been consistently reported in sperm concentration but the data on semen volume, sperm motility and morphology is inconsistent (Levine, 1999).

Men in the UK, being exposed to temperate climate may be subjected to seasonal variation in semen parameters; however studies from UK are sparse. As the study undertaken nearest to our centre geographically, in Edinburgh revealed a pattern of improved sperm counts in Winter and Spring, though not statistically significant (Mortimer *et al.*, 1983), we wondered if a similar pattern in semen parameters would emerge in men attending our centre.

If a seasonal variation in semen parameters are confirmed, it is also worthwhile investigating the influence of this on the conception rates to not only further understand the impact of season but also strengthen the proposal of seasonal recruitment of donors. Studies have shown concurrence of variation in sperm parameters with variation in natural births and also IVF conceptions (Fisch *et al.*, 1997; Ossenbühn, 1998; Levitas *et al.*, 2013). It is well known that despite the fact that only men with optimal semen quality are recruited as donors, the fecundity of sperm donors is variable (Thyer *et al.*, 1999; Navarrete *et al.*, 2000). Can this be related to the season of sperm donation?

Studies have revealed that conceptions by donor insemination were highest between October and March (Paraskevaides *et al.*, 1988; Ronnberg, 1989). In contrast, in a large French epidemiological survey of an artificial insemination programme over six years, where frozen sperm was used, there was no seasonal variation in donor conceptions (Mayaux and Spira, 1989). Studies investigating the effect of seasonality of sperm on conception are scant in the literature. I have taken the novel approach of investigating the distribution of conceptions achieved by donor insemination (DI) treatment, with regards to season of original sperm donation to find out if there was any variation.

The role of vitamin D in male reproduction has been known for decades in animal studies. vitamin D deficient rats and /or VDR knockout mice show low sperm count, decreased sperm motility, impaired fertility, decreased testicular function and adverse histological changes in the testis (Kwiecinski *et al.*, 1989a; Sood *et al.*, 1992; Kinuta *et*

al., 2000). Repletion of vitamin D in these animals restored fertility and improved testicular function (Uhland *et al.*, 1992; Sood *et al.*, 1995).

In humans wide expression of VDR and vitamin D metabolising enzymes in the male reproductive tract suggest local production of 1,25 (OH)₂D and also supports the potential for autocrine –paracrine responses (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2010; Foresta *et al.*, 2011).

In vitro human studies have shown that vitamin D enables sperm capacitation by contributing to involved processes such as increasing cholesterol efflux, protein phosphorylation and also by improving sperm survival (Aquila *et al.*, 2008). It has also been revealed that vitamin D increases intracellular calcium levels in sperm, improves sperm motility and induces the acrosome reaction (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011).

Interest in vitamin D has increased as its deficiency has now been identified as a worldwide problem, in particular a nationwide survey of middle-aged British adults revealed a high prevalence of hypovitaminosis D in the UK (Hyppönen and Power, 2007; Holick and Chen, 2008). With this background, we aimed to investigate the potential role of vitamin D in human male fertility. In addition if vitamin D is found to have a strong link with semen quality, applicants for sperm donation could either be invited for initial screening of semen quality when their vitamin D levels are high or potentially to consider vitamin D supplementation to improve parameters, thereby improving sperm donor recruitment.

I will consolidate our aims and objectives in the next section.

Chapter 2 Aims of the project

The **central aim** of the project is to optimise the use of human sperm in subfertility.

Over recent years at the Newcastle Fertility Centre (NFC) a long waiting time for donor sperm treatments has developed. Various changes both nationally and locally may have had an influence on the sperm donor programme.

Our **first aim** was

To analyse the changes or trends in various aspects of the sperm donor programme at the NFC over 11 years from 2000 to 2010, to investigate the issue of sperm donor recruitment.

If a problem in donor recruitment is present, then a more efficient use of existing resources may be of benefit. The most common reason for rejection of a potential sperm donor is suboptimal semen quality. We will review some external factors that may influence semen quality.

Our **second aim** was therefore

To investigate if there was a seasonal variation in the semen parameters of men attending NFC.

In order to understand further the influence of season, we investigated the impact of season on conception.

Our **third aim** was

To investigate the distribution of conceptions achieved by donor insemination treatment, with a view to finding out if there was any variation in relation to the seasonal timing of the original sperm donation.

In view of clinical and preclinical data suggesting that vitamin D has a role in male reproduction as well as linking this to possible seasonal effects,

Our **fourth aim** was

To investigate if there is association between serum vitamin D level and semen parameters.

We further wished to evaluate the impact of serum vitamin D on male fertility.

Our **fifth aim** therefore was

To investigate if there is correlation between serum vitamin D and

1. semen quality
2. IVF treatment outcomes.

This study will contribute to potentially increasing the efficiency of the treatment programme with donor sperm and also the understanding of male infertility

Chapter 3 Is there a problem with sperm donor recruitment currently at NFC?

3.1 Introduction

In this chapter, I have examined the sperm donor programme at NFC over a period of 11 years from 2000 to 2010 inclusive.

At NFC over recent years a gradually increasing waiting time for donor sperm treatments has been noted. In addition, there was a perception of an increase in sperm import to the centre. Significant national and local changes to the sperm donor programme in the 2000 decade may have influenced this. One of the major changes included the removal of sperm donor anonymity which allowed only donors agreeing to reveal their identity to children born by their donation (when children reached 18 years of age) to donate from 1st April 2005 (HMSO, 2004).

Following on from the adoption of EU Tissues and Cells Directive in the UK, (Directive 2004/23/EC, 2004), the SEED (Sperm Egg and Embryo Donation) review in 2005 concluded that sperm donors may be reimbursed to cover demonstrable out of pocket expenses and loss of earnings, but not for inconvenience related to donation and reimbursement was capped at £250 pounds for each course of sperm donation (SEED Report, 2005). However the recent review of HFEA policies allows a fixed sum of £35 per visit for sperm donors (HFEA, 2011a).

Local changes affecting the donor recruitment and treatment programmes include the introduction of a comprehensive and integrated andrology service in 2009 offering advanced surgical sperm retrieval techniques such as microsurgical testicular sperm extraction (Micro – TESE) which has made own gamete treatment accessible to many more couples than previously. However, a proportion of these will opt for donor sperm to be available as a back-up when they are aware of a significant risk that no sperm will be retrieved.

The problem of donor sperm shortage was recognised as a national problem by the BFS working party report in 2008 affecting most of the UK fertility centres (Hamilton *et al.*, 2008a). However, despite the absence of published evidence, it appears that centres have coped variably with the problem of sperm donor shortage. Some of the UK fertility

centres are struggling to recruit donors (Wardle, 2008), whilst other centres are successful (Adams *et al.*, 2006; Ahuja, 2008; Tomlinson *et al.*, 2010).

3.2 Aim

To analyse the changes or trends in the various aspects of the sperm donor programme between 2000 and 2010 at our tertiary care referral centre, NFC.

3.3 Objectives

To identify any changes in:

1. Sperm donor recruitment per year.
2. The number of patients treated, the number of treatment cycles performed and the indications for treatment.
3. The amount of sperm imported and exported.
4. The waiting times for treatment.

3.4 Outcome Measures

To investigate if there is a problem of sperm donor shortage and if confirmed, to devise appropriate strategies to manage the shortage.

3.5 Subjects and Methods

3.5.1 Subjects

All the patients who had treatment with donor sperm between 2000 and 2010 were included in the study i.e. heterosexual couples, same sex couples and single women. There is NHS funding available for all those childless couples where the woman is under 40, although not for same sex couples where there is no other fertility issue or single women in our region at the time of the study. However since the new NICE guidance same sex couples are eligible for treatment under NHS (NICE, 2013).

3.5.2 Recruitment of sperm donors at the Centre

All sperm donors undergo a standard screening process. Prior to 2008 this was undertaken in accordance with the British Andrology Society (BAS) guidelines for the screening of semen donors (British Andrology Society, 1999) and since 2008 in accordance with the updated UK Guidelines for the medical and laboratory screening of sperm donors 2008 (Association of Biomedical Andrologists *et al.*, 2008). This involves taking a detailed medical history (including a family and genetic history), performing a

physical examination and carrying out various screening tests (including cytogenetic tests and tests to exclude blood borne viral and sexually transmitted infections).

The assessment of semen parameters includes a freeze-thaw analysis. The acceptable standards for fresh semen parameters have changed over the years in an attempt to increase the efficiency of donor recruitment, whilst not compromising the success of donor sperm treatments. By taking a more critical view of the post-thaw parameters, more variation in pre-freeze samples has been accommodated, though still within normal limits. The current criteria include: volume \geq 1ml, initial motile concentration \geq 40 million/ml, post thaw motile concentration \geq 10million/ml, morphology \geq 10%, and mixed antiglobulin reaction (MAR) test negative.

Semen samples are frozen and quarantined for 6 months. At the end of the quarantine period the screening tests for sexually transmitted infections are repeated and confirmed negative, prior to releasing the samples for use. Once the donor samples are released, patients are allocated to a donor. A maximum of 10 patients are allocated at a time to each sperm donor (HFEA, 2011b). Once 10 families have been achieved, the semen samples are not used for further treatment unless for a sibling, up to the 10 year storage limit.

3.5.3 Donor programme

The donor programme offered at our centre begins with ultrasound monitored natural cycle insemination, commonly referred to as unstimulated donor insemination (unstimulated DI or DI) as the first line of treatment. This involves inseminating the thawed semen samples into the cervix, timed to the ovulation of a mature follicle. Despite the National Institute of Clinical Excellence (NICE) guidance on Fertility suggested IUI over intracervical insemination (NICE, 2013), as unstimulated DI treatment success rates were consistent with the national average (and at times higher) (Paul *et al.*, 2006) and particularly as the majority of women treated had no female infertility factor, this remained the first line treatment (Flierman *et al.*, 1997; Harris, 2000) during the study period and until recently.

If unsuccessful, then the more interventional techniques of ovarian stimulation with intra-uterine insemination (stimulated IUI) (Goldberg *et al.*, 1999; Carroll and Palmer, 2001; Besselink *et al.*, 2008) and in-vitro fertilization (D-IVF) are offered sequentially.

Stimulated IUI involves stimulating the ovaries with gonadotrophins aiming for no more than two mature follicles and inseminating the prepared sperm directly into the uterus, timed to the stimulated ovulation of mature follicle(s) with HCG. IVF is more complex and encompasses ovarian stimulation, egg collection, fertilization *in vitro* and the transfer of embryos into the womb.

Generally patients are offered 3 cycles of each method of treatment sequentially (a maximum of 9 donor sperm treatment cycles) until pregnancy is achieved, although the programme may be modified depending on other prognostic factors such as age and ovarian reserve or the presence of female subfertility factors. Our programme is slightly different to the recently issued NICE guidance in 2013 which suggests a minimum of 6 cycles of unstimulated IUI but up to 12 cycles are recommended prior to considering IVF (NICE, 2013).

3.5.4 Methodology

We performed a retrospective review of NFC's registers and databases of assisted conception treatments using donor sperm, between January 2000 and December 2010. These sources of information were used to collate the information needed for this study.

1. Laboratory database of donor sperm use for treatment.

The laboratory data base records the patient's details with date and type of donor sperm treatment carried out. The number of patients treated and the number and type of treatment cycle performed per year were calculated from this database.

2. Database and records maintained for sperm donor programme outcomes.

The treatment database holds patient details including the indication for treatment, patient status (eg. heterosexual couple, same sex couple or single women) with date, type of treatment and treatment and pregnancy outcomes. The indications for donor sperm treatment per year and the success rates of the treatments were calculated using this information.

3. Donor files.

All the donor files were studied for the year of HFEA registration, from which the number of donors recruited per year was calculated. Known donors have been excluded from the analysis.

4. Weekly donor sperm straw consumption sheets: These sheets are completed with the patient details and donor code of the sperm used and date of treatment just prior to carrying out treatment. The number of donors used per year could be calculated from these sheets.

5. Sperm transfer Log

The sperm transfer log records the donor code, date and number of straws of sperm transferred from NFC to other fertility centres. The sperm export per year was derived from this database.

6. Sperm import records

A separate register of sperm import is maintained with patient details, year of import, sperm bank and donor details and amount of sperm imported. From these records the number of patients importing sperm per year was calculated.

7. Waiting list for donor sperm treatment registers

This register records the date of placement on the waiting list, the date when treatment was offered and the response to this invitation. Of the patients placed on the waiting list up to 2010, the response to the invitation was studied till May 2012. This enabled the calculation of the numbers of patients placed on the waiting list per year, their waiting time and the outcome.

3.6 Statistical analysis

Statistical analysis was performed using the Minitab software (version 15). Linear Regression analysis was done to examine the trends over the years. A p value of <0.05 was considered significant.

3.7 Results

A total of 1889 cycles of treatment were undertaken during this time period for 420 patients.

3.7.1 Recruitment of donors

In the early years of the decade the recruitment rate for sperm donors on average was 4 donors per year Table 6. Around the years of change in donor anonymity (2003 to 2006), variations in the apparent number of donors recruited per year (between 1 and 6) were seen. This was in part a strategic effort to pre-empt the effects of the anonymity changes by “stockpiling” identifiable donors recruited earlier for use after the transition date (i.e. they were not released for treatment until the anonymity change was mandatory).

Although the numbers are small, Table 6 shows the decline in the number of donors recruited since 2006, with none recruited in 2009 and 2010. This decline generally reflects a reduced number of enquiries from potential sperm donors from 123 in year 2000 to 18 in 2010 (Table 6) although there was a slight rise in 2010 compared with the previous two years.

Before the removal of sperm donor anonymity the average size of the donor pool was 44 donors (data not shown in the table 6; calculated from weekly donor sperm straw consumption sheets), with no sperm donor shortage. This donor bank, adequate for demand and allowing for a choice of donor characteristics, was effectively shelved with the removal of sperm donor anonymity as the donors were not consented for later identification or for later contact, which precluded direct invitation for re-registration as identifiable donors. Despite publicity at the time none volunteered to make this change. This left a stock of donors for sibling use only at the end of the transition period and a drastic fall in the number of sperm donors available. The pool in 2010 of identifiable donors comprised 14 donors only (data not shown in table 6). The centre’s advertising strategy, which was through student magazines, local magazines and newspapers remained consistent throughout the study period.

Unsurprisingly, given the donor shortage there has been an increase in the requests for consideration of single use donations for family members (known donors) although as yet only a very small number of such donors have proved suitable for a number of reasons including poor quality sperm and not pursuing the treatment option further following counselling of the involved parties.

Year	Sperm donor enquiries	No: sperm donors registered per year	No: Straws of sperm transferred	No: patients per year importing sperm	Waiting times (months) for donor sperm treatment
2000	123	3	44	0	0
2001	60	4	85	0	0
2002	58	5	58	0	0
2003	22	1	142	1	0
2004	*	6	129	0	0
2005	*	1	24	0	0
2006	13	5	20	1	0
2007	17	2	2	2	6
2008	5	1	6	3	8
2009	4	0	0	0	10
2010	18	0	0	12	18

Table 6: Sperm donor enquiries, recruitment, sperm export / import, and waiting times for donor sperm treatment at the Newcastle Fertility Centre from 2000 to 2010.

**unable to retrieve data*

The number of straws of sperm transferred per year to other centres is shown in the fourth column.

3.7.2 Donor sperm treatments

The number of patients treated, treatment cycles carried out, indications for treatment and success of treatments during the study period are discussed in this section.

3.7.2.1 Patients treated

Figure 6 shows a declining trend in the number of patients treated. Approximately 50% fewer patients received treatment in 2010 compared to 2000 (26 versus 46). A considerable drop in 2007 was noted when the demand for donor sperm started to outstrip the supply. However a marked increase was apparent in 2008 following the release of four sperm donors at once enabling forty patient slots to be made available. These were promptly taken up by patients commencing treatment.

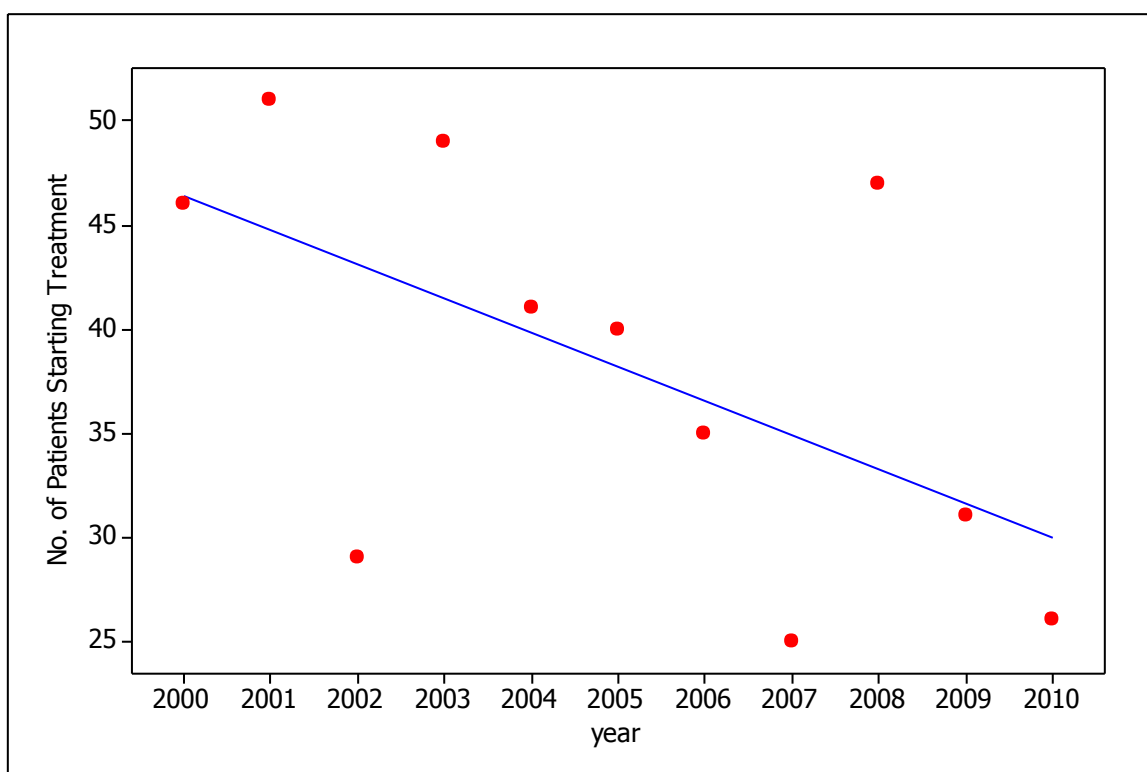


Figure 6: Number of patients starting treatment with donor sperm per year

The graph shows the regression line of the number of patients starting treatment from 2000 to 2010. Regression $p=0.06$; R^2 (adjusted) =25.3%.

3.7.2.2 Treatment cycles carried out

Figure 7 reveals a non-significant declining trend in the number of donor insemination cycles (unstimulated and stimulated) ($p=0.4$), even though only 84 cycles were performed in 2010, compared to 157 cycles in 2000. An increase in the treatment cycles in 2008 and 2009 reflected the extra patients starting treatment in 2008. Whether the marked drop in 2010 is one off or a persistent phenomenon will be revealed in future studies.

There was a significant increase in D-IVF cycles ($p= 0.002$) (Figure 7) performed over this period. The proportion of D-IVF cycles performed in relation to the total donor sperm treatment cycles also increased gradually from 5.9% in 2000 to 16.6% in 2009 (Table 7). There was a sharp increase in that proportion to 33% in 2010, most likely due to the lower total numbers of patients starting treatment in 2010 and therefore caution is advised in interpreting this rise.

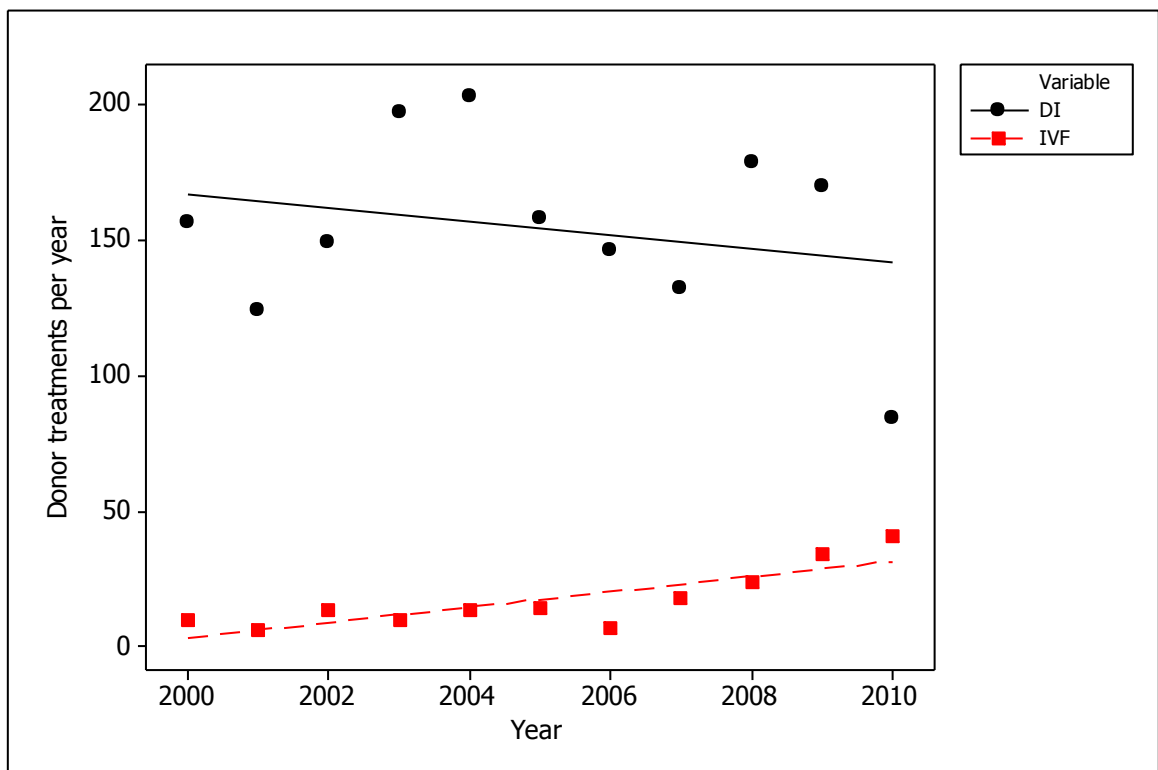


Figure 7: Number of treatment cycles with donor sperm per year

The graph shows the regression line of the number of Donor Insemination (DI) cycles (Unstimulated and Stimulated) and Donor IVF cycles per year from 2000 to 2010. For DI, Regression $p= 0.4$; R^2 (adjusted) =0.0%; For IVF, Regression $p= 0.002$; R^2 (adjusted) = 65.1%.

Year	Donor IVF cycles	Total donor sperm treatments	Proportion of Donor IVF (%)
2000	10	167	5.9
2001	6	130	4.6
2002	13	162	8
2003	10	207	4.8
2004	13	216	6
2005	14	172	8.1
2006	7	153	4.5
2007	18	150	12
2008	24	203	11.8
2009	34	204	16.6
2010	41	125	32.8

Table 7: Rising proportion of Donor IVF in donor sperm treatment cycles.

3.7.2.3 Indications for treatment with donor sperm

Figure 8 shows as expected that, male factor remained the commonest indication for treatment with donor sperm and was the indication in an average of 83% of cases over the study period, although a significant decline in that proportion was noted over the years ($p= 0.01$). There was a significant rising trend in the proportion of same sex couples attending for treatment from 4% of the total in 2000 to 11.5% in 2010 ($p= 0.01$) and a rising trend (although non-significant) in the number of single women treated (2% of the total in 2000 to 8% in 2010 ($p=0.2$)).

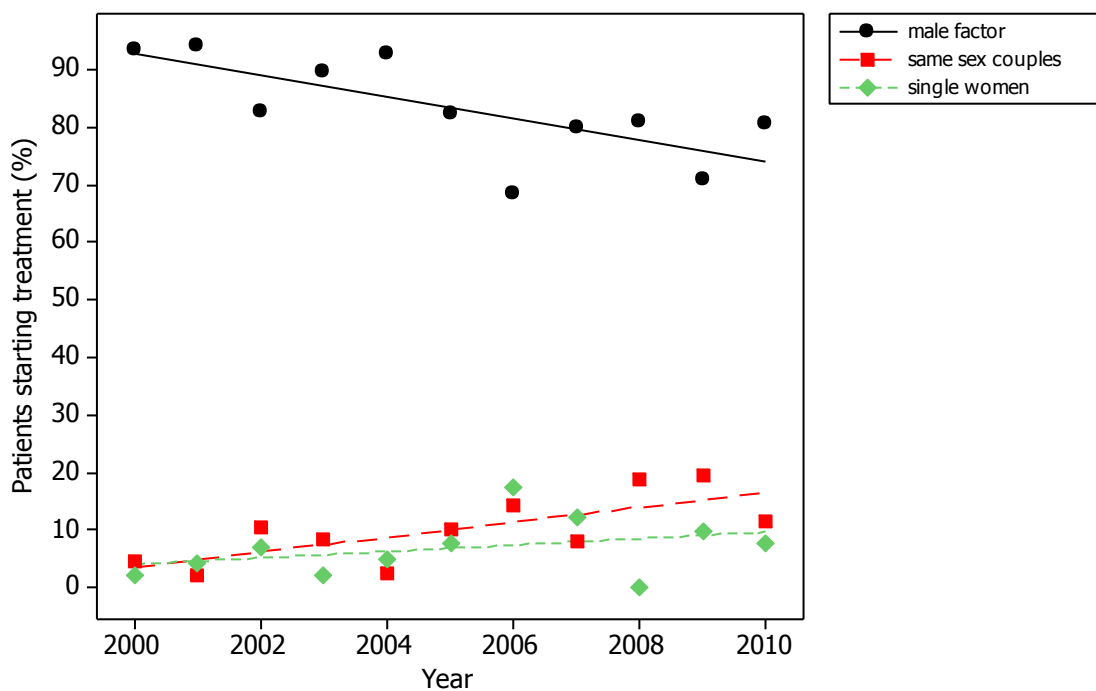


Figure 8: Indications for use of donor sperm

The graph shows the trend of the patients with Male factor infertility, same sex couples and single women starting donor treatment per year from 2000 to 2010. Patients starting treatment are expressed in percentages. For Male factor infertility, Regression $p= 0.01$; R^2 (adjusted) = 45.9%; For same sex couples, Regression $p= 0.01$; R^2 (adjusted) = 47.9%; For single women, Regression $p= 0.2$; R^2 (adjusted) = 4.7%.

3.7.2.4 Success Rates

The live birth rate per cycle with unstimulated DI, Stimulated DI and IVF were 10.8%, 16.8% and 20% respectively over the study period. More importantly the overall cumulative live birth rate with the donor programme during this time period was 55%.

3.7.3 Sperm export and imports

Table 6 shows a decline in the number of straws of sperm transferred outside the centre (44 straws in 2000 to 0 straws in 2010). Sperm was transferred at nominal cost to secondary care centres in our region that did not have a facility to recruit donors to enable them offer DI treatments locally. This has all but disappeared with the shortage of sperm such that since 2005, sperm is only passed to other Centres for the sole purpose of treating couples who had undergone prior successful treatment with our centre's sperm donor (i.e. for the purpose of siblings only).

Conversely during the same time period there was an increase in the number of patients (none in 2000 to 12 in 2010, Table 6) importing sperm from commercial sperm banks to fast track their treatment instead of waiting for the availability of sperm on the NHS. When there was no shortage of sperm donors, sperm was imported only occasionally when the centre could not provide a suitable match eg: a specific racial match to for non-Caucasian couples requesting donor sperm treatment. Whilst solving waiting issues for some patients this comes with a burden of cost to couples selecting this option and is therefore not accessible to all.

3.7.4 Waiting list issues

The waiting times and outcomes of the patients on the waiting list are described in the following section.

3.7.4.1 Waiting times

It is evident from Table 6 that the waiting list appeared in 2007, and that by 2010 the waiting time had increased to 18 months.

3.7.4.2 Patients on the waiting list

From 2007 to 2010, 186 patients were added to the waiting list. Male factor was the indication in 75.8%. 3.7% were single women and 20.4% were same sex couples amongst these patients (Table 8), similar to the trend illustrated previously in Figure 8. The waiting list is not attributable to primary care trust (PCT) funding which supports the programme appropriately. By May 2012, 166 of the 186 had been called for treatment, however treatment was commenced only in 65.6% of those patients called. Reasons for not commencing treatment in the remaining patients are shown in Table 9.

Year	Number of patients added to the waiting list	Same sex couple	Single women	Male factor infertility
2007	56	7	2	47
2008	57	11	2	44
2009	30	8	3	19
2010	43	12	0	31
Total	186 (100%)	38 (20.4%)	7 (3.7%)	141 (75.8%)

Table 8: Patients added to the waiting list for donor sperm treatment – with their indications

Patients who reached the top of the waiting list (by May 2012)	166 (100%)
Treatment commenced	109 (65.6%)
Did not attend (no obvious reason)	34 (20.4%)
Decided not to pursue further treatment	8 (4.8%)
Sought treatment in other centres including abroad	5 (3.0%)
Couple separated	3 (1.8%)
Patients turned 40 (lost NHS funding / declined self-funded treatment)	2(1.2%)
BMI over 30 (treatment could not be commenced)	5 (3.0%)

Table 9: Patients on the waiting list- Outcome

At the time of writing (May 2012) 166 patients of the 186 who were placed on the waiting list by 2010 had been called for treatment.

3.8 Discussion

In this chapter we have examined the donor programme at our tertiary centre over a period of 11 years between 2000 and 2010. There has been a significant change in the sperm donor programme at NFC over this time with fewer donors recruited, a smaller pool of donors, fewer patients receiving treatment, a decline in the number of donor insemination cycles undertaken accompanied by rising D-IVF cycle numbers, increasing sperm imports and a longer waiting time for treatments.

3.8.1 Recruitment of donors

There is an obvious decline in the number of donors registered with the HFEA at our centre since the removal of anonymity, which is similar to the experience of other fertility centres across the country (Wardle, 2008). Previous work done at our centre (between 1994 and 2003) showed a significant decline in the number of enquiries to be sperm donors. As the number predominantly declined after 2000, it was possibly related to the growing awareness of the removal of donor anonymity at the time (Paul *et al.*, 2006). However the HFEA data (HFEA, 2011d) shows a decline in the number of donors from 426 in 1993 to 239 in 2004, much before the removal of anonymity.

In a Department of Health survey of UK gamete donor's views on the removal of anonymity prior to the Regulations in 2004, only 50% of the participating 43 sperm donors expressed their willingness to continue to donate following the removal of anonymity (Frith *et al.*, 2007). In my study, there were variations seen in the numbers of donors registered around the years of introducing the disclosure of donor information regulations 2004. Apart from the centre's effort to recruit identifiable donors prior to the regulations in preparation for the potential shortage after anonymity changes, there were other factors influencing this variation. There was increased media interest heightening awareness at the time and also prospective donors' apprehensions about the potential legal and financial responsibilities and concerns about the consequences of contact with the donor offspring and their (sperm donor) rights regarding the access to information about the offspring (Frith *et al.*, 2007). Whilst some were unfounded, these concerns reveal the difficulty in decision making for potential sperm donors at the time.

In the British Fertility Society Donor Survey 2005/06 which included responses from 35 treatment and storage centres, 37% agreed that it was harder to recruit donors and in 9% of centres the service of donor sperm treatment ceased (British Fertility Society survey,

2006). In a BBC survey in 2006, it was revealed that 75% of donors were recruited from just 9 clinics and 50 out of the 74 clinics contacted either had no sperm or insufficient supplies of donor sperm (BBC survey, 2006).

In a recent study where responses from 324 UK men registering as sperm donors on Sperm Donors Worldwide website (which “facilitates free donation of sperm in private arrangements”) were included, there was decreased willingness by donors to be identified and 47% desired no involvement with children born by their donation (Riggs and Russell, 2011).

There has been conjecture about the significant rise in the cost to purchase sperm following the removal of anonymity owing to the shortage and the smaller centres which purchased sperm previously have struggled to maintain their services (Wardle, 2008). The BFS Donor survey showed that 94% of the 35 centres found it harder to purchase sperm from commercial banks and 89% reported associated increased costs involved with donor sperm treatments.

The other debated contributing factor to the low sperm donor numbers is donor payment. It was widely argued that UK sperm donors were not adequately compensated and yet clinics were using imported sperm from overseas commercial sperm banks, where the donors may have been well compensated (Heng, 2007; Hamilton, 2010; Tomlinson *et al.*, 2010). In the recent HFEA review following a 3 month public consultation including all relevant stakeholders, the policy regarding gamete donor payment was revised to compensate UK sperm donors a fixed sum of £35 per visit (which reflects their expenses) to avoid exploiting altruistic sperm donors and yet prevent financially motivated donations (HFEA, 2011a). Restrictions were also made regarding acceptable compensation to men donating in foreign centres where the sperm is later imported to the UK. This policy was introduced outside our study period and the outcomes will be investigated in a future review.

As suboptimal semen parameters is the commonest cause of rejection of sperm donors (Paul *et al.*, 2006; Hamilton *et al.*, 2008a) a general decline in the semen parameters in men over years although debatable because of the conflicting results and geographical limitations (Carlsen *et al.*, 1992; Adamopoulos *et al.*, 1996; Irvine *et al.*, 1996; Gyllenborg *et al.*, 1999; Dindyal, 2004; Sripada *et al.*, 2007; Calonge *et al.*, 2009;

Hiltrud *et al.*, 2010; Axelsson *et al.*, 2011; Olsen *et al.*, 2011; Prisant *et al.*, 2011; Priskorn *et al.*, 2012; Splingart *et al.*, 2012) may be considered as it could possibly lead to a greater rejection of potential sperm donors. The minimum standards of fresh semen parameters for acceptance have been cautiously adjusted to the current criteria (detailed in methods) from those previously published (Paul *et al.*, 2006). By taking a more critical view of the post thaw parameters, more variation in pre freeze samples has been accommodated (still within normal limits) whilst maintaining satisfactory treatment outcomes on par with national averages.

The decline in donor recruitment compounded with losing a large pool of sperm donors (anonymous donors) led to increasing waiting times for treatment, increasing sperm import and an increase in the request for known sperm donations at our centre. Interestingly the number of sperm donors registered with the HFEA has risen from 272 in 2005 to 480 in 2010 (HFEA, 2011d) despite the fact that the number of patients treated and the total number of donor treatment cycles performed were still lower in 2010 than in 2004 (HFEA, 2011c). Even though the number of sperm donors in 2010 (480) is so close to the estimated need of sperm donors in the UK per year i.e. 500 (Hamilton and Pacey, 2008b), there is evidence of unmet demand for donor sperm.

It is unclear why the rise in donors has not translated into a proportionate increase in the number of patients receiving treatment, which is also reflected in an apparent increase in patient waiting times, the amount of sperm imported and the numbers of patients travelling abroad for treatment (Hamilton, 2010; Culley *et al.*, 2011). It has been suggested that the recent rise in sperm donor numbers is contributed to by the increase in numbers of overseas sperm donors used (who need to be screened as per the UK guidelines for sperm donors and also registered with HFEA) i.e. sperm import, and the increasing use of known donations by friends and family of the recipients of donor sperm treatments (Tomlinson *et al.*, 2010). Such imports and donations are unlikely to lead to treatment of more than one couple but are registered individually hence there are fewer patients per donor overall than if each was used to the 10 family capacity available with altruistic donors who consent to it.

HFEA data shows that 11 % of donors were from overseas in 2005 and this had increased to about 24% by 2010 (HFEA, 2011e). Moreover, 16% of newly registered sperm donors in 2008 limited their donation to one family i.e. were likely to be 'known

donors' donating to family or friends (HFEA, 2011b). Janssens *et al.* (2006) reported an increase in known donors during the years of debate and following the removal of anonymity in the Netherlands.

It is worth noting that less than 1% of donors registered with the HFEA between 2006 - 2008 actually created 10 families (upper limit) (HFEA, 2011b). The average number of families created was only one or two with one or two children in each family. This was explained by the non-use of 19% of donors probably due to the withdrawal of consent or of not being chosen by the patients, clinic protocols involving under-utilisation of a donor (i.e. create only 6 families) and approximately about 20% of the donors being overseas usually being imported for only one patient.

3.8.2 Donor sperm treatments

3.8.2.1 Fewer patients receiving treatment

There has been a noticeable decline in the number of patients receiving treatment with donor sperm over the study period. The fewer number of treatment cycles undertaken and therefore patients receiving treatment is primarily the result of the sperm donor shortage which has also led to the waiting list for treatment. Furthermore it is notable and regrettable that about 30% of the patients on the waiting list never return for treatment.

Contributing to the change in treatment patterns is the fact that more couples are able to be treated with the man's own sperm owing to the success of advanced surgical sperm retrieval techniques since 2009 (At NFC eight men underwent surgical sperm retrieval in 2002 compared to 57 men in 2010 alone with successful sperm recovery in 40%, data not shown in the results).

The decline in the numbers of patients treated is unlikely to be due to reduced referrals to the Centre, even though there is no formal recording of the referrals specifically for donor sperm treatment, since the waiting list (although stabilising) has not declined to any extent.

Nationally the HFEA published figures show a decline in the number of patients receiving donor sperm treatments since 1993. This was initially thought to be due to the acceptance of ICSI which was a well-established method of treatment by the year 2000.

However the expected stabilization of this decline has not materialized (Pacey, 2010). The numbers of patients treated with DI and D-IVF only started to gradually rise again from 2007 to 2010 (from 2389 patients in 2007 to 2960 patients in 2010). Despite this encouraging trend, the number of patients treated and the total number of donor treatment cycles performed are still lower in 2010 than in 2004 (3712 patients in 2004) (HFEA, 2011c)

3.8.2.2 Treatment cycles carried out

There is a declining trend in the number of DI cycles (unstimulated and stimulated) performed at our Centre reflecting the decline in the total number of patients treated with donor sperm. Nationally, the decline in DI cycles stabilized between 2007 and 2010 (3871 cycles in 2007 to 3878 cycles in 2010) (HFEA, 2011c) as the number of patients receiving treatment rose.

Our data shows that an increasing proportion of donor treatments involve D-IVF (i.e. the ratio of D-IVF cycles to the total donor sperm treatment cycles increased) although this has been generally lower than the comparable HFEA data which remained stable between 15 to 18% of total treatments (Hamilton *et al.*, 2008a). However the recent HFEA data of 2009 and 2010 does show that the proportion of IVF treatments are on the rise at 22% (HFEA, 2011c).

It has been suggested that fertility centres may be resorting to increasing use of IVF with donor sperm, to improve the efficiency of limited sperm stores (Pike and Pacey, 2005; British Fertility Society survey, 2006). Our data show a similar increase in the proportion of donor treatments involving IVF. The likely reasons for the increase in our centre are not only because of the strategies to improve the efficiency of the treatment (particularly for women in their late thirties and forties to start IVF instead of insemination) but also an increase in NHS funding for IVF treatment from two to three cycles in the majority of primary care trusts in the North East region. Furthermore, the use of back up donor for our men undergoing surgical sperm retrieval who aren't successful also contributed to this rise.

3.8.2.3 Indications for treatment with donor sperm

In our study there was a rising trend in the proportion of same sex couples and single women treated, particularly as there was no restriction on the utilisation of sperm

services, unlike in some European countries such as France (Shenfield *et al.*, 2010). The Human Fertilisation and Embryology Act of 2008 (HFEAct, 2008), apart from recognising same sex couples as legal parents of children, also replaced the reference ‘need for a father’ with ‘need for supportive parenting’, recognising the role of all parents. This recognition in the Act is likely to further increase the numbers of single women (Jadva *et al.*, 2009) and same sex couples coming forward for treatment.

The rising trend was also seen nationally and it was reported that in 2010 40% of DI services were used by same sex couples and single women, as opposed to 20% in the year 2000 (Hamilton, 2010). It was pointed out by the author however that absolute numbers of same sex couple and single women are not significantly higher. Our data concurs that these increasing proportions of same sex couple or single women treatments are secondary to a fall in the numbers of heterosexual couples receiving treatment.

3.8.3 Sperm export and imports

The transfer of sperm to the peripheral centres within the North East region and rest of UK has essentially stopped after the removal of anonymity to try and unify the waiting lists within the catchment area, which is likely to have led to difficulties for some patients in accessing treatment, similar to the experiences of other fertility centres (Hamilton, 2010). The amount of sperm imported to NFC has increased in recent few years and this is comparable to the rising number of overseas sperm donors nationally, as per the HFEA data detailed above.

3.8.4 Waiting list issues

We have developed a waiting list for treatment similar to many other centres (Hamilton, 2010; Culley *et al.*, 2011). In the BFS Donor survey 05/06, 74% of centres reported an increase in waiting times and also commented that waiting could essentially impose infertility upon older women (British Fertility Society survey, 2006). The BBC survey also reported that many of the 74 fertility clinics surveyed had a waiting time of at least 6 months for treatment (BBC survey, 2006).

Due to the current limited pool of donors at NFC, large numbers of patients wait for treatment and there is little or no choice of donors for selection. As a maximum of only 10 patients can be allocated to a sperm donor at any time, the donors are fully saturated,

leaving the patients with no choice, in contrast to before the removal of anonymity. In the BBC survey 86% of the centres reported less choice of donor for the recipients and as Wardle reported, the majority of centres provide only a 'racial match' (Wardle, 2008). Even if NFC succeeds in reverting to donor recruitment similar to earlier in the decade (40 in 10 years i.e. about four per year) (Paul *et al.*, 2006), it will take at least ten years to build a donor bank up to a similar in size to before the removal of anonymity, to allow improved choice and immediate treatment for patients.

Significant proportions (31.3%) of patients called for treatment failed to return to the centre because of the waiting times. Whilst only 3% of the patients admitted to seeking treatment elsewhere (i.e. abroad), there is a possibility that this could be much higher (20.4% did not respond when called for treatment) and also that patients could be accessing unlicensed sperm services that are easily available over the internet and could be associated with significant risks.

Cross border reproductive care appears to be increasing globally although the empirical evidence on the incidence is scant (Hudson *et al.*, 2011). Professor Culley's group interviewed 41 couples from the UK seeking treatment abroad and found that 12% travelled for donor sperm and a further 10% for both donor sperm and egg treatments. Among the couples travelling for donor gametes which included for donor oocytes, the commonest reason mentioned was donor shortage. The factors taken into account by the couples in choosing a destination for treatment included shorter waiting times and a greater availability of donors (Culley *et al.*, 2011).

A 2008 the European Society of Human Reproduction and Embryology (ESHRE) task force study on cross border reproductive care showed that 17% of the 53 forms received from UK couples seeking treatment abroad was for donor sperm treatment; and a quarter of the patients seeking treatment with donor gametes (including eggs and embryos) wished for anonymous donors although it was unclear if this was the main reason for travelling abroad (Pennings, 2010; Shenfield *et al.*, 2010).

Single women and same sex couples are the biggest groups accessing internet based sperm services (Hamilton and Pacey, 2008b), which first appeared to be available from 2004 (Pacey, 2010). Following the conviction of the owners of *Fertility First* which couriered sperm to women trying to conceive, the HFEA has investigated such websites

(Dyer, 2010). Whilst it is illegal to obtain, test, process or issue gametes including sperm without a HFEA license, the HFEA is aware of 'grey market' sites and urges couples to be cautious in dealing with these unlicensed providers.

3.9 Strategies to cope with the sperm donor shortage

Previous authors have suggested that there are two areas which may help to improve sperm donor recruitment, which includes advertising strategies and aspects of customer care relations.

Having no specific budget for advertising for gamete donors, the sperm donor programme at NFC is primarily dependent on opportunistic publicity and word of mouth (Paul *et al.*, 2006). The previous study done at NFC on sperm donor recruitment showed that the popular sources of information for potential sperm donors were certain media, student sources and word of mouth or friends (Paul *et al.*, 2006). Amongst the various media sources, the magazines and local newspapers were found to be popular sources of information whilst television, radio and tabloids were not. As student sources contributed considerably as an information source to non-students as well, since this group appear to be the biggest group of potential sperm donors in the current non-anonymous environment identified by the HFEA database (SEED Report, 2005), it emphasises the need to continue advertising through student sources such as student magazines.

Tomlinson *et al.* (2010) showed an increase in the enquiry rate from potential sperm donors when higher-cost advertising was used. The fact that 14 donors were released in a 4 year period from 151 enquiries at a cost of 5,500 pounds each gives an estimation of the resources needed for successful recruitment. However, novel advertising ideas have also been used, such as advertising for donors on the pay slips of NHS employees (Sinclair, 2009).

Raising the awareness of the sperm donor shortage using appropriate advertising strategies, nationally rather than locally, utilises the resources efficiently (Hamilton *et al.*, 2008a). The National Gamete Donation Trust (NGDT) is a national government-funded charity set up in 1998 to try to alleviate the national shortage of donors. One of their main goals is to raise public awareness of the donor need and it has launched

several large scale public awareness campaigns with some increase in the enquiry rate about becoming a donor ([ww.ngdt.co.uk](http://www.ngdt.co.uk)).

Despite fertility centres struggling to recruit sperm donors (Wardle, 2008; Hamilton *et al.*, 2008a; Culley *et al.*, 2011), it is certainly not in the entire UK, as some centres continue to be successful in the current framework (Adams *et al.*, 2006; Ahuja, 2008; Tomlinson *et al.*, 2010). Focused advertising, a dedicated recruitment team trained to improve customer care relations and better facilities for donors including out of hours services are some of the strategies used by these centres and which are both attitude and resource dependent. The underlying principle of this training should be to acknowledge the value of sperm donors and their contribution, making the entire process more donor friendly and hence to reduce the dropout rate (Blackburn-Starza, 2009). It is known that providing written information leaflets, counselling and support to sperm donors aids the recruitment process (Frith *et al.*, 2007).

At NFC changes have been made to improve donor recruitment, efficiency of the donor programme and to support patients as detailed below.

The donor selection and recruitment criteria have been addressed to improve sperm donor recruitment. We have maintained an upper age limit for donors of 45 years, [although UK guidelines for sperm donors recommend 40 years] (Association of Biomedical Andrologists *et al.*, 2008) and broadened our donor selection criteria as we no longer routinely exclude men with a past history of treated sexually transmitted infections. In particular, prospective donors at the centre are not currently rejected unless at least two (rather than one) of their semen samples fail to meet the required standard. Our previous study revealed that 95% of the applicants rejected because of suboptimal semen parameters had only 1 semen analysis (Paul *et al.*, 2006).

The strategies to improve the efficiency of the service have included the cessation of sperm transfer to other centres in order to unify regional waiting times for treatment. Also the laboratory processing of semen samples has been modified i.e. the samples are prepared for IUI prior to cryopreservation as opposed to the previous practice of cryopreserving neat semen samples, thus enabling more straws per ejaculate to be stored.

Historically intracervical insemination was done for unstimulated cycles at the centre which were high in straw consumption particularly when multiple inseminations were needed. Instead single IUI is undertaken to reduce the utilization of straws. This is also donor friendly as it decreases the number of ejaculates needed to create 10 families where consented.

NFC, with its small knowledgeable recruitment team continued to support patients by assisting in their efforts for donor sperm treatment. The centre does not discourage known donations and known donors once referred by their General Practitioner (GP) to the centre are offered a consultation, written information, preliminary semen analysis, support and counselling to enable their decision making.

The centre also provides support (albeit limited) to couples planning to travel abroad for treatment. Even though associated with a significant administrative burden for the centre, support is also given to patients who are keen to import sperm from other UK or foreign sperm banks for treatment. Overseas donors have to be identifiable and registered with the HFEA, apart from undergoing screening as per UK guidelines before being used for treatment. Registering imported donors only for single use (12 in 2010) takes considerable staff time and resources to streamline the process and prevent errors; hence financial viability has to be kept in mind.

Nationally the proposed solutions are:

- A hub and spoke model: A BFS working party in 2008 proposed a national sperm donation service programme to improve the sperm donor recruitment. This service frame work would have the regional centres as hub centres (14) coordinating donor recruitment by facilitating sperm donation at local centres to make it donor friendly (with regards to facilities, distances to travel, and/or convenient opening hours). The resources are to be focussed at the hub centres (14 hub centres have been proposed based on the proportion of UK patients treated including 3 centres in London and 2 in Scotland (Glasgow, Dundee), which will also co-ordinate distribution of the donor samples to the peripheral fertility centres to enable patients to access treatment locally (Hamilton *et al.*, 2008a) . This proposal from the BFS was piloted by Department of Health in Manchester, however the results have not yet been made public.

- A 2011 HFEA review of gamete donation policies to improve sperm and egg donation services in UK confirmed that the current family limit of 10 is to remain (HFEA, 2011b). The family limit of 10 was initially intended to avoid inadvertent consanguinity, however it was not evidence based. There is a wide safety margin with this family limit considering the geographical distribution and population density in UK (Janssens, 2003). However, taking into consideration the donors' perspective (particularly with the open identity system) and the psychosocial interests of all involved in donor insemination treatment, it was decided to maintain the same family limit (Hamilton and Pacey, 2008b; Janssens *et al.*, 2011).
- Sperm sharing schemes are also proposed whereby the fertile male partner of the couple needing IVF could donate sperm for other treatment benefits, such as the funding of treatment or movement up the waiting list (Hamilton and Pacey, 2008b).

Sperm donor shortage at a national level is a complex issue with limited empirical evidence. The possibility that the loss of anonymity in 2005 contributed to the current shortage of donors has been extensively argued with contradicting views, but is confounded by the fact that the decline in donor recruitment was reported even before that came into effect (Pike and Pacey, 2005; BBC survey, 2006; British Fertility Society survey, 2006; Witjens, 2007; Blyth and Frith, 2008; Wardle, 2008; Tomlinson *et al.*, 2010). Furthermore donor shortage is a global issue also affecting countries where donor anonymity is protected (Blyth and Frith, 2008).

A review in 2008 of the regulations, to consider revoking donor anonymity did not receive much support for change (Hamilton, 2010). Whilst some bodies like the NGDT strongly believe that removal of anonymity is not responsible for the decline in the numbers of sperm donors in the UK, others acknowledge its contributory role (Witjens, 2007; Tomlinson *et al.*, 2010). Most likely the removal of anonymity, along with the issue of payment to donors, (Tomlinson *et al.*, 2010) combined with the lack of awareness of the problem (Witjens, 2007) have contributed to the current shortage. Although there is no clear 'cause and effect' relationship, the removal of anonymity appears to have had a significant impact particularly due to the abrupt loss of large pool of anonymous sperm donors and the fragility of low donor recruitment since (Blyth and Frith, 2008).

3.10 Limitations of the study

Apart from the inherent limitations of a retrospective analysis, the study findings are from a single centre in the UK and cannot be generalised, but importantly the authoritative HFEA data does not seem to reflect the practice at the centre. In the absence of published empirical evidence apart from the national HFEA data, we used media surveys (BBC survey) and web news (Bionews) to understand the impact of removal of donor anonymity on various fertility centres in the UK.

3.11 Conclusion

Overall the sperm donor programme at our centre has been significantly altered in the past decade with fewer donors recruited, a smaller pool of donors, fewer patients receiving treatment, increased sperm import and increased waiting times for the patients.

The majority of the solutions proposed to manage the problem are reliant on funding, which can be difficult to obtain in the current economic climate. Use of existing resources to improve the efficiency of use of donor sperm may be helpful. Sub optimal semen parameters were the commonest reason to reject a potential sperm donor. We will next therefore consider the influence of external factors on semen parameters with a view to investigate if it can be positively used to improve the use of donor sperm. The next section will examine the impact of 'Season' on semen parameters.

Chapter 4 Seasonal variation in semen parameters

4.1 Introduction

Seasonal variation in semen parameters has been extensively described in literature, although the findings have been variable and inconsistent. Seasonal variation does not appear to be universal, however the majority of studies revealed a significant seasonal variation with improved parameters in Winter/Spring (or worsening parameters in Summer/Autumn) (Tjoa *et al.*, 1982; Levine *et al.*, 1988; Reinberg *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Jørgensen *et al.*, 2001; Chen *et al.*, 2003; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005; Calonge *et al.*, 2009) whilst some revealed an absence of seasonal variation (Mortimer *et al.*, 1982; Mallidis *et al.*, 1991; Ombelet *et al.*, 1996; Chia *et al.*, 2001; Carlsen *et al.*, 2004). Furthermore varied patterns such as progressive sperm motility being lower in Summer and also Spring (Centola and Eberly, 1999) or that morphology was better in Summer and Winter (Detti *et al.*, 2006) have also been reported.

There are few longitudinal studies and therefore most of the data is gained from retrospective cross sectional observational studies. Although the majority of the studies were conducted in the Northern hemisphere (Levine, 1999), a study done in the Southern hemisphere (Sobreiro *et al.*, 2005) revealed a similar seasonal variation of significant decline in all semen parameters in Summer. Seasonal variation is more commonly seen in temperate than tropical climates (Chia *et al.*, 2001), though not always. Seasonal variation in sperm concentration has been consistently reported whilst the data on semen volume, sperm motility and morphology is contradictory (Levine, 1999).

Although men in the UK, being in the Northern hemisphere and exposed to a temperate climate could be subjected to seasonal variation in semen parameters; this has not been confirmed because of limited number of studies from the UK. A study undertaken nearest to our centre geographically, in Edinburgh, revealed a non-significant improvement in sperm counts in Winter and Spring (Mortimer *et al.*, 1983).

In this chapter we studied all the semen analyses performed at the centre over 3 years.

4.2 Aim

We aimed to investigate the seasonal variation in semen parameters.

4.3 Objectives

The objectives were to investigate if there was any variation in semen parameters (semen volume, sperm concentration, total concentration, motile concentration, progressive motile concentration, progressive motility, total motility and morphology) between different seasons.

4.4 Outcome measures

To determine if the semen parameters were significantly better or poor in any particular season.

4.5 Subjects and Methods

4.5.1 Subjects

Men who had their semen analysis performed at NFC as part of their preliminary fertility investigations were included in the study. Some men attended the centre for more than one semen analysis.

4.5.2 Methods

A retrospective review of all the semen analyses done at NFC between December 2006 and November 2009 was performed. Semen analysis reports were routinely saved in Microsoft access. All the relevant data was transferred to an EXCEL spread sheet. Semen analyses were divided into 4 groups by season.

4.5.3 Study size

There were 4081 semen analyses undertaken during the study period after excluding samples showing azoospermia (absence of sperm in the ejaculate). Eleven further semen analyses were not included as the reports had more than one missing value. Three semen analyses were not included as the sample volumes were very high (36ml, 47ml, 68ml) with poor counts indicating that whilst not specifically recorded, they were likely to have been retrograde samples. A single missing semen volume was entered as 2ml as the rest of the parameters were available and normal. Two of the semen volumes were corrected to 10.8 and 4.9 from 108ml and 49ml respectively, as the rest of the semen

parameters were normal. After excluding or amending the above, a total of 4067 semen analysis results were included in the study.

4.5.4 Semen Analysis

4.5.4.1 Sample collection

Men attending for semen analysis were generally given written instructions regarding abstinence (2-7 days) and the method of collection. Men produced samples by masturbation into a clean wide mouthed container. Men producing the sample at home or outside the centre were advised to bring the sample to the centre within 90 minutes of production, keeping the sample warm during transit.

4.5.4.2 Liquefaction

Semen samples were left to liquefy for a maximum of 30 minutes at ambient temperature. The samples were analysed soon after liquefaction or at 30 minutes.

4.5.4.3 Volume

The volume of the ejaculate was measured using an appropriate sized, graduated, disposable serological pipette with 0.1ml accuracy.

4.5.4.4 Sperm concentration and motility

The sperm concentration and motility were routinely performed via computer-assisted semen analysis (CASA) using the Hobson Sperm Tracker (Hobson Tracking Systems Ltd, Sheffield, United Kingdom). An IBM (International Business Machines Corporation, Armonk, New York) compatible computer equipped with Hobson sperm tracker image analysis software (version 7.08) and hardware connected to a phase contrast microscope (Nikon Labophot -2) via a video camera (Sony CCD-IRIS) was used. 10µl of the semen sample was loaded into one of the two chambers of a Leja 20 micron glass counting chamber (Nieuw Venne, The Netherlands) using a Gilson P20 pipette. The chamber was filled and placed on a warm microscope stage whilst the microscope was focussed on the heads of the sperm. The sample was allowed to settle and the analysis commenced. The computer analysed the images acquired by the video monitor, 200 sperm were tracked averaging 100 sperm tracks per minute for total of 2 minutes. The minimum track time was at least 3 seconds for each sperm and 30 frames were analysed per field. Sperm were quantified and the results printed out. The Hobson Sperm Tracker was pre-set by the manufacturer to grade motility parameters based on

average path velocity (VAP) as follows - Grade A (VAP>25 $\mu\text{m/s}$), Grade B (VAP 10-25 $\mu\text{m/s}$), Grade C (VAP <10 $\mu\text{m/s}$) and Grade D (VAP = 0 $\mu\text{m/s}$). When the motile sperm concentration was less than 5 million/ml manual assessment was done. Semen samples were diluted when the counts were over 80 million/ml.

4.5.4.5 Sperm morphology

Morphology assessment was done manually on unstained preparations. 10 μl of the semen sample was pipetted onto a microscope slide and a coverslip applied. At a magnification of $\times 400$ ($\times 40$ objective), using a phase contrast microscope, 100 different motile sperm were recorded for normality using the Celltrac counting chamber. The classification of sperm morphology was based on the 1999 World Health Organisation criteria (World Health Organisation, 1999).

4.5.4.5.1 Classification of sperm morphology (WHO criteria – 4th edition)

4.5.4.5.1.1 Normal morphology

A Sperm consists of a head, neck, mid piece and tail.

Head – The head should be oval in shape. The length of the head should be 4 -5 μm and the width 2.5 – 3.5 μm . There should be well defined acrosome region comprising 40 – 70% of the head area.

Mid piece –The mid piece should be slender, less than 1 μm in width, about one and half times the length of the head and attached axially to the head. Cytoplasmic droplets should be less than half the size of the normal head.

Tail – The tail should be straight, uniform, thinner than the mid piece, uncoiled and approximately 45 μm long.

4.5.4.5.1.2 Abnormal morphology

This classification categorised all the ‘borderline’ forms as abnormal. It was considered unnecessary to routinely distinguish between all the variations in head, midpiece or tail defects. Apart from the head, neck and mid piece and tail defects, the abnormalities included cytoplasmic droplets (located in the midpiece) larger than half of the sperm head size.

4.5.5 Quality assurance

NFC labs routinely participated in regular 3 monthly National External Quality Assurance assessments (NEQAS) in addition to internal quality assessments to ensure that standards in semen analyses were maintained. The quality assurance methods are detailed in Chapter 6 (Section 6.5.8.2.4).

4.5.6 Semen Parameters

The variables noted were semen volume (ml), sperm concentration (millions/ml), the percentage of motile sperm and percentage of normal morphology. Grade A and B were added together to obtain the percentage of progressively motile sperms as per the World Health Organisation (2010) criteria and the percentage of total motile sperm was calculated by adding grades A, B and C together. The total sperm concentration was calculated by multiplying semen volume and sperm concentration. The motile sperm concentration was obtained by multiplying sperm concentration and the percentage of total motile sperm, divided by 100. Similarly progressive motile sperm concentration was obtained by multiplying sperm concentration and the percentage of progressively motile sperm, divided by 100.

4.5.7 Seasons

The seasons during which the semen samples were obtained were classified as Winter (December –February), Spring (March-May), Summer (June-August) and Autumn (September-November).

4.5.8 Subsets

It is believed that men with persistent severe oligospermia are likely to have a pathology contributing to the semen quality although this is not always proven. It is likely that the semen quality in these men may be unaffected by seasonal changes (Centola and Eberly, 1999; Levitas *et al.*, 2013). Analysing the entire study group, which included normal semen analyses and all grades of severity in oligospermia, could potentially fail to identify subtle seasonal variation in semen parameters (if there was any). Therefore to minimise selection bias and improve the efficiency of the evaluation of the effect of season on the semen parameters, we analysed the study group in the following subsets.

1. Total study group (samples with azoospermia excluded) - 4067 samples
2. Samples with sperm concentration ≥ 1 million / ml - 3905 samples
3. Samples with sperm concentration ≥ 5 million / ml - 3748 samples

4. Samples with sperm concentration ≥ 10 million / ml - 3600 samples

4.6 Statistical Analysis

Statistical analysis was performed using IBM SPSS (Version19). All the semen parameters are expressed as median + centiles. As the study size was large, the Kolmogorov – Smirnov test of normality was applied which showed that the data was not normally distributed ($p=0.00$). Variables were not transformed to normalise the data. The non-parametric Kruskal Wallis test with a post hoc multiple comparisons test (Siegal and John jr, 1988) was applied to find any significant difference in semen parameters between the seasons.

4.7 Results

The age and semen parameters in each group were expressed as median + centiles. The median male age was 34 years (ranging from 15 to 67 years) and comparable in the four groups. The median (inter-quartile range) for semen volume (ml), sperm concentration (million/ ml), total motility (%) and normal morphology (%) for the entire study size (4067 samples) were 3 (2.1-4.1), 56 (26-99), 62 (46-74) and 6 (2-10) respectively. As shown in Table 10, there was no significant difference in semen volume, sperm concentration, total concentration, total motile concentration, progressive motile concentration, total motility and progressive motility detected between the seasons ($p > 0.05$). Sperm morphology was significantly better in Winter and Spring compared to Summer and Autumn, although the actual improvement was low. Furthermore all the subsets i.e. sperm concentration ≥ 1 million/ml, ≥ 5 million/ml and ≥ 10 million/ml showed similar results with sperm morphology being the only semen parameter with a significant seasonal variation (Table 11, Table 12 and Table 13).

Semen parameters N=4067 samples; Median (25 th -75th centile)	Winter (N=1015)	Spring (N=966)	Summer (N=1006)	Autumn (N=1080)	Kruskal Wallis test
Age (years)	34 (30-38)	34 (30-38)	34 (30-38)	34 (30-38)	0.5
Semen volume (ml)	3.0 (2.1-4.1)	3.0 (2.0-4.1)	3.0 (2.1-4.2)	3.0 (2.2-4.1)	0.4
Sperm concentration (millions/ml)	54 (26-98)	59 (26-99)	56 (29-103)	54 (23-95)	0.2
Total sperm count (millions/ml)	166 (68-308)	165 (66-320)	173 (77-306)	157 (64-297)	0.3
Total motile concentration (millions/ml)	34 (13-70)	37 (10-68)	35 (13-72)	34 (10-67)	0.3
Progressive motile concentration (millions/ml)	26 (9-54)	29 (8-55)	27 (9-57)	26 (7-53)	0.3
Total motility (%)	64 (50-74)	61 (45-74)	63 (46-74)	62 (44-74)	0.2
Progressive motility (%)	49 (36-59)	48 (33-60)	48 (35-59)	48 (32-59)	0.3
Morphology (%)	7 (2-12)	6 (2-11)	5 (2-10)	6 (1-10)	<0.005

Table 10: Characteristics of the semen samples by seasons in the total study group (N= 4067 samples)

Semen parameters N= 3905 samples; Median (25th -75th centile)	Winter (N=978)	Spring (N=920)	Summer (N=966)	Autumn (N=1041)	Kruskal Wallis
Age (years)	34 (30-38)	34 (30-38)	34 (30-38)	34 (30-38)	0.5
Semen volume (ml)	3.0 (2.1-4.1)	3.0 (2.0-4.1)	3.0 (2.1-4.2)	3.0 (2.2-4.1)	0.5
Sperm concentration (millions/ml)	57 (30-100)	53 (29-102)	59 (32-105)	57 (26-97)	0.09
Total sperm count (millions/ml)	174 (77-314)	176 (83-330)	181 (90-314)	164 (70-302)	0.17
Total motile concentration (millions/ml)	36 (15-71)	40 (15-71)	38 (15-75)	35 (12-68)	0.1
Progressive motile concentration (millions/ml)	28 (11-56)	31 (11-56)	28 (11-59)	27 (9-54)	0.1
Total motility (%)	64 (50-74)	62 (48-75)	64 (50-75)	63 (46-74)	0.4
Progressive motility (%)	49 (37-59)	49 (35-60)	48 (36-60)	48 (33-69)	0.4
Morphology (%)	7 (4-12)	6 (2-12)	6 (2-10)	6 (2-10)	<0.005

Table 11: Characteristics of the semen samples by seasons in samples with sperm concentration ≥ 1 million / ml (N= 3905 samples)

Semen parameters N=3748 samples; Median (25th -75th centile)	Winter (N= 942)	Spring (N= 881)	Summer (N=934)	Autumn (N= 991)	Kruskal Wallis test
Age (years)	34 (30-38)	34 (30-38)	34 (30-38)	34 (30-38)	0.6
Semen volume (ml)	3.0 (2.1-4.1)	3.0 (2-4.1)	3.0 (2.1-4.2)	3.0 (2.2-4.1)	0.5
Sperm concentration (millions/ml)	60 (33-103)	65 (34-104)	60 (35-108)	59 (31-100)	0.1
Total sperm count (millions/ml)	180 (86-324)	186 (90-336)	188 (98-319)	172 (83-308)	0.3
Total motile concentration (millions/ml)	39 (17-73)	42 (17-73)	38 (18-77)	38.1 (15-71)	0.3
Progressive motile concentration (millions/ml)	29 (13-57)	33 (13-58)	30 (13-60)	29 (11-55)	0.2
Total motility (%)	65 (50-75)	63 (50-75)	64 (50-75)	64 (50-75)	0.7
Progressive motility (%)	50 (39-59)	49 (37-61)	49 (37-60)	49 (36-60)	0.6
Morphology (%)	8 (4-12)	7 (4-12)	6 (2-10)	6 (2-10)	<0.005

Table 12: Characteristics of the semen samples by seasons in samples with sperm concentration ≥ 5 million / ml (N=3748 samples)

Semen parameters N= 3600 Median (25th -75th centile)	Winter (N=902)	Spring (N=846)	Summer (N= 900)	Autumn (N=951)	Kruskal Wallis test
Age (years)	34 (30-38)	34 (30-38)	34 (30-38)	34 (30-38)	0.5
Semen volume (ml)	3.0 (2.1-4.1)	3.0 (2.0-4.1)	3.0 (2.1-4.2)	3.0 (2.2-4.1)	0.5
Sperm concentration (millions/ml)	64 (37-106)	68 (38-107)	62 (37-110)	62 (34-103)	0.1
Total sperm count (millions / ml)	190 (98-333)	193 (100-344)	194 (104-329)	183 (92-319)	0.3
Total motile concentration (millions/ml)	41 (20-75)	44 (19-76)	40 (19-78)	40 (18-72)	0.3
Progressive motile concentration (millions/ml)	31 (15-59)	34 (14-60)	32 (14-62)	30 (13-58)	0.2
Total motility (%)	65 (50-75)	64 (50-75)	65 (50-75)	65 (50-75)	0.8
Progressive motility (%)	51 (40-60)	50 (38-61)	50 (38- 60.5)	50 (37-60)	0.7
Morphology (%)	8 (4-12)	8 (4-12)	6 (2-11)	6 (2-11)	<0.005

Table 13: Characteristics of the semen samples by seasons in samples with sperm concentration ≥ 10 million / ml (N= 3600 samples)

4.8 Discussion

We conducted a retrospective database review of all the semen analyses performed at NFC between December 2006 and November 2009 which revealed sperm morphology to be significantly better in Winter and Spring compared to Summer and Autumn, but there was no significant seasonal variation in other semen parameters.

Although the criteria for classification of sperm morphology used in our study was in accordance with WHO 1999 criteria, the methodology to assess this was not.

Morphology was assessed on unstained motile sperm samples while the recommendation was to assess on stained samples which are inevitably immotile.

Several surveys have been done to check the compliance with the WHO recommendations of laboratories regularly performing semen analyses. In a global questionnaire survey regarding sperm morphology assessment by Ombelet *et al.* (1997) wherein they received the responses from 170 centres in 40 different countries, a wide variation in staining methods and use of classification criteria were noted. While staining of the specimens was not performed in 7.6%, 11 different staining methods were used in the rest of the 157 centres. It is well known that different staining methods have variable effects on sperm dimensions and therefore none would be considered accurate even if the WHO sperm classification criteria were used without the recommended staining procedure. In the UK, similar to our centre, it was noted that a significant proportion of centres (47% - 8/17) did not use any staining method though the WHO criteria for sperm classification were often used (57.8%).

To assess sperm morphology 100 motile sperm were counted in our centre. In a survey by Keel *et al.* (2002) in the United States, responses from 536 centres were received and 15% of the centres did not routinely perform sperm morphology. In the centres that undertook sperm morphology assessment, 83% counted less than the recommended 200 sperm to report normal morphology.

More recently in the UK, a survey done by Riddell *et al.* (2005) has revealed that only 5% (2/37) of the centres which participated were fully compliant with WHO recommendations for sperm morphology assessment. Unstained preparations were used in 43% of the centres, concurring with Ombelet's survey findings. Among the labs

which used stained preparations only 68% used the WHO recommended staining methods. Despite the common use of unstained smears or staining methods outwith the WHO recommendations, the majority of the centres (32/37) followed the WHO criteria for sperm classification. It was interesting to note that majority of the centres (69%) chose to classify 100 or fewer sperm.

There was a global lack of compliance by laboratories with the WHO recommendations for sperm morphology in the 1992 guidelines (Ombelet *et al.*, 1997) and the problem continued to persist in UK with the 1999 guidelines, (Riddell *et al.*, 2005) when again the importance of compliance and the need for education and training initiatives were emphasized.

As our centre's specialist laboratory undertakes semen analyses for assisted conception treatments in addition to routine semen analysis for preliminary infertility assessment, we noted with interest in the UK survey suggestion that District General Hospital (DGH) laboratories were arguably closer to compliance with WHO recommendations than specialist laboratories. It was suggested that the difference in approach was possibly due to the type of staff performing the analyses (mainly embryologists and PhD scientists in specialist laboratories versus mainly biomedical scientists at the District General Hospitals) who had different training pathways, or alternatively to the culture in the specialist laboratories of avoiding the use of unnecessary volatile solvents to prevent contamination of the atmosphere where embryos are cultured. The precise reason is not entirely clear.

It is accepted that the methodology for sperm morphology for samples included in our study was not the most accurate available, however was approved at the time based on the centre's logistics. It is well known that the Quality Assurance (QA) is an essential method of maintaining the accuracy of semen analyses (Pacey, 2006). The centre has regularly participated in the National External Quality Assurance Scheme (NEQAS) and as the results were comparable to national standards, the approved methodology in the centre continued to be used.

Sperm morphology was the only semen parameter which showed significant seasonal variation (i.e. better in Winter and Spring) in our study. Levine *et al.* (1992) in their prospective longitudinal study revealed that there was considerable deterioration of

sperm concentration, motile concentration and percentage of morphologically normal sperm in Summer (in both outdoor workers and indoor workers) compared to Winter, however after adjusting for the confounders, there was statistically significant reduction only in the percentage of morphologically normal sperm, similar to our study findings.

The actual improvement was however low in our study, whilst in Levine *et al.*'s study the sperm morphology deteriorated by 14% in Summer compared to Winter (Unadjusted values of percentage of morphologically normal sperm in Winter vs. Summer was 52.4% vs. 45.1%). The percentages of normal morphology in both seasons are strikingly very high compared to our study as there was a significant difference in the sperm morphology classification used. In Levine *et al.*'s study 10 distinct abnormal forms such as large head, two heads, two tails were described and the remaining were labelled as normal. In contrast, in our study only sperm that fulfilled WHO criteria for normal sperm morphology as described in the methods were assigned as normal.

Few studies similarly reported improved sperm morphology in Winter and/or Spring (Levine *et al.*, 1988; Andolz *et al.*, 2001; Chen *et al.*, 2003; Sobreiro *et al.*, 2005), however an improvement in other semen parameters (sperm concentration and or motility but not semen volume) was also reported. Swatowski *et al.* (1994) in their retrospective study including 200 semen samples showed that sperm morphology was the only parameter showing seasonal variation. However in contrast, they found that the proportion of abnormal sperm was higher in Spring compared to Summer and Winter. Notably many studies investigating seasonal variation in semen parameters did not study sperm morphology (Tjoa *et al.*, 1982; Reinberg *et al.*, 1988; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Gyllenborg *et al.*, 1999; Chia *et al.*, 2001).

Interestingly in the study by Centola and Eberly (1999), where men attending a fertility clinic and also semen donors were recruited, the pattern of seasonal variation varied between the two groups. Whilst in the semen donors the sperm count was lower in Summer, the motility was lower in Summer and also Spring. However similar to our study, in men attending the fertility clinic there was no seasonal variation in semen volume, count or motility but the percentage of tapered forms were significantly higher in Autumn than Spring. As the semen parameters in fertile semen donors was subject to greater seasonal variation than 'suspected infertile' men the authors suggested that possible pathology might not be affected by season. Furthermore in a recent study by

Levitas *et al.* (2013), where semen samples from men undergoing basic fertility investigations were included, the authors analysed the seasonal variation in normozoospermic samples (≥ 20 million/ml) separately to oligozoospermic samples and noted that the significant seasonal variation seen in normozoospermic samples (sperm concentration, rapid motility and morphology were better in Winter and Spring) was not seen in oligozoospermic samples (sperm concentration and rapid sperm motility did not reveal seasonal variation).

However other studies conducted on men attending a fertility clinic did show seasonal variation (Andolz *et al.*, 2001; Chen *et al.*, 2003). Observed seasonal changes in semen parameters are not restricted to semen donors (Saint Pol *et al.*, 1989; Gyllenberg *et al.*, 1999) or men attending infertility clinics, but have also been seen in volunteers from the general population (Levine *et al.*, 1990; Levine *et al.*, 1992), men prior to vasectomy (Tjoa *et al.*, 1982; Sobreiro *et al.*, 2005) and infertile men (Politoff *et al.*, 1989).

Our study showed that there was no seasonal variation in semen volume which is consistent with several other studies (Levine *et al.*, 1992; Andolz *et al.*, 2001; Chen *et al.*, 2003; Sobreiro *et al.*, 2005), although Reinberg *et al.* in their study on pre-vasectomy men reported higher semen volumes in Spring (Reinberg *et al.*, 1988).

A retrospective study including semen analyses from men attending an infertility clinic in Edinburgh which has similar temperate climate to the catchment area of our centre in the North East of the UK, revealed an absence of seasonal variation in all semen parameters, but noted a pattern of increased sperm counts in late Winter and early Spring (Mortimer *et al.*, 1983). Furthermore the majority of studies in temperate climates did show seasonal variation with a trend towards improved sperm parameters in Winter and Spring (Levine *et al.*, 1988; Gyllenberg *et al.*, 1999; Levine, 1999; Calonge *et al.*, 2009).

In view of the lack of seasonal variation in sperm count and motility seen in our study the following reasons may be considered.

There are some methodological differences in semen analysis in studies investigating seasonal variation. Two methods, manual and CASA are widely used. CASA is objective and more reproducible than the manual method (Centola, 1996; Larsen *et al.*,

2000) and the variation in semen parameters between the two methods is well known (Macleod *et al.*, 1994). It is well known that CASA moderates extreme values of sperm count and therefore has the potential to minimise the differences in the counts between seasons, leading to a false conclusion of an absence of seasonal variation (Levine *et al.*, 1990).

A previous study done at our centre confirmed the variation between CASA and the manual method in sperm count and motility assessment. There was significant underestimation of sperm count and over estimation of motility when the sperm concentration was above 80 million/ml (Spiropoulos, 2001). CASA methodology in our study may have contributed to the lack of seasonal variation in sperm count and motility, however other studies which used CASA still revealed considerable seasonal variation in sperm count and/or motility (Levine *et al.*, 1990; Centola and Eberly, 1999; Chen *et al.*, 2003; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005). Finally the effect of genetics, lifestyle factors and environmental factors on regional trends cannot be ruled out.

The large study size and duration over three years may have contributed to minimise the effect of confounders on the results. In addition the semen analysis methodology remained consistent throughout the study period and only five laboratory technicians (who all regularly participated in Quality Control checks with results confirming the maintenance of standards) were involved in the analysis of semen samples.

4.9 Limitations of the study

There are several limitations to the study.

The lack of information on abstinence and sample loss at collection may confound the association between season and semen parameters. This relevant data could not be retrieved from the database and therefore was unavailable for the analysis.

It is well known that abstinence affects not only semen volume, sperm count and motility, but also morphology with a longer than 7 day duration (Mortimer *et al.*, 1982; Pellestor *et al.*, 1994). However as studies have shown that period of abstinence did not vary with season (Levine *et al.*, 1988; Gyllenborg *et al.*, 1999), even though this data cannot be extrapolated universally, it seems likely that it was consistent. The effects of abstinence on the semen analysis may be minimal as the men are also given written

instructions on recommended period of abstinence (2-7days) prior to sample production and the instructions are followed majority of the times. Although no internal audit has been done, observations by the senior Andrology staff at NFC confirm this. In addition in our vitamin D study (Chapter 6, Section 9), out of the 87 participants who returned for follow up only 1 did not follow the recommended abstinence, confirming suitability of the instructions given.

It was interesting to note in a study done by Levine *et al.* (1988) to assess the seasonal variation of semen parameters in men attending an infertility clinic, the reduction of semen parameters in Summer remained the same before and after adjusting for abstinence.

Some men in the study have produced more than one semen sample, which is presumed to be a small proportion. Although the exact percentage of this is unknown, it is unlikely to have adversely affected the conclusions.

The inherent limitations of a retrospective study include being unable to retrieve information on other factors (i.e. smoking and recent fever), which may potentially affect semen parameters, is applicable to our study.

Intra-individual variation in semen parameters is well known and therefore a single semen sample is considered insufficient to estimate semen quality (Carlsen *et al.*, 2004; World Health Organisation, 2010). As it is a cross sectional association study, this element, of not adjusting for intra-individual variation of semen parameters affecting the results, cannot be ruled out.

The samples included in the study were from men being investigated for infertility and despite including fertile and infertile men, may not be representative of general population.

All semen parameters essentially follow non-Gaussian distribution. In the statistical analysis, semen parameters were not transformed to normalise the data although it is generally recommended (Berman *et al.*, 1996; Handelsman, 2002; Sánchez-Pozo *et al.*, 2013). Application of non-parametric tests is a reasonable approach for non-normally distributed data, although powerful and flexible parametric tests following

transformation are commonly preferred (Handelsman, 2002; Sánchez-Pozo *et al.*, 2013). We note that in similar retrospective studies to our study, with a large sample size (1159 samples) (Levine *et al.*, 1988) 10,877 samples (Andolz *et al.*, 2001), and also in prospective longitudinal studies involving up to 142 men (Levine *et al.*, 1990; Levine *et al.*, 1992), the findings following the application of non-parametric tests on untransformed, non-normally distributed variables were similar to the findings following the application of parametric tests on transformed variables. Non-parametric tests were generally carried out to avoid the secondary effect of the artefact of transformation on the results.

Based on the above limitations we recommend caution in interpreting the results.

4.10 Conclusion

In men attending our fertility centre, seasonal variation is seen only in sperm morphology. This was significantly better in samples produced in Winter and Spring, however this is not a clinically significant improvement. No difference was seen in other semen parameters.

It is well known that despite the fact that only men with optimal semen parameters are recruited as donors, the fecundity of sperm donors is variable. Is this related to the month or season of donation? With regards to season of original sperm donation, we further investigated, and described in the next section, the distribution of conceptions achieved by donor insemination (DI) treatment, with a view to find out if there was any seasonal variation.

Chapter 5 Seasonal variation in donor conceptions

5.1 Introduction

Apart from the seasonal variation in semen parameters, it is worthwhile investigating the effect of seasonality of sperm on the chances of conception as this would reinforce the argument for the seasonal recruitment of sperm donors. Studies investigating this are sparse in literature. In this chapter we studied DI treatments with a view to identify any seasonal influence on the outcome.

5.2 Aim

We aimed to analyse the distribution of conceptions achieved by DI treatments, with a view to ascertain if there was a variation in relation to the seasonal timing of the original sperm donation.

5.3 Objectives

The objectives were to determine

1. The distribution of conceptions by the season of sperm production.
2. The distribution of conceptions by the season of treatment.
3. The association between the number of inseminations and pregnancy outcome.
4. Seasonal variation in donor semen parameters.
5. The association between season of sperm production and cryopreservation.

5.4 Outcome measures

The outcome measures were

1. Is sperm functionally better in any particular season? i.e. are more pregnancies achieved by sperm produced in any particular season?
2. Is cryopreservation of sperm (number of straws of sperm frozen and post thaw parameters) improved in any particular season?

5.5 Subjects and Methods

5.5.1 Subjects

This includes patients and donors as described below.

5.5.1.1 Patients

All the patients who had DI (natural cycle intra-cervical insemination) treatment from 2005 to 2010 are included in the study. This study period was selected as prior to 2005 the records did not allow us to track the season of semen sample production. DI treatment was offered to couples where the male partner had azoospermia and surgical sperm retrieval was either not an option, was unsuccessful or was declined by the patient. DI treatment was also offered to same sex couples and single women. The treated female partners were generally healthy, ovulating regularly and at low risk for any tubal disease.

5.5.1.2 Donors

All the sperm donors of NFC whose sperm was used for the treatments in the study period mentioned above were included. The recruitment of sperm donors at the centre was detailed in Chapter 3 (Section 3.5.2).

5.5.2 Donor insemination treatment (DI)

For natural cycle intra-cervical donor insemination women are monitored by trans-vaginal ultrasound to track follicular growth. Once the dominant follicle reaches a mature size (≥ 16 mm) arrangements are made for insemination. Two straws of the appropriate donor sperm are removed from storage and thawed at room temperature for 5 minutes. The patient's name is entered in the donor sperm straw consumption sheet, when the sperm to be used for treatment is handed over to the nurse. The ends of the straws are cut and the sample emptied into a container to be loaded into the insemination catheter (Insemicath, Cook). Intracervical insemination is carried out by inserting the tip of the catheter into the endo-cervical canal and injecting the fluid. The woman returns the following day for ultrasound follicular tracking. The inseminations are done every day till ovulation is proven (disappearance of the follicle and/or presence of free fluid) by ultrasound or a maximum of 4 inseminations are done.

When a batch of straws of donor sperm is released, the release date is recorded in the donor release file adjacent to the date of sample production. A new batch is released only after all the straws are used for the treatment. Therefore all the patients treated until a new batch is released, are treated with sperm produced in the same season.

5.5.3 Laboratory processing of the semen from recruited sperm donors

5.5.3.1 Pre freeze analysis

When donors produce a semen sample, a routine semen analysis is performed as previously detailed in Chapter 4 (Section 4.5.4), unless there is gross evidence of contamination or abundant leucocytes. If the semen parameters are inferior to the eligibility criteria for a sperm donor, the samples are deemed not suitable for freezing. The parameters recorded are semen volume, sperm concentration, motile concentration, progressive motile concentration, progressive and total motility. Sperm morphology was recorded for donor samples produced at the centre only from 2006 onwards. As the donors in this study were producing samples from 1995, morphology was not recorded for 915 out of 1114 samples. Therefore sperm morphology could not be included in the statistical analysis.

5.5.3.2 Process of cryopreservation

If the semen parameters meet the criteria, the semen volume is diluted with cryo-protectant (1:0.7 ratio, volume:volume), containing physiologic salts, glycine, dextrose monohydrate, lactate, glycerol, sucrose, and human serum albumin (3.95g/litre) (SpermFreeze, FertiPro) and the total volume is divided into 250 µl straws (Rocket Medical) each. Once the straws are sealed they are placed in vapour of liquid nitrogen for 20 – 30 minutes. Finally they are labelled, divided into a number of holding tubes and transferred to liquid nitrogen tanks (-196° C) (Statebourne Cryogenics (Model; Bio 36) for storage.

5.5.3.3 Post thaw analysis

At the time of cryopreservation, a single straw is reserved for post-thaw analysis and is thawed at room temperature for 5 minutes. Routine semen analysis is performed and if the sample reveals poor survival, all the straws frozen from that semen sample are discarded. If there is good post thaw survival, a record of the date of sample production and the number of straws frozen and stored is made.

5.5.4 Methodology

We performed a retrospective review of all the DI treatments for a period of 6 years. The following sources of information were used to collate the information needed for this study:

1. Laboratory database of donor sperm use for treatment

The database of donor treatments comprises the patient details, donor code, cycle of treatment, first date of insemination for each cycle and outcome. All the records previously erroneously entered in the database (13 records) were identified and excluded by cross checking with the database and records maintained for the sperm donor programme outcomes and consumption sheets (described below). Likewise missing records (5 records) were entered onto the database retrospectively. The number of patients, number of DI cycles, season of treatment and outcome were obtained from this database.

2. Database and records maintained for sperm donor programme outcomes

The treatment database holds patient details with date and type of the treatment and treatment and pregnancy outcomes. This database was used to cross check the information obtained from laboratory database mentioned above.

3. Weekly donor sperm straw consumption sheets

The weekly donor sperm straw consumption sheets were used to cross check the date of commencing treatment, to obtain the additional dates of treatment and also the number of inseminations. The season of treatment and number of inseminations per treatment cycle were obtained from this information.

4. A donor release file

The donor release file records the donor code, donation date of each sample, the number of straws frozen and also the release date of the sperm straws. This was used to track the sample used for treatment back to the date and season of sample production. Where this information was not recorded, an andrology database of donors (described below) was used to retrieve the information. The season of original sperm production of the thawed sperm used for treatment was obtained from this information.

5 An andrology database of donors.

The andrology database of donors holds information about donor code, semen parameters (both pre freeze and post thaw) with the date of sample production, number of straws of sperm frozen and the release date of the straws for use. The information on donor semen parameters and the number of straws of sperm cryopreserved by season were obtained from this database.

5.5.5 Study size

All the information was collated in an EXCEL file for analysis. A total of 496 DI treatments were recorded on to the database. The seasons are classified as Winter (December –February), Spring (March-May), Summer (June-August) and Autumn (September-November). When semen samples produced in different seasons was used for sequential inseminations in the same treatment cycle (10 cycles), the sample used for the last day/days of treatment which most likely led to the conception in pregnancy positive cases was recorded as the season of sperm sample production. The outcome of the treatment cycle was taken as pregnant if the pregnancy test was positive.

5.6 Statistical Analysis

The variables are expressed as mean \pm SD and median \pm centiles. The distribution of all the continuous variables was checked with the Anderson Darling normality test. To investigate the association between DI treatment pregnancy outcome and season of sperm sample production / season of treatment / number of inseminations, chi square tests were applied. As the donor semen parameters, both pre freeze and post thaw were not normally distributed (Anderson Darling test >0.05), the Kruskal Wallis test with post hoc multiple comparison test was applied to investigate the difference between the seasons. The association of season of semen sample production with efficiency of cryopreservation (number of straws of sperm frozen) was verified using analysis of variance ANOVA.

5.7 Results

A total of 241 patients underwent 496 DI cycles of treatment between 2005 and 2010. The age range of the women was 22 to 43 years with a median at 33 years. Each patient had an average of 2 DI cycles of treatment which is consistent with the centre's donor programme (detailed in Chapter 3, Section 3.5.3) which generally offers 3 cycles of DI treatment.

5.7.1 Is the pregnancy rate following DI treatment higher when sperm produced in a particular season was used?

There were 496 DI treatments undertaken between 2005 and 2010. 16 out of 496 cycles of treatment could not be included in the section. The reason for this was more than one batch of straws were released at the same time with different dates or seasons of semen sample production and the records did not allow further clarification to track down the precise season of sample production for these treatments. Therefore 480 DI treatment cycles were included in this section.

Most of the treatment cycles (42.2%) were carried out with semen samples produced in Spring (probably because most of the donor semen samples were produced in Spring as elaborated later in section 5.7.4.1) and only 15.2% were carried out with samples produced in Autumn. 22.2% and 20.2% of the treatment cycles were with semen samples produced in Winter and Summer respectively.

55 pregnancies were recorded including biochemical pregnancies (4), miscarriages (10), still birth (1), live birth (36) and also unknown outcomes following positive pregnancy test (4). The positive pregnancy rate per treatment cycle was 11.3% during this time period (56 pregnancies from 496 cycles). The pregnancy rate per cycle varied from 9.3% from semen samples produced in Winter to 15.1% in Autumn but there was no statistical difference in the outcome of DI treatment by the season of semen sample production ($p=0.67$) (Table 14).

Season of semen sample production		Pregnant		Total
		No	Yes	
Winter	Count	97	10	107
	% within	90.7%	9.3%	
Spring	Count	179	24	203
	% within	88.2%	11.8%	
Summer	Count	87	10	97
	% within	89.7%	10.3%	
Autumn	Count	62	11	73
	% within	84.9%	15.1%	
Total	Count	425	55	480
	% within	88.5%	11.5%	

Table 14: Donor insemination treatment outcome by the season of semen sample production

5.7.2 Is the pregnancy rate following DI treatment higher when treatment occurs in a particular season?

All 496 DI cycles were included in this section and 56 pregnancies were recorded. The DI treatment cycles performed were lowest in Winter (21.5%) with a slight compensatory rise seen in the Autumn (28.2%) earlier. DI cycles were carried out uniformly through the rest of the year with 24.7% and 25.4% of cycles carried out in Spring and Summer respectively. The lowest number of treatment cycles in Winter is likely to be due to the 3 week shutdown of laboratories for maintenance at Christmas. The rise just before Christmas is likely due to patients' preference to avoid treatment at Christmas.

The pregnancies included biochemical pregnancies (4), miscarriages (10), still birth (1), live birth (37) and also unknown outcomes following positive pregnancy test (4). The pregnancy rate per treatment cycle varied from 10.3 % with treatments carried out in Winter and Summer to 13.8% in Spring with 10.7% in Autumn. There was no significant difference in the outcome of the DI treatment by the season of treatment ($p>0.79$). The results are shown in the Table 15.

Season of DI treatment		Pregnant		Total
		No	Yes	
Winter	Count	96	11	107
	% within	89.7%	10.3%	
Spring	Count	106	17	123
	% within	86.2%	13.8%	
Summer	Count	113	13	126
	% within	89.7%	10.3%	
Autumn	Count	125	15	140
	% within	89.3%	10.7%	
Total	Count	440	56	496
	% within	88.7%	11.3%	

Table 15: Treatment outcome by the season of donor insemination

5.7.3 Is the outcome of DI treatment dependent on the number of inseminations?

Out of the 496 DI cycles, most of the treatment cycles (40.3%) received 2 inseminations (22.6% had one insemination, 27.2% had 3 and only 9.9% had 4 inseminations). In other words 63% of the treatment cycles had 1-2 inseminations as expected for DI treatments with ultrasound monitoring. The pregnancy rate per cycle varied from 7.1% with one insemination to 14.3% with four inseminations, although the number of treatment cycles with 4 inseminations was low (12.5% pregnancy rate per cycle with 2 inseminations and 11.9% with 3 inseminations). As expected with ultrasound monitored DI cycles there were no statistical difference with number of inseminations and DI treatment pregnancy outcome (p 0.44). The data are shown in Table 16.

			No: of inseminations				Total	Chi square
			1	2	3	4		
Pregnancy	No	Count	104	175	119	42	440	0.44
		%	92.9%	87.5%	88.1%	85.7%	88.7%	
	Yes	Count	8	25	16	7	56	
		%	7.1%	12.5%	11.9%	14.3%	11.3%	
Total		Count	112	200	135	49	496	

Table 16: Association between number of inseminations and pregnancy outcome of DI treatment

5.7.4 Donor semen parameters

Samples from 32 donors were used for DI treatments between 2005 and 2010. These donors produced a total of 1114 samples from 1995 to 2009 (Table 17). Sperm was frozen from 906 samples. No attempt was made to freeze 124 samples as the parameters were either sub-optimal with regard to the freezing criteria or an excess of round cells or leucocytes were seen. 84 samples were discarded as the samples did not thaw well. There was missing data on the number of straws frozen for 2 samples.

Total semen samples	1114
Samples frozen (No: of frozen sperm straw information was not recorded in 2 samples)	906
Samples not suitable for freezing	124
Samples discarded because of poor survival on thawing (Thaw analysis was recorded only for 75 samples)	84

Table 17: Details of the donor semen samples

5.7.4.1 Is there a seasonal variation in the pre-freeze donor semen parameters?

Out of the 1114 samples produced for donation over the study period, 6 samples did not have a record of full semen analysis. Four of them were discarded because of a high percentage of round cells or leucocytes, one for low volume and one for low motility. Therefore 1108 samples were investigated for seasonal variation in semen parameters.

Most of the semen samples were produced in Spring (33.8%), whilst the number was least in Winter and Autumn with 19.9% and 19% respectively. The Summer was the second most popular season in which to donate samples with 27.3% of samples produced (Table 18).

The semen volume was not recorded for 71 samples out of which 65 samples were eventually not frozen. The decimal point was adjusted for five semen volumes as the volume was recorded as above 10mls (10 to 113mls) despite the number of straws frozen being between 6 and 20.

Semen volume was significantly higher in Spring compared to Winter but not compared with Summer or Autumn. Sperm concentration, total motile concentration and progressive motile concentration in Summer and Autumn were significantly lower compared to Winter and Spring. The total motility in Autumn was significantly lowest than the other three seasons. The progressive motility was again significantly lower in Autumn compared to Spring and Summer but not Winter Table 18.

Semen parameters N=1108 samples; Median (25-75 centile)	Winter (N=220)	Spring (N=374)	Summer (N=303)	Autumn (N=211)	Kruskal Wallis test
Semen volume (ml)	2.2 (1.8-2.8)	2.5 (1.9-3.0)	2.4 (1.8-3.5)	2.2(1.6-3.0)	0.04
Sperm concentration (millions/ml)	132 (97-186)	127 (89-163)	103 (72-137)	100 (80-141)	<0.005
Total Motile concentration (millions/ml)	96 (67-142)	92 (63-125)	76 (51-109)	73 (55-103)	<0.005
Progressive motile concentration (millions/ml)	89 (65-131)	86 (57-118)	69 (45-99)	65 (49-95)	<0.005
Total motility (%)	74 (66-81)	74 (68-81)	75 (68-82)	72 (66-78)	0.01
Progressive motility (%)	66 (58-74)	68 (60-74)	69 (60-76)	65 (57-71)	0.001

Table 18: Pre freeze semen parameters of donor samples by season of sample production (N= 1108 samples)

5.7.4.2 Is there a seasonal variation in the post thaw semen parameters?

There was no difference in the outcome of DI treatment by the season of semen sample production ($p=0.67$; Table 14) even though the pre-freeze donor semen parameters showed significant seasonal variation. Therefore we analysed the post-thaw semen parameters of the cryopreserved samples which could have potentially been used for treatment. 906 semen samples were included in this section Table 19.

Similar to the pre-freeze sperm concentration, post-thaw sperm concentration continued to be significantly lower in Summer and Autumn compared to Winter and Spring. The post-thaw total motile and progressive motile concentrations also are significantly lower in Summer and Autumn compared to Winter, but not Spring. There is no statistical difference in the post-thaw progressive and total motility between the seasons.

Semen parameters N=906 samples; Median (25-75 centile)	Winter (N=190)	Spring (N=317)	Summer (N= 227)	Autumn (N= 172)	Kruskal Wallis test
Sperm concentration (millions/ml)	81 (62-114)	77 (59-107)	65 (49-88)	65 (50-91)	<0.005
Total Motile concentration (millions/ml)	42 (29 -72)	40 (24-68)	36 (20-56)	32 (23-52)	0.001
Progressive motile concentration (millions/ml)	36 (23-56)	32 (20-53)	29 (18-43)	27 (19-44)	0.001
Total motility (%)	54 (42-67)	53 (39-67)	57 (42-68)	52 (39-63)	0.07
Progressive motility (%)	45 (34-53)	44 (35-52)	46 (37-53)	44 (33-52)	0.09

Table 19: Post thaw semen parameters of cryopreserved donor samples by season of sample production (N= 906 samples)

5.7.5 Is the cryopreservation of sperm more effective in any particular season?

There were 906 semen samples frozen from the 32 donors. The number of straws frozen was not documented in 2 samples. Semen volume was not recorded in a further 5 samples. Overall we had 899 semen samples with season of sample production, semen volume and number of straws of sperm frozen recorded. The details are shown in Table 20.

Semen volume was similar in Spring and Summer but was significantly higher compared to Winter (Post-hoc Tukey test $p=0.03$ for Winter vs. Spring and 0.01 for Winter vs. Summer). As expected by the semen volume, there was a significantly higher number of straws of sperm frozen in Summer compared to Winter (Post-hoc Tukey test 0.01 for Winter vs. Summer) but there was no statistical difference between Winter and Spring in the number of straws frozen.

N=899 Median (25th -75th centile)	Winter N=189	Spring N=314	Summer N=226	Autumn N=170	ANOVA test
Semen Volume (ml)	2.2 (1.8-2.8)	2.5 (2.0-3.1)	2.4 (1.8-3.2)	2.2 (1.8-3.0)	0.01
Cryopreservation (No: of straws of sperm)	14 (12-19)	16 (12-20)	16 (12-22)	14 (11-20)	0.01

Table 20: Association between season of sample production and cryopreservation

5.8 Discussion

In this chapter, we investigated 496 DI treatment cycles performed between 2005 and 2010. Also the donor semen parameters were evaluated for seasonal variation.

Most of the donor semen samples were produced in Spring (33.8%) and therefore most of the treatment cycles (42.2%) were done with semen samples produced in Spring. Likewise the lowest number of semen samples was produced in Autumn with the least number of treatments carried out with Autumn semen samples.

As the semen samples produced in one particular season have not yielded better pregnancy rates, it implies there is no seasonal variability in the function of sperm. Very few studies in the literature investigated the seasonal variation in donor conceptions. A study by Paraskevaides *et al.* (1988), showed that donor conceptions in the artificial insemination programme of their clinic were highest between October and March. However in this study both fresh and frozen sperm was used for treatment and results were not presented separately for each method. As the highest sperm counts occurred from February to May in their study (and not October to March), the authors suggested the possible role of female factors to explain the seasonal variation.

In another study by Ronnberg (1989), including 677 donor insemination cycles conducted in Northern Finland (sub-Arctic region) using only frozen sperm, found a similar increase in conceptions from October to March. As frozen sperm was used the authors did not consider the contribution by seasonality of sperm parameters to the conception and suggested a possible variation in female factors again.

In contrast to the mentioned two studies and similar to our study, study by Mayaux and Spira (1989), a large French epidemiological survey on couples treated by artificial insemination (using only frozen sperm) did not find any seasonal variation in donor conceptions. This is the only study in literature which investigated donor conception by the month or season of sperm collection. In this study there was no seasonal variation in donor conceptions either by the season of sperm collection or season of treatment, similar to our study.

There is an assumption in our study that the female factors are reasonably constant. Even though the women are spontaneously ovulating and at low risk of tubal disease,

factors such as variability in egg quality and endometrial receptivity cannot be ruled out. However, as there is no significant difference in the outcome of the DI treatment by the season of treatment, it suggests that there were no significant season dependent female factors (such as egg quality and endometrial receptivity) influencing the success of treatment.

There was no association between the number of inseminations and pregnancy outcome in DI cycles, which is similar to other studies (Paraskevaides *et al.*, 1988). In contrast, some studies investigating intra-cervical insemination revealed an increased chance of conception with increasing number of inseminations (Deary *et al.*, 1997; Carroll and Palmer, 2001). Among other variables (such as concentration of inseminated sperm), the methods used to decide on the day of ovulation (ultrasound, urinary LH) for timing of insemination may have contributed to the variation in results.

Although donor semen parameters appear to be optimal in all seasons and semen parameters exceed the minimal accepted standards for a sperm donor considerably, significant seasonal variation has been noted in this study. The semen volume was significantly higher in Spring compared to Winter. The concentration parameters (sperm concentration, total motile and progressive motile) also are significantly better in Winter and Spring compared to Summer and Autumn. The motility parameters appear to be lowest in Autumn.

There are major differences in these results compared to our previous results from the retrospective study on seasonal variation in semen parameters (Chapter 4, Section 4.7), which revealed a seasonal variation only in sperm morphology but not volume, count and motility. However there are significant differences between the two studies. The study group in the retrospective study was men undergoing preliminary fertility workup whilst this cohort study consists of donors with proven fertility. Although the cohort study was not as large as retrospective study (4067 samples) it is still large with 1108 semen samples and had the advantage of accounting for intra-individual variation (as the samples were from 32 donors only with average of 34 semen samples from each donor). Of note, there was no semen analysis methodology related bias between the studies.

Seasonal variation in semen parameters has been discussed extensively (Chapter 1, Section 1.3; Chapter 4, Section 4.8). Briefly, similar to our study, seasonal variation was noted more in sperm donors than men attending an infertility clinic suggesting that the possible pathology in the ‘suspected infertile’ may not be affected by the season to the same extent (Centola and Eberly, 1999). Our retrospective study included oligozoospermic samples whilst in the cohort study despite semen donors having had few sub-optimal semen samples the majority were normal. It was shown by Levitas *et al.* (2013) that significant seasonal variation exists in normozoospermic samples compared to oligozoospermic samples.

Seasonal variation noted in donor semen parameters is similar to the seasonal variation reported in several temperate climates with poorer sperm parameters (sperm count and or motility) in Summer and or Autumn (Tjoa *et al.*, 1982; Levine *et al.*, 1988; Reinberg *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Jørgensen *et al.*, 2001; Chen *et al.*, 2003; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005; Calonge *et al.*, 2009; Levitas *et al.*, 2013).

Seasonal variation however, is also seen in semen volume in our study which has not been reported much in the literature except in a couple of studies which revealed similar finding to our cohort study of increased semen volume in Spring in semen donors (Zhang and Yao, 2010) and also men pre-vasectomy (Reinberg *et al.*, 1988). It has to be acknowledged though that semen volume was missing in 71 of our samples and may have affected the results.

Even after thawing, donor sperm concentration appears better in Winter and Spring, but there is no difference in the motility between the seasons after thawing. Similar to our study, Yogev *et al.* (2004), reported significantly higher post-thaw progressive motile sperm concentration in Winter compared to Autumn and Zhang and Yao (2010) reported higher progressive motile sperm counts in Spring. Zhang *et al.* (2012) in their further study revealed that progressive motility recovery rate (defined as % post-thaw progressive motility / % pre-freeze progressive motility x 100%) was lowest in semen samples donated in Summer compared to other seasons. Interestingly as a higher progressive motility recovery rate was noted in the most fertile donors compared to least

fertile donors, it was suggested that it is an indicator of high fertility (Paraskevaides *et al.*, 1991).

In the Yogev *et al.* (2004) study, there was no seasonal variation in percentage of post-thaw sperm motility; however there was no seasonal variation in pre-freeze motility either. Although in our study there was a statistically significant seasonal variation in pre-freeze motility with lower parameters in Autumn, the difference clinically between seasons was small. As cryopreservation does decrease sperm motility (Sharma *et al.*, 1997; Stanic *et al.*, 2000), the seasonal differences in pre-freeze sperm motility appear to be lost with thawing.

In our study a higher number of straws were frozen in Summer. In the study by Yogev *et al.* (2004), freezability of sperm was more effective in Winter and Spring. However the cryopreservation method in this study is different to our study. As neat specimens were cryopreserved in our study with no pre-determined progressive motile concentration of sperm per each straw, cryopreservation straw number is dependent on the semen volume. Whilst in Yogev *et al.* (2004) study, the aim of cryopreservation was to obtain 8-12 million/ml of progressive motile sperm concentration in each straw and the freezability was judged on the total cryopreserved, progressive motile sperm count (i.e. product of number of straws and progressive motile sperm concentration). Surprisingly in their further study (including a much longer study period), they reported the absence of a consistent seasonal trend in the freezability of sperm (Yogev *et al.*, 2012).

5.9 Limitations of the study

It has been shown that different methods of cryopreservation have varying recovery rates (sperm motility / vitality) and effect on functional characteristics of sperm (Vutyavanich *et al.*, 2010; Isachenko *et al.*, 2011). Therefore our results are limited to the cryopreservation method used in this study.

The ultimate aim of our project is to investigate if seasonality of sperm can improve recruitment of sperm donors and whether it provides increased conceptions (i.e. with frozen sperm), however it is not valid to extrapolate the results of this frozen data to fresh semen samples and their contribution to seasonality of natural conception.

5.10 Conclusion

Despite a significant seasonal variation in donor semen parameters (both pre-freeze and post-thaw), there was no seasonal variation in the outcome of donor conception by the season of original sperm donation.

In the next section we will consider another potential external factor influencing semen parameters i.e. vitamin D.

Chapter 6 Association of vitamin D and semen parameters

6.1 Introduction

For decades, animal studies have shown a role for vitamin D in male reproduction. Vitamin D deficient rats and VDR knockout mice show reduced sperm counts, motility, and adverse effects on the testis and fertility (Kwiecinski *et al.*, 1989a; Sood *et al.*, 1992; Kinuta *et al.*, 2000). Replacement of vitamin D in these animals restores fertility and improves testicular function (Uhland *et al.*, 1992; Sood *et al.*, 1995).

It has been suggested that vitamin D contributes to spermatogenesis by various mechanisms, including by normalising calcium levels (Uhland *et al.*, 1992), affecting oestrogen synthesis in the testis via its influence on aromatase activity and gene expression (Kinuta *et al.*, 2000); and by up-regulating testis-specific genes in Sertoli cells (Hirai *et al.*, 2009) although the precise mechanism(s) remains unclear.

In humans, VDR and vitamin D metabolising enzymes are widely expressed in the male reproductive tract suggesting that in sperm, 1,25(OH)₂D is locally produced, emphasizing the potential for autocrine –paracrine responses (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2010; Foresta *et al.*, 2011).

In vitro human studies have shown that vitamin D enables sperm capacitation, improves sperm survival, increases intracellular calcium levels in sperm, improves sperm motility and induces the acrosome reaction (Aquila *et al.*, 2008; Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011).

6.2 Aim

To investigate the hypothesis that vitamin D has an important role in male fertility.

6.3 Objectives

1. To investigate the correlation between serum levels of VD and semen parameters.
2. To investigate the effect of a seasonal rise in VD levels in men without VD deficiency on semen parameters.
3. To investigate the effect of VD supplementation in VD deficient patients, on semen parameters.

6.4 Outcome measures

To determine

1. If there is a link between serum vitamin D levels and semen parameters.
2. If a rise in serum vitamin D levels in vitamin D deficient participants with supplementation or in vitamin D non deficient participants with seasonal rise improves semen parameters.

6.5 Subjects and Methods

6.5.1 Subjects

Men attending NFC for investigations and/or fertility treatment were recruited to the study. Ethical approval for the study was obtained from County Durham and Tees Valley Regional Ethics Committee.

6.5.2 Study design / size

Male partners of the couples attending NFC from November 2010 to May 2011 were approached to participate in a prospective, longitudinal, observational study and 125 were recruited.

6.5.3 Recruitment

Couples attending for IVF / ICSI treatment were primarily targeted. The reason for this is that couples are usually seen at least 5 times over a 15 days period (for ultrasound monitoring of ovarian stimulation, egg collection and embryo transfer). Figure 9 shows the sequence of events in an IVF treatment cycle. These visits enabled us not only to gradually introduce the idea of participation in the research, but also to give patients adequate time to reflect on participation. Moreover the participants would produce a semen sample on the egg collection day for their treatment, which could also be used for research and thereby avoiding the need to produce a semen sample specifically for research.

A patient information sheet containing a brief introduction to the study (Appendix A) was given to the couple when they attended for the down regulation scan. When the couple attended for the next scan about a week later, a research nurse counselled them with respect to enrolling in the study. When the couple attended for egg collection, this researcher (MG) saw them if they had already expressed interest in participating in the study. 117 men from couples following this pathway were recruited.

Information leaflets were given to the men when booking their semen analysis at the centre and 4 further men were recruited when they attended the centre for semen analysis. 1 patient was recruited from clinic who had submitted the semen sample for analysis on the same day. 2 men were recruited when attending with their partners for IUI (intra-uterine insemination) treatment. 1 man was recruited when his partner attended for follicle reduction prior to IUI treatment.

Overall, 125 men were recruited to the study. One participant had to be excluded later (participant 14: 25(OH)D=179nmol/l) when he revealed that he had been taking cod liver oil supplements at the time of recruitment.

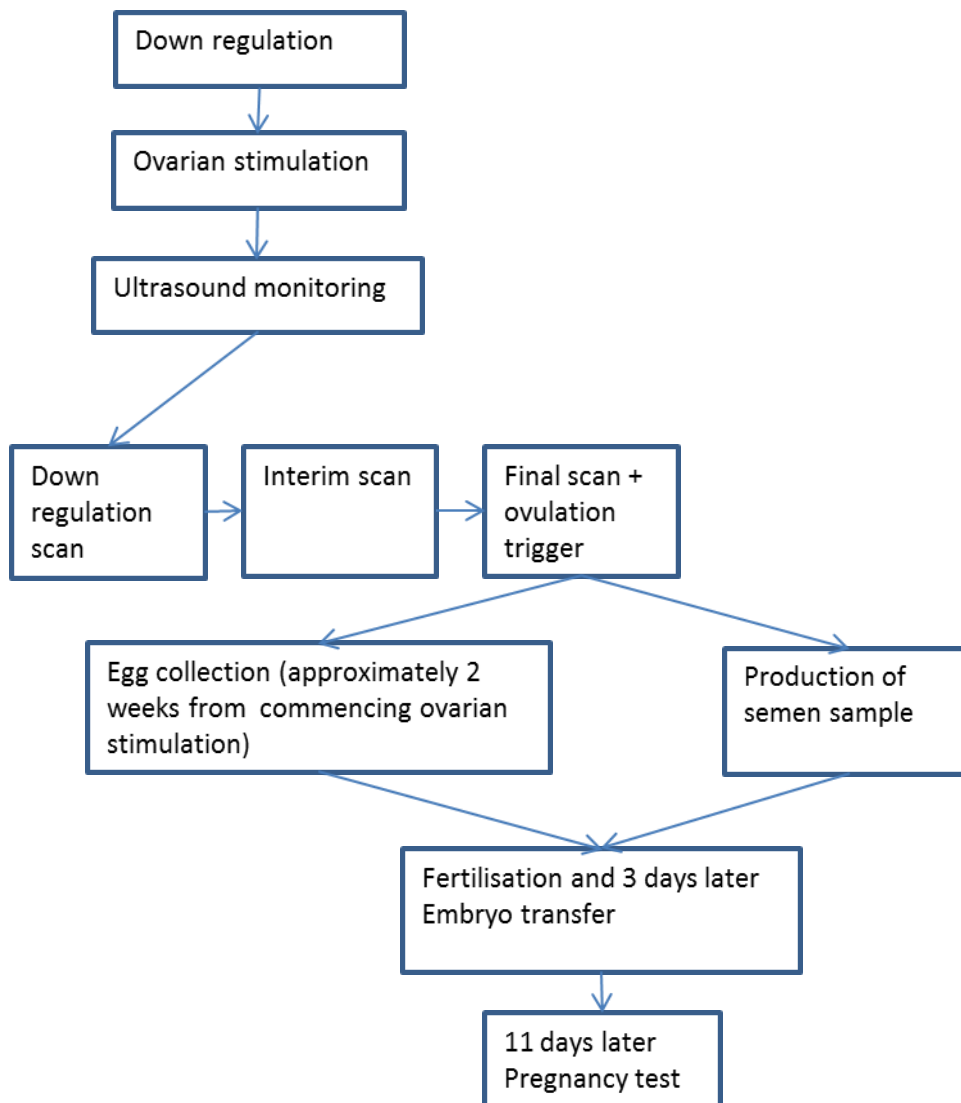


Figure 9: Sequence of events in IVF

6.5.4 Eligibility

Males aged over 18 years who were competent to give informed consent were eligible to participate in the study. Patients from all ethnic groups were included.

6.5.5 Exclusion Criteria

Men with azoospermia and those taking vitamin D supplements were excluded from the study.

6.5.6 Procedure

All the men who verbally consented to participate were seen for a consultation where the research study was discussed in detail by MG and a questionnaire about demographics, fertility, diet and lifestyle was completed (Appendix B).

Ethnicity included only 2 groups: Caucasian and non-Caucasian. Regarding fertility, information on the type of subfertility (primary/secondary) and the cause of subfertility was obtained. The cause of subfertility was divided into 3 groups - male factor only (idiopathic sub-optimal semen quality, primary testicular failure etc.), combined male and female factors (sub-optimal semen quality and tubal or ovarian reserve/ovulation problems) and non-male factor (which included unexplained subfertility and sole female factor subfertility).

The presence of possible risk factors for male subfertility [such as medical conditions, previous genital surgery, sexually transmitted infections, use of recreational drugs (including anabolic steroids)], consumption of alcohol, smoking, fever in the previous 3 months, intake of regular medications and intake of vitamins or nutrients other than vitamin D were noted. The average intake of alcohol in units per week was noted. Men smoking occasionally were also included as smokers. However we do acknowledge that it has been shown that common life style factors do not significantly affect semen parameters (Povey *et al.*, 2012).

Diet and lifestyle questions included factors which influence serum vitamin D levels such as being a vegetarian, intake of margarine and oily fish, recent travel to hot countries or tropical places in the previous 3 months, hours of indoor activity (such as time spent watching TV or using a personal computer (PC) etc.) per day, hours spent outdoor per day in the previous month and use of sun protection in the form of the topical application of creams on sunny days either in the UK or on holiday.

The questions on frequency of the consumption of vitamin D rich food (such as daily, weekly or less than weekly), the use of sun protection and the timings spent indoors and outdoors (<2h, 2-3h, and >3h) were derived from the questionnaire used in a nationwide cohort study on diet and lifestyle predictors of vitamin D status in the UK (Hyppönen and Power, 2007).

The participant's weight and height were measured and BMI calculated. Obesity was defined as a BMI above 30. Written informed consent (Appendix C) was obtained. A copy of the consent form was filed in the notes and a second in the research file and a copy was given to the patient. A blood sample was taken to assess serum 25(OH) D levels and the participant informed that the results of the analysis of the semen sample produced by him for their treatment (IVF/ ICSI/ IUI) or investigation would be utilised for the research study, apart from samples analysed further to obtain additional necessary parameters as required.

The season when the samples (i.e. blood and semen) were obtained was also noted and the classification of seasons used was as detailed in the Chapter 4 (Section 4.5.7). The participant was informed that a reminder for their next appointment 6 months later would be sent to them by their preferred contact method as indicated in the consent form, about 3 weeks prior to the scheduled date.

6.5.7 Follow up clinics

An invitation letter (Appendix D), e-mail or text message was sent to remind the patient to attend the follow up clinic appointment. There were 2 follow up clinics every week (Monday and Friday). The aim of the follow up clinics was to review men seen initially in the Autumn, 6 months later in Spring. Similarly men seen in Winter and Spring were to be reviewed in Summer and Autumn respectively as shown in the following Table 21.

Season	Recruitment phase	No: of men recruited	Season	Follow up phase
Autumn	November	21	Spring	May
Winter	December	9	Summer	June
	January	17		July
	February	28		August
Spring	March	34	Autumn	September
	April	14		October
	May	2		

Table 21: Follow up clinics

If the couple had a scheduled routine clinic appointment or were coming to the centre for a further treatment cycle around the time of their planned research follow up, the couple were advised to inform MG so that the arrangements to see them regarding the research at the same time were made.

When in the first couple of weeks, significant numbers of men either did not attend or rescheduled their appointments, changes to the follow up approach were made to improve the efficiency. The changes were –

1. The option of attending the centre at weekend or evening clinics was offered.
2. The option of producing the sample at home was given, although none of the participants utilised this option.
3. Invitation letters were sent out 4-6 weeks before the scheduled appointment, with a reminder by text message the day before the appointment.

Men who did not attend the clinic appointment were telephoned on the same day for a discussion. This replaced the earlier plan of sending standard ‘did not attend’ letters.

Prior to the review clinic appointment the couple’s notes were checked for further information which included the medical history for any risk factors for sub-fertility which the participant may not have volunteered, the indication for fertility treatment, the details (such as fertilisation and embryo quality) and the outcome of the IVF treatment cycle (120 participants) undertaken at their initial recruitment.

At the follow up appointment the questionnaire was reviewed again to note any major changes in the previous 6 months. Blood and semen samples were collected on the same day. All the semen samples were analysed by MG within 60 min of production. The blood and semen samples were dealt with as described below.

6.5.8 Laboratory Procedures

6.5.8.1 Blood sample

4.5mls blood was taken by standard venepuncture technique into a plain tube (yellow top where in the blood is clotted) and labelled with participant’s unique identification code and date of birth to obtain serum 25(OH)D levels. 25(OH)D was measured by an automated assay at the Biochemistry laboratories, Royal Victoria Infirmary Hospital, Newcastle upon Tyne (RVI). The Liaison 25-OH Vitamin D TOTAL assay (DiaSorin Liaison 2) method is a competitive chemiluminescent immunoassay (CLIA) where a

polyclonal goat antibody to 25(OH)D is used to coat magnetic particles and 25(OH)D is linked to an isoluminol derivative. During the incubation, 25(OH)D is dissociated from its binding protein and competes with isoluminol-labelled 25(OH)D for binding sites on the antibody (Roth *et al.*, 2008). The unbound material is removed with a wash cycle and reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured by photomultiplier and is inversely proportional to the concentration of 25(OH)D present in calibrators, controls or samples.

The measuring range of the Liaison 2 assay is from 10-375nmol/L with linearity up to at least 155nmol/l. The intra-assay coefficient of variation (CV) is 7.2% and 5.1%, at 25(OH)D levels of 37.57 and 125.7nmol/l respectively. The inter-assay CV is 12.6% and 7.4% at 25(OH)D levels of 36.9 and 123.1nmol/l. The assay has a cross reactivity of 100% with 25-hydroxyD3 and 25-hydroxyD2 on an equimolar basis.

6.5.8.1.1 External Quality Assurance

The variability in the results of different vitamin D assays is well known. Since 1989, the Vitamin D external Quality Assessment scheme (DEQAS) has been monitoring the performance of vitamin D assays internationally with more than 100 participants registered in 18 countries. The scheme offers the opportunity to determine the accuracy of the various methods used (Carter *et al.*, 2004). The participants are sent 5 samples of serum every 3 months for analysis of 25(OH)D levels. The participants return the results within 6 weeks. From all the results received, statistical analysis is performed and an all-laboratory trimmed mean (ALTM) is calculated. Results are also pooled for each method and a method mean (MM) calculated. Accuracy of the result is defined by percentage bias, which is the deviation from the ALTM. Assessment of the overall accuracy of the method used is calculated by the percentage bias of the method mean from the ALTM $(MM-ALTM / ALTM) \times 100$. The labs at the RVI are enrolled with DEQAS for quality checks which confirm that standards are maintained and are done once every 3 months.

6.5.8.1.2 Vitamin D status classification

The differing recommendations in defining adequate levels of vitamin D and the classifications are well known and are detailed in the Chapter 1 (Section 1.5.9). The

most widely recognised and used classification of D status as described below is used for our study (Pearce and Cheetam, 2010; Blomberg Jensen *et al.*, 2011)

- < 25nmol/l - Deficiency
- 25-50nmol/l –Insufficiency
- 51-75nmol/l – Adequate
- >75nmol/l – Optimal

6.5.8.2 Semen sample

6.5.8.2.1 Reasons for semen sample production

In the recruitment phase of the study, the majority of the participants (120/125) produced the sample for their treatment (IVF/ICSI/IUI) while the rest (5/125) produced the sample for their routine preliminary investigations. The semen analysis results were utilised for the study. In the follow up phase, the majority of the participants (73/87) produced the semen sample only for the research study while the rest (14/87) produced the semen sample for their further treatment cycle.

6.5.8.2.2 Staff performing the semen Analysis

Semen samples produced for treatment are routinely analysed by embryologists while the Andrology laboratory technicians perform the routine semen analysis submitted for investigations.

All the semen samples done solely for the research study were analysed by MG. Furthermore, the samples submitted for the fertility treatment in the recruitment phase (113) and follow up phase (14) were also analysed by MG for the sperm count using a Neubauer counting chamber as per the WHO 2010 recommendation, as otherwise sperm counts are usually done on a wet preparation slide for the treatment purposes. Wet preparation analysis is less accurate as it does not include the dilution technique (described in Section 6.5.8.2.3.2.) or utilise fixed volumes compared to a sperm count performed using a counting chamber, however it suffices for IVF/ICSI treatment. Wet preparation sperm count was the only method possible in 7 participants in the recruitment phase where the entire sample had to be used for their treatment. Once semen analysis was done, MG was blinded to the results till the second semen analysis was completed at the follow up 6 months later.

All the staff performing the semen analyses, which were included in the research study (including MG) undertook regular quality control checks, both internal and external (UK National External Quality Assessment service UK NEQAS) on a regular basis.

6.5.8.2.3 Semen analysis

The details of initial assessment; sample collection, liquefaction and volume are described in Chapter 4 (Section 4.5.4). All participants produced their semen sample at the centre and semen analysis was completed within one hour of sample production, although in the majority of cases analysis was undertaken soon after liquefaction.

6.5.8.2.3.1 Abstinence and sample loss

Whilst all participants were advised to avoid ejaculation for between 2 and 7 days prior to sample production, as per the WHO laboratory manual – fifth Edition (World Health Organisation, 2010), the actual period of abstinence for each was documented only in the follow up phase when recommended abstinence was noted in 86/87 patients. Sample loss was also documented in the follow up phase only. 3/87 participants in the follow up phase recorded partial sample loss. Presumably there would have been similar proportion of participants with sample loss in the first phase as well however, and more importantly as this information was not available for one phase of the study, it was not taken into consideration in the statistical analysis.

6.5.8.2.3.2 Sperm concentration

10µl of the semen sample was loaded onto a fixed depth micro-cell slide. After allowing it to settle for few minutes the number of sperm per field of view was noted using a phase contrast microscope at a magnification of $\times 400$ ($\times 40$ objective). The criteria in Table 22 were used to calculate the required dilution. The semen sample was diluted with a specific volume of sterile distilled water in an Eppendorf tube.

The sample was agitated using the vortex for 10 seconds to ensure homogeneity. Using a positive displacement pipette, both sides of the C-Chip disposable Neubauer slide were loaded with sample from the Eppendorf tube. Whilst pipetting, care was taken to avoid under or over filling the chambers. Once the chamber was full, it was allowed to settle for 5 minutes. The number of complete sperm (heads and tails) in focus in one

large square of the Neubauer counting chamber was counted using the phase contrast microscope at a magnification of $\times 400$ ($\times 40$ objective). Using the criteria in the Table 23, the number of large squares to be counted was determined.

Apart from the sperm completely in the square, the sperm lying on the left and the lower edge of the square were also counted. The average of counts from the two sides of the Neubauer was calculated. This number was divided by the conversion factor which can be derived from Table 24 to give the sperm concentration of the original semen sample in millions per millilitre.

Sperm per field of view	Dilution (Semen + diluent)	Volume of water μl	Volume of sample μl
<15	1:5 (1+ 4)	100	25
15-40	1:10 (1+ 9)	225	25
40-200	1:20 (1+ 19)	475	25
>200	1:50 (1+ 49)	1225	25

Table 22: Calculation of dilution for sperm concentration

Number of sperm seen in one large square	Number of large squares to count
<10	25
10-40	10
>40	5

Table 23: Determination of squares to count by number of sperm seen

Dilution applied to the sample	Number of large squares counted		
	25	10	5
1:5	20	8	4
1:10	10	4	2
1:20	5	2	1
1:50	2	0.8	0.4

Table 24: Conversion factor to obtain sperm concentration in millions per millilitre

6.5.8.2.3.3 Sperm motility

Sperm motility is analysed soon after liquefaction as it deteriorates with time. If the liquefaction had not occurred within half an hour, the sperm motility assessment was still completed. 10µl of the undiluted semen sample was loaded on to a fixed depth micro-cell slide and allowed to settle. Using a phase contrast microscope at a magnification of ×400 (×40 objective) 100 sperm were counted as per the following criteria using a Celltrac counting chamber to derive the percentages of sperm motility.

Progressive motility (PM): Actively moving spermatozoa either linearly or in big circles irrespective of the speed.

Non-motile (NM): All other forms of motility with lack of forward progression for instance moving in small circles or when only the flagellar beat is observed.

Immotile (IM): Sperm with no movement.

6.5.8.2.3.4 Sperm Morphology

The methodology is described in detail in the Chapter 4 (Section 4.5.4.5.) For this study however, the recent classification of sperm morphology based on 2010 World Health Organisation criteria is used (World Health Organisation, 2010). Similar to WHO 1999 criteria, sperm are classified as normal or abnormal and all borderline sperm are considered abnormal.

6.5.8.2.3.4.1 Normal Sperm

This is described as follows-

Sperm head: The head should be regular contoured and oval in shape. A well-defined acrosome occupying 40-70% of the sperm head area should be present. The acrosome should not contain any large vacuoles but small vacuoles, 2 or less occupying less than 20% of the head are considered normal. There should not be any vacuoles in the post acrosomal region of the head. The form of the head is more important than the dimensions, except when these are grossly abnormal.

Mid piece: This should be regular, slender, approximately same length as the sperm head and should be aligned to the longitudinal axis of the sperm head. Residual cytoplasm is considered abnormal if it is more than 30% of the sperm head size.

Principle piece: This should be uniform along the length, thinner than mid piece, 45µm long with no sharp angle.

6.5.8.2.3.4.2 Abnormal sperm

Any deviations of normal forms are considered abnormal. Apart from head, neck and mid piece and principal piece defects, another abnormality, excess residual cytoplasm is described (ERC). ERC is a result of defective spermatogenesis and is characterised by large amounts of irregular cytoplasm, one third or more of the head size and usually associated with mid piece defects.

6.5.8.2.4 Quality Control

The intra- and inter-individual variation in the analysis of semen samples is well known (Auger *et al.*, 2000). The reproducibility of a semen analysis can be assessed by analysing the same sample several times. This principle is used in quality control to assess intra-technician variability (same technician performing several analyses on the same blinded sample) and inter-technician variability (various technicians performing the analysis on a single sample).

The laboratory at our centre runs an internal quality control programme and also participates in the UK National External Quality Assessment service (UK NEQAS). UK NEQAS is a scheme facilitated by the Department of Reproductive Medicine, St. Mary's Hospital, Manchester since 1994 (World Health Organisation, 2010). Aliquots of liquid semen (fixed in 10% formalin) are sent from Manchester to the participating fertility centres for the assessment of sperm concentration and sperm morphology. For the assessment of sperm motility a DVD and online web link [in collaboration with Gamete Expert (www.gamete-expert.com), an online training website for the assessment of gametes] are sent. The online web link is also used to assess sperm morphology. Four samples are sent to the labs every 3 months and the results are returned to NEQAS within 3 weeks.

The percentage of bias and the bias index score (BIS) are calculated for each semen parameter as detailed in Appendix E. Accuracy of the test result performed can be derived from BIS score. If the BIS score is outside -100 to 100, it indicates an unacceptable result. The details of interpretation of the results are available in Appendix E. All laboratory staff performing the semen analyses including MG participated in the

internal quality control which follows the principles of external quality for assessment, to ensure that the standards are maintained. UK NEQAS results reveal the centre's satisfactory performance during the study period.

6.5.8.2.5 Training in Semen Analysis

I had formal hands on training in semen analysis, under the supervision of two embryologists, over a period of 3 months. Training commenced with watching online videos (for sperm parameters). During the training various steps in performing the semen analysis were observed and the results of the sperm parameters were compared with that of the trainers. A training check list (Appendix F) which is a thorough systematic approach to conduct a semen analysis was completed. When the procedure was performed as per the requirements on more than 5 occasions, and the results of the sperm parameters (semen volume, sperm count, motility and morphology) tallied with those of the trainers (<5% variation), I was deemed competent to perform the semen analysis independently. Although the training requirements for routine semen analysis were completed, the trainers continued to check my results with theirs for longer at my request which as shown in Table 25. The NEQAS accreditation paper work for MG is shown in Appendix G.

Sperm parameter	No: of occasions checked with trainer
Semen volume	7
Sperm count	26
Motility	31
Morphology	17

Table 25: Training in semen analysis

6.5.9 Data Handling

All the questionnaires and consent forms were placed in the research file. The participants were given unique identifier codes. The register maintaining the patient identifying information and their planned follow up visits was also in the research file. The research file was put in a secure and confidential place within the department. The request form for the blood test was labelled with the participant's code and date of birth but no other identifying information and all the vitamin D reports were filed in the research file (as per the Trust Research and Development advice). Only blood results with vitamin D deficiency were decoded and filed in the couple's case notes.

6.5.10 Handling of results

The results of serum 25(OH)D and semen analysis were entered in the questionnaire. All the questionnaire answers and results were uploaded on to the electronic database excluding any patient identifying information.

6.5.11 Vitamin D deficiency

Participants with low vitamin D level ($< 25\text{nmol/l}$) were informed of the result by letter (Appendix H) and advised to approach their General Practitioner (GP) for vitamin D supplementation therapy. The participant's GP was also sent a letter (Appendix I) to request that they organise treatment with the general recommendation of a 6-8 week course of high dose Colecalciferol 20,000 IU, 3 capsules per week. Thereafter, men were advised either to go onto a maintenance vitamin D regime of 1,000 IU per day, or to modify their diet to increase vitamin D intake, e.g. to eat oily fish twice weekly. A vitamin D information leaflet (Appendix J) was also enclosed with the GP's letter. Where possible the participants were informed of the result face to face. The letters were filed in the notes along with the decoded laboratory report.

6.5.12 Vitamin D compliance

Participants were sent a reminder (e-mail or text) 6 weeks after the initial letters were sent out regarding their low vitamin D levels with recommended treatment. Meanwhile if the couple were seen for a routine fertility clinic appointment, participants were encouraged to contact their GP for vitamin D supplementation if they had not already done so. The importance of vitamin D supplementation for bone and general health was discussed.

6.6 Statistical Analysis

Statistical analysis was performed using SPSS (version 19) and Minitab (version 16). Variables were expressed as mean \pm SD or SEM and median with centiles.

The distributions were checked using the Anderson Darling normality test. The continuous variables BMI, vitamin D levels and semen parameters showed non-normal distribution whilst age showed normal distribution. The study group was divided into 4 groups based on their vitamin D status. The significance of indicators of vitamin D status (recent travel, vegetarian, intake of margarine, oily fish intake, indoor activity, outdoor activity and use of sun protection) among the vitamin D groups was checked using chi square test. Vitamin D was used as a continuous variable and checked in the sub-categories of vitamin D indicators using either the Mann Whitney test (2 categories) or the Kruskal Wallis test (>2 categories). If significance was noted by the Kruskal Wallis test, the Levene statistic (Homogeneity of variance) was checked to ensure >0.05 to apply one way ANOVA with post-hoc analysis.

To compare the characteristics of men in the vitamin D groups, chi square was used for categorical variables (type of subfertility, cause of subfertility, presence of risk factors, recent fever, consumption of alcohol, smoking, intake of regular medication and of vitamins or nutrients) and for continuous variables such as age and BMI, the ANOVA and the Kruskal Wallis test was used respectively. For the association study the significance of semen parameters between the vitamin D groups was checked using the Kruskal Wallis test. Spearman correlation test was used to analyse the correlation between vitamin D and the semen parameters.

For each semen parameter, multiple regression modelling was employed to establish the significance of vitamin D when viewed alongside selected covariates. Candidate covariates chosen for the modelling procedure had a univariate association with an outcome of $p < 0.15$ – recognising the fact that a covariate showing a weak univariate association with outcome may have a stronger association with outcome when viewed alongside other predictors (Hosmer and Lemeshow, 2000; Labopin and Iacobelli, 2003; Blomberg Jensen *et al.*, 2011). The candidate covariates were then entered into a variable selection procedure alongside vitamin D entered as a continuous variable ($p = 0.05$ was chosen for variable entry and $p = 0.1$ for variable removal). The covariates considered were age, BMI, season, ethnicity, type of subfertility, cause of subfertility,

presence of risk factors, recent fever, alcohol, smoking, intake of regular medication and of vitamins or nutrients. All the covariates were categorised as described in Table 28. for regression analysis except cause of subfertility and alcohol consumption. These were re-categorized to 2 groups instead of three [cause of subfertility (male/non-male) and alcohol consumption (<21 units/wk or >21 units/wk)] for regression analyses.

For vitamin D versus treatment regimens, since the distribution of vitamin D was normal, one way ANOVA was applied to compare the improvement between the treatment groups. In the longitudinal study, the association of vitamin D levels and semen parameters was verified by applying Wilcoxon signed ranks test.

The comparison of vitamin D between seasons was tested using Kruskal Wallis and Multiple comparison tests. A p value of <0.05 was considered significant.

6.7 Results

6.7.1 Recruitment

A total of 273 patients were approached during the study period. 42 (15.3%) patients could not be recruited as they were on vitamin D supplements whilst 106 patients (38.8%) declined to participate in the study. The main reasons quoted for declining to participate were: not interested in the study (76/106, 71.6%), unable to return for follow up (17/ 106, 16%) and up (17/ 106, 16%) and needle phobia (13/106, 12.2%). 125 (45.7%) patients were recruited to the study. The recruited to the study. The recruitment details are shown in the

Figure 10.

Although all men were partners in a subfertile couple, 54 (43.2%) had male factor subfertility whilst 71(56.8%) had non-male factor subfertility, therefore the study group was heterogeneous. The male factor subfertility details are shown in Table 26.

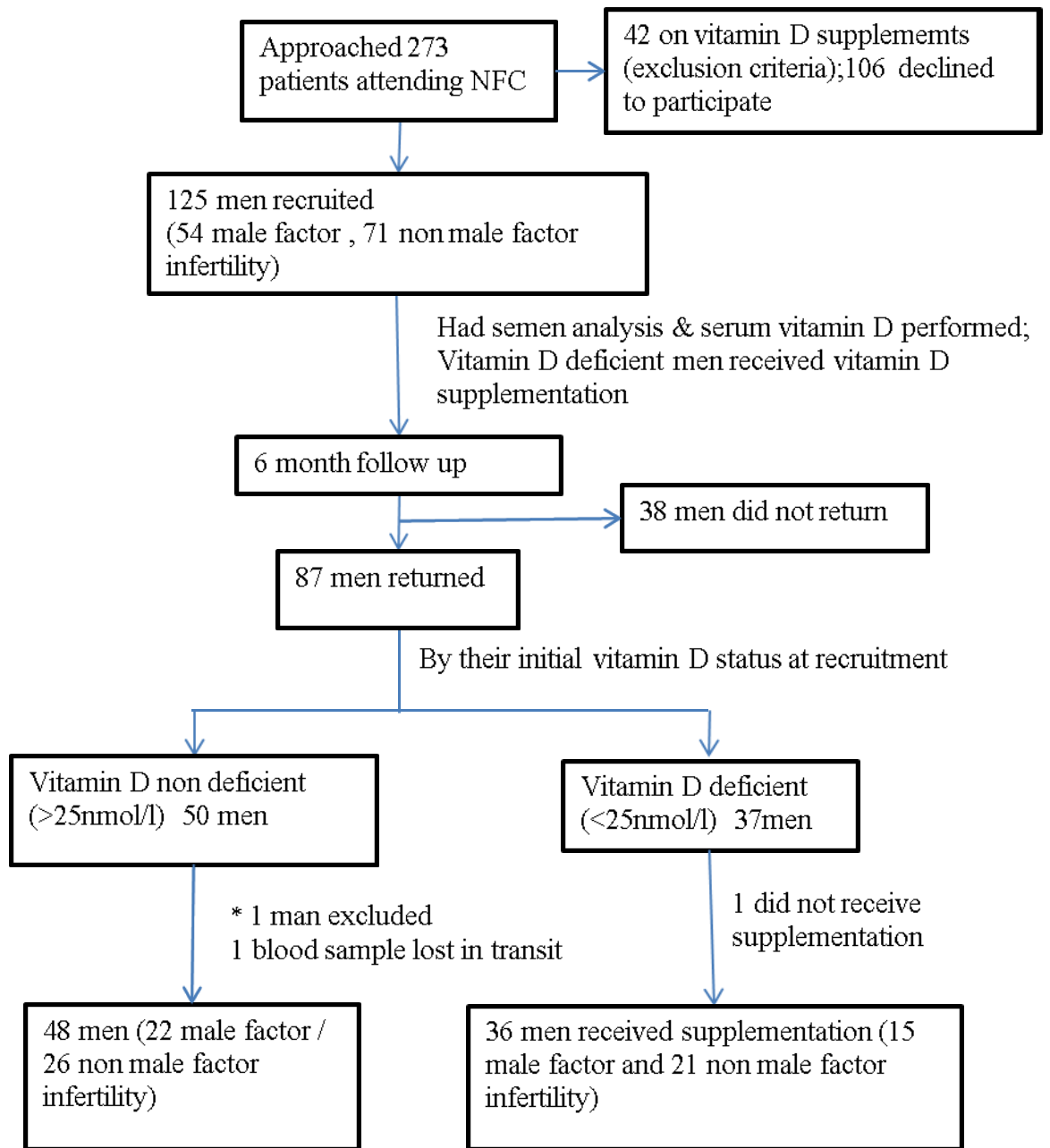


Figure 10: Participant’s recruitment and follow up

** Revealed at the follow up phase to be on cod liver oil since prior to recruitment. Investigation results were excluded from the analysis, therefore only 124 results available for cross sectional association study.*

Cause	No: of participants
Idiopathic male factor	41
Primary testicular failure	1
Undescended testis (includes unilateral and bilateral)	7
Unilateral Orchiectomy	2
Previous chlamydia (with sub optimal semen quality)	2
Testicular cancer, unilateral orchiectomy, chemotherapy	1

Table 26: Male factor subfertility

6.7.2 Prospective observational association study

Vitamin D levels were deficient in 52 men (41.9%), insufficient in 51 (41.1%), adequate in 15 (12.1%) and optimal in 6 (4.8%). These four vitamin D groups are used for the majority of the analysis. A review of the participants in the optimal vitamin D group revealed that 3 of them had been on recent holidays to sunny places and one participant used a sunbed once a week. Only 2 of the participants had true optimal vitamin D levels. The prevalence of vitamin D deficiency was 47.1% (49/104) during Winter and Spring in the study group whilst 42% (38/90) in Caucasians only.

Hypovitaminosis D as defined in the large scale British survey (i.e. <than 40 nmol/l of serum vitamin D) was also calculated for comparison. In Winter the level of hypovitaminosis D in was 79.6% (43/54) while it was 64% (32/50) in Spring. Only if Caucasians were included it was 77.7% (35/45) and 60% (27/45) respectively.

6.7.2.1 Vitamin D and Ethnicity

109 (88%) participants were Caucasians whilst 15 (12%) were non-Caucasians. The prevalence of vitamin D deficiency in non-Caucasians was significantly higher compared to Caucasians [80 % (12/15) versus 36.7% (40/109); Fisher's exact p value = 0.002].

6.7.2.2 Vitamin D and BMI

There was no statistical difference in vitamin D levels in the obese group (BMI>30) 30 (14-41) and non-obese group 29 (15-43.5) nmol/l [Median (25th-75th centiles); Mann Whitney p=0.19]. Furthermore BMI is comparable in all the vitamin D groups (Kruskal Wallis test p= 0.51). Though the actual BMI was not recorded for 18 participants, the category (obese or non-obese) was recorded for all the participants.

6.7.2.3 Factors influencing vitamin D levels

The results are shown in Table 27.

Were the vitamin D levels higher in participants who had travelled abroad within the previous 3 months? Recent travel was significantly different between the vitamin D groups (Chi square p value = 0.03; Table 27). Participants who had travelled in the previous 3 months to sunny places had significantly higher levels of vitamin D than those who had not [41.5nmol/l (30.5- 52.4) versus 30.1nmol/l (26.3-33.8); mean (95% CI); Independent samples test p=0.03)].

Was vitamin D deficiency more common in vegetarians? There were only 2 vegetarians in the entire study group and both had vitamin D deficiency. One participant was Caucasian whilst the other non-Caucasian. As expected there was no significant difference in the number of vegetarians seen in the 4 vitamin D groups (p=0.65; Table 27) but the numbers were too low to investigate the effect of being vegetarian on vitamin D status.

Was intake of oily fish and or margarine higher in participants with adequate / optimal vitamin D levels? The intake of oily fish and margarine were uniform among the vitamin D groups (p=0.76;Table 27). Also the vitamin D levels were comparable in men consuming margarine (n=84, daily, weekly or less than weekly) and men who had it occasionally or never (n=40); [32 ± 21.6 vs.32 ± 20.1, (Mean ± SD; Mann Whitney p=0.8)]. Furthermore the vitamin D levels were comparable in men consuming oily fish regularly (weekly and less than weekly, n=97) and who had it rarely or never (n=27) [31.4 ± 22.5 vs.31.4 ± 20, Mean ± SD; Mann Whitney p=0.6]

Was vitamin D lower in participants with longer hours of indoor activity? Hours of indoor activity were significantly different between the vitamin D groups (p=0.01;Table 27). Participants who spent more time indoors watching television or using a PC (> three hours per day) had significantly lower levels of vitamin D nmol/l compared to participants who spent less than three hours doing the same [28.3, (24.1 – 33.5) versus 38.2, (31.5 - 44.9) Mean, (95% CI); Independent sample test p=0.01].

Was vitamin D higher in participants with longer hours of outdoor activity? Daily hours of outdoor activity were significantly different between the vitamin D groups (p=0.001; Table 27). Participants who spent less than 1 hour outdoors had significantly lower levels of vitamin D nmol/l compared to spending one to three hours [Mean, (95% CI) 21.9 (30-42.7) versus 36.9 (30- 43.7); ANOVA post-hoc tukey p0.002] or more

than three hours at 36.3 (30-42.7) (ANOVA post-hoc tukey $p=0.004$). There was no significant difference in vitamin D levels between participants who spent 1 to 3 hours outdoors (per day) and those who spent over 3 hours.

Was vitamin D lower in participants who used sun protection? The use of sun protection was similar in all the vitamin D groups ($p=0.71$; Table 27) and did not alter the vitamin D status. Men who used sun protection ($n= 73$) had comparable vitamin D levels to men who rarely or never use sun protection ($n=51$) [31.7 ± 18.7 vs. 32.4 ± 23 , Mean \pm SD; Mann Whitney $p=0.6$).

Indicators of vitamin D status / Vitamin D group		Vitamin D Deficiency <25nmol/l	Vitamin D Insufficiency 26-50nmol/l	Vitamin D Adequate 51-75nmol/l	Vitamin D Optimal >75nmol/l	P value Chi ²
Recent travel	Yes	6 (28.5%)	7 (33.3%)	5 (23.8%)	3 (14.2%)	0.03
Vegetarian	Yes	2 (100 %)	0	0	0	0.65
	No	50 (40.9%)	51 (41.8%)	15 (12.2%)	6 (4.9%)	
Margarine	Daily	13 (35.1%)	18 (48.6%)	4 (10.8%)	2 (5.4%)	0.94
	Weekly	8 (36.3%)	11 (50.0%)	2 (9.0%)	1 (4.5%)	
	Less than wkly	13(52.0%)	8 (32.0%)	3 (12.0%)	1 (4.0%)	
	Never	18 (45.0%)	14 (35.0%)	6 (15.0%)	2 (5.0%)	
Oily fish intake	Weekly	14 (36.8%)	17 (44.7%)	4 (10.5%)	3 (7.8%)	0.76
	Less than wkly	26 (44.0%)	25 (42.3%)	7 (11.8%)	1 (1.6%)	
	Occasional/	12 (44.4%)	9 (33.3%)	4 (14.8%)	2 (7.4%)	
Indoor activity	< 3 hrs / day	13 (28.2%)	21 (45.6%)	9 (19.5%)	3 (6.5%)	0.01
	> 3 hrs / day	39 (50.0%)	30 (38.4%)	6 (7.6%)	3 (3.8%)	
Outdoor activity	<1 hr / day	27 (69.2%)	9 (23.0%)	3 (7.6%)	0	0.001
	1 -3 hrs / day	12 (27.2%)	24 (54.5%)	4 (9.0%)	4 (9.0%)	
	> 3 hrs / day	13 (31.7%)	18 (43.9%)	8 (19.5%)	2 (4.8%)	
Sun protection	Usually	16 (51.6%)	9 (29.0%)	4 (12.9%)	2 (6.4%)	0.71
	Sometimes	15 (35.7%)	20 (47.6%)	6 (14.2%)	1 (2.3%)	
	Rare / never	21 (41.1%)	22 (43.1%)	5 (9.8%)	3 (5.8%)	

Table 27: Indicators of vitamin D status in the various groups

(Recent travel was to sunny places in the previous 3 months)

6.7.2.4 Characteristics of men in the vitamin D groups

The characteristics of the men in the four vitamin D groups are shown in the Table 28. Age and BMI were comparable between vitamin D groups. The type of subfertility (primary or secondary), cause of subfertility (male factor, combined male and female factors and non-male factor), fever in the previous 3 months, alcohol consumption, smoking and intake of regular medication were comparable between the 4 vitamin D groups ($p>0.05$). 33 men in the study had risk factors potentially affecting semen parameters (Table 29) and some of them had more than one risk factor, however they were comparable in numbers in the four vitamin D groups. 14 men took vitamins or nutrients other than vitamin D, but none were in the vitamin D optimal group, however number of participants in this group was low. Vitamins C, E, selenium and zinc were the most common supplements, whilst the intake of omega fatty acids, carnitine, larginine, lycopene, B complex vitamins and folic acid was also noted. Eight men (8/14; 57%) were on more than one nutrient, yet the frequency of intake of nutrients was not statistically different between the vitamin D groups.

Variable		Vitamin D Deficiency <25nmol/l	Vitamin D Insufficiency 26-50nmol/l	Vitamin D Adequate 51-75nmol/l	Vitamin D Optimal >75nmol/l	P value
Participants N=124		52 (41.9%)	51 (41.1%)	15 (12.1%)	6 (4.8%)	
Age (years) (Mean ± SD)		34 ± 4.3	35 ± 6.6	32.8 ± 5.5	30.8 ± 5.4	0.24 (ANOVA)
BMI (Kg/m ²) (Mean ± SD)		27.3 ± 3.6	28.2 ± 3.8	26.3 ± 2.9	24 ± 1.8	0.51 (Kruskal Wallis)
Subfertility n (%)	Primary	38 (39.5%)	39 (40.6%)	14 (14.5%)	5 (5.2%)	0.44 (chi ²)
	Secondary	14 (50.0%)	12 (42.8%)	1 (3.5%)	1 (3.5%)	
Cause of subfertility n (%)	Male factor	14 (36.8%)	16 (42.1%)	5 (13.1%)	3 (7.8%)	0.91 (chi ²)
	Male + female	7 (46.6%)	6 (40.0%)	1 (6.6%)	1 (6.6%)	
	Other factors	31 (43.6%)	29 (40.8%)	9 (12.6%)	2 (2.8%)	
Recent fever		5 (71.4%)	2 (28.4%)	0	0	0.38 (chi ²)
Alcohol	None	18 (54.5%)	13 (39.3%)	2 (6.0%)	0	0.22 (chi ²)
	<21 IU / wk	32 (37.6%)	34 (40.0%)	13 (15.2%)	6 (7.0%)	
	>21 IU / wk	2 (33.3%)	4 (66.6%)	0	0	
Smokers		8 (50.0%)	5 (31.2%)	3 (18.7%)	0	0.54 (chi ²)
Intake of Regular medication		9 (40.9%)	7 (31.8%)	4 (18.1%)	2 (9.0%)	0.50 (chi ²)
Intake of vitamins / nutrients		5 (35.7%)	8 (57.1%)	1 (7.1%)	0	0.53 (chi ²)
Other risk factors n (%)		13 (39.3%)	14 (42.4%)	4 (12.1%)	2 (6.0%)	0.98 (chi ²)

Table 28: Characteristics of 124 men by serum vitamin D groups

BMI was missing in 18 participants; Sub fertility – primary / secondary; Recent fever– in the past 3 months; Intake of vitamins / nutrients: other than vitamin D; continued...

(continued: Other risk factors include – medical problems like primary testicular failure, cystic fibrosis carrier, surgical problems like orchidopexy, orchidectomy, vasectomy reversal, varicocele inguinal hernia repair, sexually transmitted infections like chlamydia, gonorrhoea, non-specific urethritis and use of recreational drugs in the recent past).

Risk factors	Number of participants
Past chlamydial infection	11
Undescended testis / Orchidopexy	11
Inguinal hernia operation	5
Orchidectomy	3
Previous gonorrhoea	1
Nonspecific Urethritis	1
Cystic fibrosis carrier	1
Vasectomy reversal	1
Varicocele	1
Previous chemotherapy	1
Primary testicular failure	1

Table 29: Risk factors in the participants.

6.7.2.5 Vitamin D and semen parameters

The semen parameters of men in all the vitamin D groups are shown in Table 30. There was no significant difference in the various parameters i.e. semen volume, sperm concentration, total sperm count, motile concentration, progressive motile concentration, progressive motility, total motility and morphology between the vitamin D groups.

Semen parameters Median (25 -75 percentile)	Vitamin D Deficiency <25nmol/l	Vitamin D Insufficiency 26-50nmol/l	Vitamin D Adequate 51-75nmol/l	Vitamin D Optimal >75nmol/l	Kruskal Wallis test P value	Spearman Correlation P value	Multiple linear regression P value
Participants N=124	52 (41.9%)	51 (41.1%)	15 (12.1%)	6 (4.8%)			
Semen volume (ml)	2.9 (2.0-3.8)	2.5 (2.0-3.5)	3.3 (2.0-4.5)	2.6 (1.8-3.8)	0.75	0.69	0.89
Sperm concentration (millions/ ml)	71 (32- 133)	54 (24-112)	78 (3-123)	19 (2- 51)	0.12	0.13	0.41
Total sperm count (millions/ml)	233 (77- 356)	144 (55-260)	220 (7-416)	42 (6- 204)	0.12	0.15	0.49
Motile concentration (millions/ml)	51 (25- 92) *	36 (10-77)	37 (2 – 89)	12 (1-37)	0.11	0.08	0.41
Progressive motile concentration (millions/ml)	44 (17-79) *	31 (9-65)	31 (1-66)	9 (1-32)	0.10	0.07	0.37
Progressive motility (%)	62 (43-69) *	55 (45-64)	49 (24-65)	55 (29-68)	0.39	0.29	0.58
Total motility (%)	72 (55-79) *	67 (58-75)	67 (53-72)	63 (49-79)	0.57	0.36	0.57
Morphology (%)	9 (3-11)	8 (3-11)	7 (0-9)	4 (0-8)	0.15	0.21	0.17

Table 30: Semen parameters of 124 men divided by the vitamin D groups

(* motility parameters were missing for 1 participant in vitamin D deficient group); Details of the co-variates in the following page.

The relevant co- variates (i.e. $p \leq 0.15$) for each semen parameter used in the regression models are: **semen volume:** age and BMI; **sperm concentration:** ethnicity, cause of sub fertility, presence of risk factors, and alcohol consumption; **total sperm count:** age, cause of sub fertility, presence of risk factors, alcohol consumption, smoking, and intake of vitamins/nutrients; **total motile concentration:** ethnicity, cause of sub fertility, presence of risk factors, alcohol consumption and intake of vitamins/nutrients; **Progressive motile concentration:** ethnicity, type of subfertility cause of sub fertility, presence of risk factors, alcohol consumption and intake of vitamins/nutrients; **progressive motility:** type of subfertility, cause of sub fertility, presence of risk factors, and recent fever; **total motility:** type of subfertility, cause of sub fertility recent fever and intake of vitamins/nutrients; **morphology:** age, type of subfertility, cause of sub fertility, presence of risk factors and alcohol.

6.7.3 Follow up

87/125 (69.6%) of the participants returned for follow up. 8/87(9%) participants were reviewed when they attended their scheduled clinic follow up and 13/87 (15%) participants were on their subsequent IVF treatment cycle at follow up and were seen on their egg collection day. The remaining participants 66/87 (76%) were reviewed in the research clinic.

6.7.3.1 Participants who did not return for follow up

38 participants did not return for follow up. On reviewing the notes the factors identified are detailed in Table 31.

Overall, 31 (81.5%) of the participants who failed to return for the follow up appointment did not need, decided against or could not afford further IVF treatment and so decided to drop out of the study. 2 participants (5.2%) moved out of the area for work reasons. Only 5 (13.1%), were still receiving treatment at the centre, but decided not to return for the research follow up.

Reason	Number of participants
Partner pregnant	11
Completed NHS funding	8
Couple on a break from treatment	4
Lost to follow up from clinic	3
Self-funded treatment	3
No response when contacted	3
Couple split up	2
Others*	4

Table 31: Participants failing to return for follow up

** Return to Army-1, moved to China-1, Opted out of the research because of needle phobia – 1, Unable to attend -1.*

6.7.4 Cohort study

For further analysis, all the participants who returned for follow up were stratified into two groups based on their initial vitamin D level. These were: vitamin D deficient group i.e. <25nmol/l (37/ 87) and vitamin D non-deficient group i.e. > 25nmol/l (50/87). The return rate was comparable between vitamin D deficient participants (37/52) and vitamin D non-deficient participants (50/73) (71% vs. 68.4% Fisher's exact test: p value = 0.8)

6.7.4.1 Vitamin D deficient participants

6.7.4.1.1 Improvement in vitamin D levels with various treatment regimens

37/52 (71%) of vitamin D deficient participants returned for follow up. Amongst the 37 participants, 36 received vitamin D supplementation. The one participant who did not receive supplementation had a seasonal improvement in his vitamin D level from 11nmol/l (November) to 29nmol/l (May). The average interval between the two visits in this group was 175.6 ± 14.3 days (Mean \pm SD).

The GPs of the vitamin D deficient participants were recommended to prescribe a supplementation regime with loading dose of 20,000 IU Cholecalciferol three times a week for 6 – 8 weeks, followed by a maintenance dose of Cholecalciferol 1000 IU per day (Treatment Group 1, 12 participants). The suggested regime was only a recommendation and some of the GPs preferred their own regime which was essentially prescribing the loading dose only (Treatment Group 2, 16 participants) or eliminating high dose vitamin D loading treatment and prescribing vitamin D 800IU per day with calcium (Treatment Group 3, 8 participants). Some participants preferred to buy their own over the counter vitamin preparations and are also included in treatment group 3.

All treated participants had an improved vitamin D with supplementation, with levels rising to above 25nmol/l except one participant, where the rise was from <10nmol/l to 17 nmol/l only. This participant was a non-Caucasian student who used over the counter preparations. The vitamin D levels nmol/l (Mean \pm SEM) were significantly lower in treatment group 3 (45.7 ± 7.9) compared to treatment group 1 (105 ± 5.8) and treatment group 2 (93.1 ± 9.4) (One way ANOVA p <0.005) as shown in Figure 11. There was no

significant difference between treatment group 1 and treatment group 2 participants in each of which received the vitamin D loading treatment.

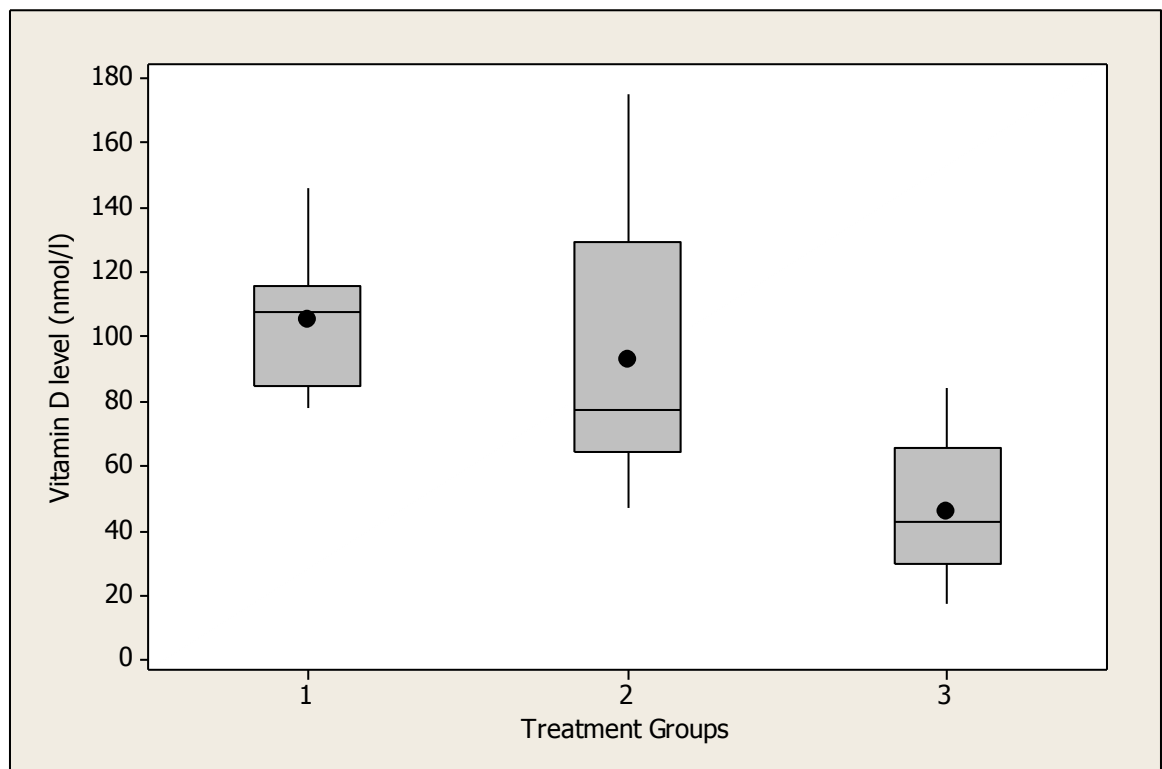


Figure 11: Serum vitamin D levels achieved in vitamin D deficiency participants in various treatment groups

Shows serum vitamin D levels achieved in vitamin D deficiency participants in various treatment groups. Mean levels of each group are shown by shaded circle. Each box plot (First Quartile to third quartile) shows the median and whiskers represent lowest to highest values. (Treatment group 1: Vitamin D loading with maintenance dose, Treatment group 2: Vitamin D loading without maintenance dose and Treatment group 3: GP regime or over the counter vitamin preparations)

6.7.4.1.2 Semen parameters following vitamin D supplementation in vitamin D deficient participants

In the 36 men who received vitamin D supplementation, serum 25(OH)D levels improved significantly from 14 (9.9-16.7) to 82 (63.2-114.2) (nmol/l; Median with 25th and 75th centiles $p < 0.005$). The semen parameters at the diagnosis of vitamin D deficiency and after vitamin D supplementation are shown in the Table 32. Sperm concentration, total count, motile concentration, progressive motile concentration, progressive motility, total motility and morphology declined significantly with supplementation whilst there was no significant difference in semen volume. A single participant reported an abstinence of less than 24 hours in the follow up phase. After excluding the participant from the analysis the conclusions still remained the same as above.

N=36; Variables	First phase Median (25 -75 percentile)	After vitamin D supplementation Median (25 -75 percentile)	Wilcoxon signed rank test
Vitamin D (nmol/l)	14.0 (9.9-16.7)	82.0 (63.2-114.2)	<0.005
Semen volume (ml)	2.5 (2.0-4.0)	2.4 (1.5-3.5)	0.33
Sperm concentration (millions/ml)	85 (34-136)	52 (16-88)	<0.005
Total sperm count (millions/ml)	262 (88-416)	108 (24-233)	0.001
Motile concentration (millions/ml)	63 (26-97)	31 (7-58)	<0.005
Progressive motile concentration (millions/ /ml)	53 (18-79)	25 (4-53)	<0.005
Progressive motility (%))	62 (43-69)	49 (31-60)	0.002
Total motility (%))	72 (53-79)	60 (43-71)	0.001
Morphology (%))	8 (2-11)	5 (1-10)	0.001

Table 32: Vitamin D and Semen parameters in the 36 vitamin D supplemented men

Table shows serum vitamin D and semen parameters at initial recruitment in 36 vitamin D deficiency participants and following vitamin D supplementation 6 months later are shown.

(Motility parameters were missing for participant 68 in the first phase i.e. at the diagnosis of vitamin D deficiency, therefore N=35 for motility parameters)

6.7.4.2 Vitamin D non-deficient participants

68.4% (50/73) of vitamin D non-deficient participants returned for follow up. Two participants had to be excluded from the analysis as one follow up vitamin D sample was lost in transit to the laboratories and the other participant revealed that he was on cod liver oil capsules at the time of initial recruitment (although he had stopped by the time of follow up). The average time interval between the first (Winter/Spring) and follow up (Summer/Autumn) visit was 180 ± 23 days (Mean \pm SD). There were 7 participants whose duration between the appointments was outside the mean \pm 1SD (157 to 203 days). The duration between the appointments for these participants was 70, 133, 153, 156, 207, 209 and 217 days respectively.

Semen parameters with seasonal rise in vitamin D levels in Vitamin D non deficient participants

There was a significant seasonal rise in serum 25(OH)D levels (Median, 25th-75th centile) from Winter/Spring to Summer/Autumn (41 (31-59) vs. 62 (52-76) nmol/l $p < 0.005$). The results of the semen parameters following the seasonal rise in vitamin D levels are shown in the Table 33.

Sperm concentration, total count, motile concentration, progressive motile concentration, progressive motility, total motility and morphology declined significantly with seasonal rise in serum vitamin D levels whilst semen volume did not change.

N=48	Season 1 (Winter / spring)	Season 2 (Summer / Autumn)	Wilcoxon signed rank test
Variables	Median (25 -75 percentile)	Median (25 -75 percentile)	
Vitamin D (nmol/l)	41 (31-59)	62 (52-76)	<0.005
Semen volume (ml)	2.6 (2.0-3.4)	2.6 (2.0-4.0)	0.97
Sperm concentration (millions/ml)	54 (16-92)	29 (4-67)	<0.005
Total sperm count (millions/ml)	162 (37-279)	97 (10-228)	0.002
Motile concentration (millions/ml)	36 (10-70)	16 (2-41)	<0.005
Progressive motile concentration (millions/ ml)	31 (7-63)	12 (2-36)	<0.005
Progressive motility (%)	54 (45-68)	43 (34-59)	0.01
Total motility (%)	68 (57-74)	56 (49-70.7)	0.01
Morphology (%)	7 (3-9)	5 (2-8)	0.008

Table 33: Semen parameters with seasonal rise in vitamin D levels

Serum vitamin D and semen parameters at the initial recruitment in winter / spring in 48 vitamin D non deficient participants and 6 months later in summer / autumn are shown.

All the participants had two sets of vitamin D levels and semen variables in Winter/Spring (season 1) and Summer/Autumn (season 2) each, except for four participants (seasonal mismatch). Two participants who were first seen in Autumn (November) were followed up with a slight delay in Summer instead of Spring (June instead May). The other two who were first seen in Winter (December, February) were followed up slightly earlier in Spring (May) instead of Summer. The samples from these four participants were allocated to the appropriate seasons (1/2) depending on the maximum exposure of the season. Statistical analysis lead to similar conclusions even after excluding these four participants from the analysis (n=44) (Results not shown).

Ten participants went on holidays abroad to sunny places in the previous 3 months of their recruitment which could have potentially increased their serum vitamin D levels at the time. Exclusion of these 10 participants from the analysis (n=38) also revealed similar results. Similarly excluding participants with seasonal mismatch and who had been on holidays concurrently (n=35) also revealed results as above.

6.7.5 Annual seasonal variation of vitamin D

We combined the results of the longitudinal study to investigate annual seasonal variation of vitamin D. Potentially we had 176 results, 125 results from the initial recruitment study and 51 results from the follow up study after excluding men who received vitamin D supplementation (36). The participant who had been on cod liver oil was excluded, even though he informed that he discontinued the preparation for at least 4 months prior to the follow up. There was one missing vitamin D level lost in transit to the laboratories. Finally there were 173 vitamin D results from 124 participants.

The monthly vitamin D levels gradually increased from January to July and decreased thereafter till January as shown in Figure 12. The serum vitamin D levels are significantly higher in Summer compared to Winter and Spring ($p < 0.005$) [Table 34].

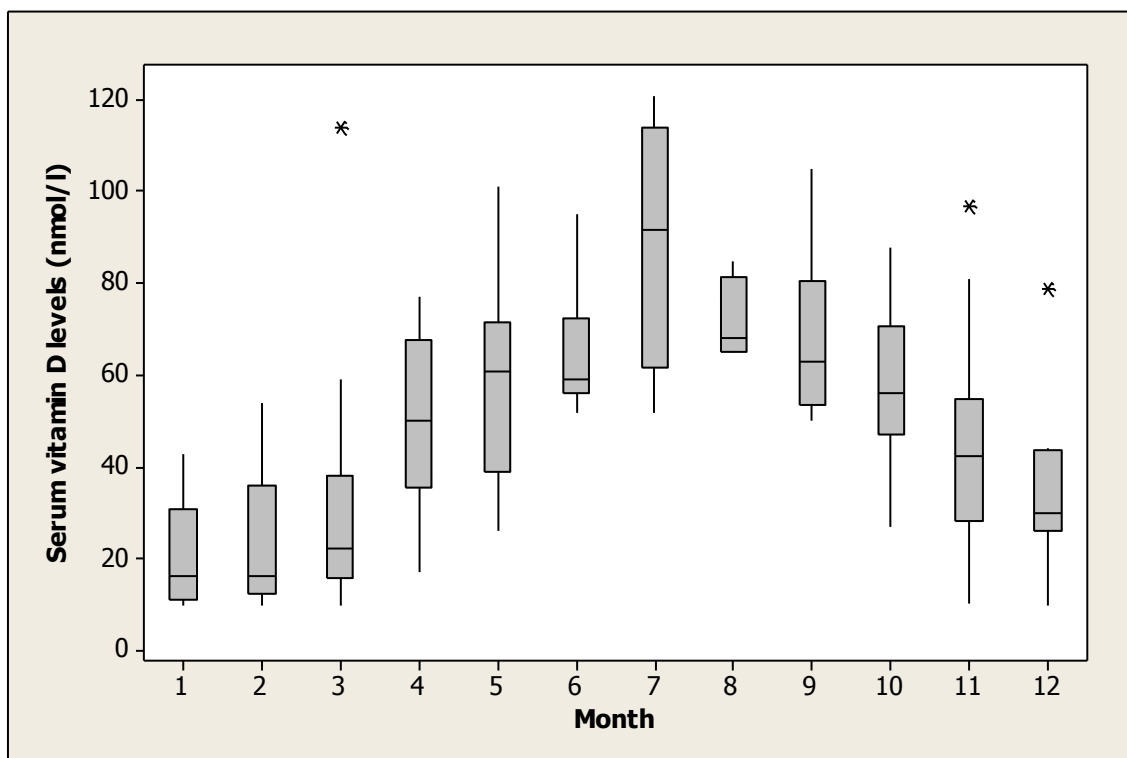


Figure 12: Monthly variation of serum vitamin D levels

Monthly variation of the median vitamin D is shown in box-plot graph. Each box plot (First Quartile to third quartile) is bisected by the median. The upper whisker extends to the highest data value within the upper limit [$Q3 + 1.5(Q3 - Q1)$] and the lower whisker extends to the lowest value within the lower limit [$Q1 - 1.5(Q3 - Q1)$].

The details of the outliers are Month 3: participant used a sunbed once a week; Month 11: Holiday within 3 months to a sunny place; Month 12: no obvious cause.

Season	Number of vitamin D results	Serum vitamin D level (nmol/l) Median (25 th -75 th centiles)
Winter (Dec- Feb)	54	17.5 (12.7-34)
Spring (Mar-May)	61	36 (21-58.5)
Summer (Jun-Aug)	17	65 (57.5-87.5)
Autumn (Sep-Oct)	41	52 (39.5-67)

Table 34: Seasonal variation of vitamin D levels – Annual pattern

6.7.6 *Is there a threshold for Vitamin D to be achieved to influence semen parameters?*

We hypothesized that serum vitamin D may need to improve to optimal levels to affect semen parameters. We did a subgroup analysis of the vitamin D deficient participants whose vitamin D levels improved to optimal levels ($\geq 75\text{nmol/l}$), however the limitation was a smaller sample size. There were 23 participants in this group. The vitamin D levels [nmol/l, Median (25th -75th centile)] in this group improved significantly from 16 (12-18) to 106 (84-128). There was a significant deterioration in the sperm concentration, motile concentration, progressive motile concentration, progressive motility, total motility and morphology with improvement in vitamin D levels to optimal levels (Table 35). There was no significant difference in the semen volume. Although the total count deteriorated with the improvement in vitamin D levels, this did not reach statistical significance which is most likely due to the small sample size.

N=23 Variables	First phase Median (25 th -75 th centile)	After vitamin D supplementation Median (25 th -75 th centile)	Wilcoxon Signed Ranks test
Vitamin D (nmol/l)	16 (12-18)	106 (84-128)	<0.005
Semen volume (ml)	2.3 (2.0-3.5)	2.5 (1.5-3.6)	0.70
Sperm concentration (millions/ml)	89 (47-134)	53 (19-103)	0.01
Total sperm count (millions/ml)	261 (81-335)	161 (48-234)	0.06
Motile concentration (millions/ml)	66 (27-101)	34 (12-66)	0.004
Progressive motile concentration (millions/ml)	63 (19-96)	27 (8-62)	0.01
Progressive motility (%)	62 (47-72)	19.5 (31-60)	0.01
Total motility (%)	74 (58-79)	64 (47-71)	0.009
Morphology (%)	9 (3-12)	6 (1-10)	0.01

Table 35: Semen parameters in men with vitamin D deficiency that improved to optimal levels with supplementation

6.7.7 *Is high vitamin D (>125nmol/l) associated with a negative impact on semen parameters?*

A study from USA has shown that high serum vitamin D levels (>125nmol/l) were associated with poor semen parameters (Hammoud *et al.*, 2012). There were 6 participants in the vitamin D supplemented men with serum vitamin D levels above 125nmol/l. As analysing the variation in semen parameters in this small group before and after vitamin D supplementation will not give us any valid conclusions, we instead excluded them from the vitamin D supplemented men (n=36, section 7.4.1) to investigate if the deterioration in semen parameters seen in this group of men was not so obvious.

We therefore had 30 participants in this analysis who had a rise in serum vitamin D levels with supplementation for deficiency, but not to above 125nmol/l. The serum vitamin D level [nmol/l, Median (25th -75th centile)] improved significantly from 13 (9.9-16.2) to 76.5 (53.7-95.7). All the semen parameters still deteriorated significantly (except semen volume) with rising vitamin D levels as shown in the Table 36.

N=30 Variables	First phase Median (25 th -75 th centile)	After vitamin D supplementation Median (25 th -75 th centile)	Wilcoxon Signed Ranks Test
Vitamin D (nmol/l)	13.0 (9.9-16.2)	76.5 (53.7-95.7)	<0.005
Semen volume (ml)	2.5 (2.0-4.0)	2.2 (1.4-3.2)	0.053
Sperm concentration (millions/ml)	81 (34-131)	49 (14-66)	<0.005
Total sperm count (millions/ml)	236 (80-382)	104 (20-215)	<0.005
Motile concentration (millions/ml)	54 (25-93)	24 (6-45)	<0.005
Progressive motile concentration (millions/ml)	47 (17-77)	20 (2-43)	<0.005
Progressive motility (%)	59 (42-67)	46 (28-61)	0.008
Total motility (%)	71 (53-79)	61 (41-70)	0.005
Morphology (%)	8 (2-11)	4 (1-10)	0.001

Table 36: Vitamin D and Semen parameters in the 30 vitamin D supplemented men

Table shows the serum vitamin D and semen parameters in 30 vitamin D supplemented men with improvement in vitamin D levels up to 125nmol/l.

(Motility parameters were missing for participant 68 in the first phase, therefore N=29 for motility parameters); (First phase is at the diagnosis of vitamin D deficiency)

6.8 Discussion

We investigated the correlation between semen parameters and serum vitamin D in a cross sectional association study involving 124 participants attending our infertility clinic and also examined the change in semen parameters with a rise in serum vitamin D level, both with seasonal rise and vitamin D supplementation. This is the first longitudinal study involving serum vitamin D and semen parameters.

It is well known that participation in semen studies is generally low (Meeker *et al.*, 2006) and sometimes necessitates financial incentives to improve recruitment (Hammoud *et al.*, 2012). Furthermore longitudinal semen studies are sparse as they can be expensive and challenging with high dropout rates as they need substantial participant time and cooperation (Mortimer *et al.*, 1983; Sánchez-Pozo *et al.*, 2013). 45.7% of the men approached for participation in this study were recruited which is considered high for semen studies (Ramlau-Hansen *et al.*, 2011) as generally the recruitment rate is in the range of 13-19% (Cooper *et al.*, 2010). The return rate for the study population was just under 70%. As the majority of our participants were National Health Service (public) patients and were not paid expenses to cover the costs of the visits, we feel that our return rate is creditable. A couple of longitudinal seasonal semen studies have shown return rates of up to 85%, where financial incentives were used (Levine *et al.*, 1990; Levine *et al.*, 1992).

The only two exclusion criteria for recruiting men were azoospermia or already using vitamin D supplements. Men with severe oligospermia were included as a change in semen parameters in this group could be clinically significant.

There was significant vitamin D deficiency in men attending our fertility centre. The prevalence of vitamin D deficiency in Winter and Spring was almost three times the UK national prevalence of 16% (Hyppönen and Power, 2007). Although the prevalence of hypovitaminosis D increases as one moves north in the UK, with the highest rates seen in Northern Ireland, Scotland and northern England (including the catchment area of our clinic) (Hyppönen and Power, 2007), the prevalence of hypovitaminosis D (i.e. serum vitamin D <40nmol/l) is higher in men attending the fertility clinic in Winter and Spring (79.6% and 64%) when compared to the reported regional prevalence (50-60% in winter and 30-40% in spring). A confounding factor however is that the national British survey included only white British participants, whilst in our study 12% were

non-Caucasians. However after excluding the at risk non-Caucasian population, we still identified a very high prevalence of hypovitaminosis D in the Caucasian population at 77.7% and 60% in Winter and Spring respectively. Whether men attending the fertility clinic are also a risk group for hypovitaminosis D needs further study.

As expected men who spend less time indoors (<than 3 hours a day), more time outdoors (>1 hour) had significantly higher levels of serum vitamin D. It is estimated that two to three exposures to sun light of thirty minutes duration per week to the face and forearms at midday between April to October is sufficient to achieve healthy vitamin D levels (Department of Health, 1998). However, as the majority of the participants were recruited in Winter and Spring, there was no difference in the vitamin D levels in participants who spent between 1 and 3 hours outdoors, and those who spent over 3 hours. In our study participants who had travelled to sunny places in the recent past had significantly higher levels of serum vitamin D, similar to previous findings (Gagnon *et al.*, 2010).

As oily fish is a rich source of vitamin D, its consumption can significantly raise vitamin D levels (Holvik *et al.*, 2004), however this was not shown in our study. This is most likely due to small sample size. The consumption of margarine (fortified with vitamin D) also did not significantly improve the levels, which was consistent with a large scale British survey, although the survey did note that the risk of vitamin D deficiency was much less in the cohort consuming margarine daily (Hyppönen and Power, 2007). The use of sun protection did not alter the vitamin D status in our study similar to other studies where sunscreen use was self-reported (Gagnon *et al.*, 2010). Sun protection is generally associated with reduced vitamin D synthesis (Matsuoka *et al.*, 1987). In contrast, studies have shown increased vitamin D levels in the cohort using sun protection (Hyppönen and Power, 2007), suggesting that the level of sun exposure is also high in individuals using sun protection in turn contributing to increased production. Also improper (not thick) or infrequent sunscreen use may also not block vitamin D production (Macdonald, 2013).

It has to be acknowledged however that the information on some factors influencing vitamin D levels (time spent indoors or outdoors, intake of vitamin D foods, use of sun protection) are a crude estimation by the participant at the time of the questionnaire and this may have affected the results. Furthermore, the study size is also small which again

may have affected the results. A couple of participants used a sunbed once per week and had good levels of serum vitamin D (74 and 114nmol/l). Studies have shown that regular use of a sun bed that emits UVB between 290-315nm increases vitamin D levels and bone mineral density (Tangpricha *et al.*, 2004; Armas *et al.*, 2007).

Vitamin D levels improved gradually from January to July and thereafter gradually declined to December. The highest vitamin D levels were seen in July, whilst in the British survey it was September (Hyppönen and Power, 2007). This variation is possibly due to smaller sample size in our study, although seasonal variation of vitamin D was consistent with the survey.

Participants with vitamin D deficiency (<25nmol/l) were treated with supplementation. Vitamin D levels above 75nmol/l are optimal for bone health and profound vitamin D deficiency can lead to osteomalacia (Souberbielle *et al.*, 2006; Hyppönen and Power, 2007; Pearce and Cheetam, 2010). As patients with vitamin D deficiency are generally advised supplementation to protect against bone disease (DeLuca, 2004; Dawson-Hughes *et al.*, 2005), so were the participants in this study. Vitamin D levels improved significantly when a loading dose was given with or without a maintenance regime, as this restores body stores. In the absence of a maintenance dose, participants appear to have utilised lifestyle advice as serum levels of vitamin D were maintained. The loading dose of vitamin D significantly improved the serum levels compared to just receiving maintenance vitamin D therapy, although a maintenance dose also improved the levels considerably.

There is no previous completed study which has investigated the effect of vitamin D supplementation on semen parameters. There has been a single randomised, double blind, placebo controlled study investigating the potential use of vitamin D on semen parameters using Elocalcitol, a vitamin D3 analogue intended primarily to treat benign prostatic hyperplasia and the overactive bladder. As the manufacture of Elocalcitol was terminated by the company producing it, (in view of the disappointing results found in its treatment of the over active bladder), the trial for male subfertility ended early in phase IIa (Tiwari, 2009). A clinical trial is currently underway by Blomberg *et al.*'s group to investigate the effect of vitamin D supplementation on semen parameters in subfertile men (Clinical Trials.gov Identifier: NCT01304927).

In the cohort study, vitamin D levels improved not only with vitamin D supplementation but also with the seasonal rise. The vitamin D levels with supplementation improved to 82 (63.2-114.2) as opposed to the seasonal improvement of 62 (52-75.7) (nmol/l; Median with 25th and 75th centiles). As supplemented men also would have benefitted with season and generally there is an increase of 1nmol in 25(OH)D for each μg of supplementation (Heaney, 2008), it appears that the men in the supplemented group received approximately an additional 20 μg (800IU) per day of vitamin D compared to seasonal rise group.

In the association study there was no significant difference in the various semen parameters between the vitamin D groups, although the numbers of men with adequate (15) and optimal levels (6) of vitamin D were low. Sperm counts and morphology were lower in the optimal (>75nmol/l) vitamin D group compared to other three groups but this did not reach statistical significance. These findings are similar to the cross sectional association study by Ramlau-Hansen *et al.* (2011) on 307 young healthy Danish men aged between 18-21 years, which showed no association between serum vitamin D levels and semen parameters.

Conversely in another association study on Danish men of similar study size and group, there was a weak positive but significant association between serum vitamin D level and sperm motility (both progressive and total motility). The motility was significantly better in men with optimal levels of vitamin D compared to vitamin D deficient men, but not compared to men with insufficient or adequate vitamin D levels. It appeared that vitamin D deficient men had a low percentage of normal sperm, but after adjusting for the confounders this was non-significant. There was no association between vitamin D and semen volume or sperm count in this study (Blomberg Jensen *et al.*, 2011).

Of note, our association study has a higher number of participants with vitamin D deficiency when compared to subjects recruited in other vitamin D/semen parameters association studies (Blomberg Jensen *et al.*, 2011; Ramlau-Hansen *et al.*, 2011; Hammoud *et al.*, 2012). The semen parameters in this vitamin D deficient group were of higher quality compared to the participants in the rest of the three vitamin D groups. Ramlau-Hansen *et al.* (2011) study suggested that low vitamin D (i.e. between 8-62nmol/l in their study group) was not a risk factor for poor semen parameters, however the study only had 19 participants with vitamin D deficiency i.e. <25nmol/l in this

group. From our study it does appear that serum vitamin D deficiency (<25nmol/l) is not associated with reduced semen parameters.

Sperm count, motility and morphology deteriorated significantly with increased serum vitamin D levels secondary to vitamin D supplementation and seasonal rise. Vitamin D supplementation improved vitamin D status essentially from deficient to optimal levels and with seasonal rise, the vitamin D levels improved essentially from insufficiency to adequate vitamin D status in a gradual manner. However both these interventions led to a similar deterioration in semen parameters.

There is some evidence that higher serum vitamin D levels are associated with lower sperm parameters. In Ramlau-Hansen *et al.* (2011) study it appeared that men with high vitamin D (i.e. between 94- 227nmol/l in their study group) had lower sperm counts and a lower percentage of normal sperm morphology, but after transforming the data and adjusting for the confounders as mentioned previously there was no association between serum vitamin D and semen parameters. In another association study by Hammoud *et al.* (2012), conducted in Salt Lake, Utah involving 147 healthy men with no male factor subfertility; sperm concentration, progressive sperm motility, sperm morphology and the total progressive motile sperm count were lower in men with high concentrations of vitamin D, but this group included only men with a serum vitamin D levels above 125nmol/l. Unlike our study, they also found worse sperm parameters (total progressive motile sperm count and total sperm count) in men with a serum vitamin D of less than 50nmol/l. They suggested a 'U' shaped association between vitamin D levels and semen parameters similar to the association seen between vitamin D and some non-skeletal conditions such as cardiovascular disease and pancreatic cancer (Ross *et al.*, 2011; Hammoud *et al.*, 2012; Rosen *et al.*, 2012).

All the above association studies recruited healthy young men whereas we recruited men attending an infertility clinic. Some form of male factor (by itself or combined with a female factor) was noted in 43% of the study group, and whether this is the reason for such a marked deterioration in most of the semen parameters is unclear. Analysing this group separately would further decrease the study size, making the validity of any results uncertain. Studies have shown that subfertile men display variability in the expression of VDR or response to vitamin D when compared to fertile men. Subfertile men have a lower expression of VDR and vitamin D metabolizing enzymes in testis and

sperm compared to normal men (Aquila *et al.*, 2009; Foresta *et al.*, 2011; Blomberg Jensen *et al.*, 2012). In the study by Foresta *et al.* (2011) men with normal testicular function had significantly higher levels of CYP2R1 gene expression in the testis compared to men with oligozoospermia.

In a further small cross sectional association study by Blomberg Jensen *et al.* (2012), of 70 men (involving 25 sub-fertile and 45 young men), progressive sperm motility was significantly higher in the vitamin D non-deficient group (>25nmol/l) at 60% compared to the vitamin D deficiency group at 51%. There was no statistical difference in other semen parameters (i.e. sperm concentration, total motility and morphology) between the groups. Although they had only 2 vitamin D groups in this study and included fertile and subfertile men, the results corroborated with their previous study involving 300 healthy young men (Blomberg Jensen *et al.*, 2011).

In a large recent cross sectional association study by Yang *et al.* (2012) both subfertile (314) and fertile (195) men were included. Subfertile men included men who never fathered a child with time to pregnancy (TTP) longer than 12 months. Men whose partners had female factor subfertility were excluded and therefore this group comprised of subfertile men. Of note, idiopathic male factor subfertility was not an exclusion criterion. This is different to infertility clinic recruits who are a heterogeneous cohort of normal and subfertile men, as recruited in our study and Blomberg's subset of participants in their immunocytochemistry study as mentioned above (Blomberg Jensen *et al.*, 2012).

Contrary to expectation from a potential lower VDR/enzyme expression in subfertile men, a significant positive association between serum vitamin D and sperm motility, sperm morphology, but not sperm concentration was seen in the subfertile men group. Surprisingly this positive association was seen only between vitamin D and sperm motility in fertile men. There was no association between sperm concentration and vitamin D in the fertile male group either. However in another small study recruiting 90 men attending an infertility clinic, there was no correlation between vitamin D and semen parameters (Knopf *et al.*, 2011).

In a recent immunocytochemistry study by Blomberg Jensen *et al.* (2012), 77 men from infertility clinics and 53 healthy nineteen year old males (unknown fertility) were

recruited. Vitamin D inactivating enzyme (CYP24A1) expression in sperm correlated positively with semen parameters. A significantly higher proportion of sperm (25%) expressed CYP24A1 in the healthy control group compared to only 1% in the infertility clinic recruits. Furthermore a threshold of 3% sperm with positive CYP24A1 expression distinguished young men from infertility clinic recruits with a sensitivity of 66%, specificity of 78% and positive predictive value (PPV) of 98%. When men from couples with female factor subfertility were excluded from the infertility clinic recruits, the sensitivity, specificity and PPV increased even further.

CYP24A1 expression gives an indirect estimate of adequate vitamin D levels for spermatogenesis as 1,25(OH)₂D leads to transcription of CYP24A1 (by binding to VDRE's in the CYP24A1 promoter region) as a part of the feedback mechanism (Haussler *et al.*, 2011). However as serum vitamin D was similar in men with low (<3%) and high (>3%) CYP24A1 expression, it was suggested that the local vitamin D metabolism may influence CYP24A1 expression in sperm rather than systemic vitamin D levels (Blomberg Jensen *et al.*, 2012). Cellular vitamin D responsiveness however is complex and not as tightly regulated as systemic vitamin D metabolism (Blomberg Jensen, 2012). Furthermore, in *in vitro* experiments conducted by Blomberg Jensen *et al.* (2012) on 40 men, (22 young, 18 sub-fertile men) revealed that 1,25(OH)₂D increased sperm motility in young men but not in sub-fertile men.

The significant proportion of subfertile men (42.7%) may explain the lack of a positive association between vitamin D and semen parameters seen in our study. However, this still cannot explain the significant deterioration of all the semen parameters seen with a rise in serum vitamin D levels.

Overall from the association studies published so far, the association between vitamin D and semen parameters appears to be inconsistent, varying from a positive association (Blomberg Jensen *et al.*, 2011; Blomberg Jensen *et al.*, 2012; Yang *et al.*, 2012) to a 'U' shaped association (Hammoud *et al.*, 2012) or to no association (Knopf *et al.*, 2011; Ramlau-Hansen *et al.*, 2011). Although association studies on subfertile men also showed a positive association, there is some *in vitro* experimental evidence that there is less expression of VDRs and related enzymes and a lack of improvement in sperm motility with vitamin D in subfertile men (Aquila *et al.*, 2009; Foresta *et al.*, 2011; Blomberg Jensen *et al.*, 2012).

Concomitant expression of receptors and metabolizing enzymes in Leydig cells suggests a possible role for vitamin D in hormone production (Blomberg Jensen *et al.*, 2010). As there was a positive association shown between serum vitamin D levels and androgens (Wehr *et al.*, 2010a) it is possible that the effect on semen parameters could be androgen mediated. However four other studies showed no link or a negative association between serum vitamin D levels and androgens (Blomberg Jensen *et al.*, 2011; Ramlau-Hansen *et al.*, 2011; Hammoud *et al.*, 2012; Yang *et al.*, 2012). In Knopf's study a positive correlation between vitamin D and testosterone was noted, but only in men with low testosterone levels (Knopf *et al.*, 2011).

There is some supporting evidence for the negative association seen in our study from lab based studies. The association between vitamin D and sperm function (acrosin activity or sperm motility) in the *in vitro* studies appears to be dose dependent and importantly with higher concentrations of vitamin D these responses became either ineffective or inhibitory (Aquila *et al.*, 2008; Blomberg Jensen *et al.*, 2011). Although the vitamin D concentrations inducing the positive reaction in sperm in the *in vitro* experiments are close to physiological levels of serum vitamin D levels, it is possible that different concentrations of vitamin D can elicit varied responses in sperm. A possibility of receptor down regulation with higher levels of vitamin D hormones has also been suggested (Aquila *et al.*, 2009).

Importantly the functional significance of vitamin D induced sperm motility and acrosome reaction depends on the concentrations of vitamin D in seminal plasma (Blomberg Jensen, 2012) and little is known about seminal plasma vitamin D concentrations and their correlation to serum vitamin D levels. A discrepancy between serum and semen vitamin D cannot be ruled out.

The variation in 25(OH)D measurements due to differences in the utilized assay technology is well known (Roth *et al.*, 2008; Thienpont *et al.*, 2012). Cut off measurements for vitamin D groups vary between studies also. Some divide study groups by the commonly used clinical classification of vitamin D status (Blomberg Jensen *et al.*, 2011) as used in our study, whilst the study by Hammoud *et al.* (2012), merged vitamin D deficiency and insufficiency groups together and also created another group with a vitamin D higher than 125nmol/l. Ramlau-Hansen *et al.* (2011) divided men by tertiles of vitamin D levels. So variations in measurement techniques and

vitamin D group stratification may also have contributed to the variation in results seen between previous studies. As spermatogenesis starts about 72 days prior to semen sample collection, it has been suggested that serum vitamin D levels be checked a few months prior to the semen analysis to investigate the association (Blomberg Jensen *et al.*, 2011).

The deterioration in semen parameters seen in our study associated with a rise in serum vitamin D level could be just due to a seasonal effect on semen parameters, independent of vitamin D, although whether semen parameters is subject to seasonal variation remains controversial and is discussed in detail in Chapters 4 and 5. The negative association seen in our study is intriguing and warrants future research to clarify. The presence of VDR and metabolising enzyme expression in the male genital tract raises the possibility of a role for vitamin D in human male reproduction, however the importance of these receptors and the role of vitamin D in spermatogenesis and the function of the male reproductive tract is still not completely understood and therefore the biological mechanisms are speculative.

One of the hypotheses tested was that vitamin D should cross a threshold ($\geq 75\text{nmol/l}$ i.e. on optimal level) before semen parameters improved, but a significant deterioration in semen parameters was still seen in patients achieving this level.

6.9 Limitations of the study

1. Semen sample: A high intra-individual variation in semen parameters (Carlsen *et al.*, 2004; World Health Organisation, 2010) and particularly in male partners of sub-fertile couples (Leushuis *et al.*, 2010) is known. Only one semen sample from each participant on each occasion (i.e. at recruitment and follow up) was collected, therefore potential intra-individual variability was not accounted for. Considering the difficulty in recruiting men for semen studies and particularly cohort studies (Sánchez-Pozo *et al.*, 2013), we anticipated problems with recruitment if four semen samples were to be requested from participants.

The information on abstinence was not collected at the recruitment phase, although all men were given verbal and written instructions to maintain an abstinence of 2-7 days. It is important to acknowledge that couples attending for IVF treatment are known to follow the instructions given to them effectively. This is proven in the follow up phase,

as even though they were mainly attending for the research (where you can expect non-compliance with instructions) and not their treatment, 86 of 87 participants maintained the recommended abstinence. Despite the effect of abstinence on semen volume, sperm concentration, motility and possibly morphology (Mortimer et al., 1982; Carlsen et al., 2004), as it is not correlated to vitamin D levels, it was argued that abstinence is not a true confounder in this association (Hammoud et al., 2012).

Sample loss at the collection of semen was not recorded at the recruitment, however when recorded at the follow up phase was noted only in a small percentage of participants (3/87). The duration between times from ejaculation to sperm motility assessment was not recorded, however all the samples were assessed for sperm motility soon after liquefaction or at half an hour at the latest. As described in Methods, the samples were completely analysed (all the parameters) within one hour starting with semen volume and sperm motility.

2. The observational association study had four groups divided by their vitamin D status (deficiency, insufficiency, adequate and optimal), however in the cohort study, the study group was divided in to only 2 groups (vitamin D deficient and non-deficient). This was done to avoid very small groups and results which would have been difficult to validate.
3. The vitamin D supplementation treatment protocol was not uniform in the study as at the time, there were no licensed high dose vitamin D preparations and different GPs preferred their own regime, despite a standard recommendation from us.
4. As the participants are male partners of sub fertile couples, the results may not be extrapolated to the general population.

6.10 Conclusion

The study suggests that vitamin D deficiency is very common in men attending our infertility clinic, but is not associated with poor semen parameters. Despite limitations, a negative association between vitamin D and semen parameters is noted. Based on the results from our preliminary study vitamin D supplementation may be harmful to semen parameters for male partners of subfertile couple and therefore we advise caution and

suggest monitoring the semen parameters when supplemented. A well-designed RCT to evaluate this further is warranted.

In the next chapter we investigated the association between vitamin D and semen quality, and also IVF treatment outcomes.

Chapter 7 Vitamin D and male fertility

7.1 Introduction

The WHO criteria for semen parameters are used worldwide and the recent evidence based WHO 2010 reference values for semen characteristics are based on a large cohort of men whose partners had a time to pregnancy (TTP) of less than 12 months (Cooper *et al.*, 2010). Table 37 shows the 5th centiles of the semen parameters with their 95% confidence intervals that provided the lower reference limits (World Health Organisation, 2010).

Semen Parameters	5th centiles (95% confidence intervals)
Semen volume (ml)	1.5 (1.4-1.7)
Total sperm number (million/ejaculate)	39 (33-46)
Sperm concentration (million/ml)	15 (12-16)
Vitality (% live)	58% (55-63)
Progressive motility (%)	32 (31-34)
Total motility (%)	40 (38-42)
Morphologically (%)	4 (3-4)

Table 37: WHO 2010 criteria for semen parameters

For this chapter, if semen parameters met the WHO 2010 requirements, the sample was defined as being of “optimal semen quality” and if it failed to meet at least one of the above criteria it was defined as “sub-optimal semen quality”. This was done to investigate the association between vitamin D and “semen quality” (in addition to the association between serum vitamin D and individual semen parameters in Chapter 6).

A significant positive or negative correlation between individual semen parameters and serum vitamin D may not alter the overall semen quality from a clinical perspective, if the individual semen parameters remain within the normal range. For instance if the sperm concentration alone deteriorated from 120 million/ml to 60 million/ml (bearing in mind that other parameters were normal) the “semen quality” would still remain optimal (WHO 2010 criteria) despite this deterioration and therefore may not adversely affect the chances of conception. Hence it is worth investigating the impact of serum vitamin D on overall “semen quality” and not just individual semen parameters.

Many studies have investigated the association of serum vitamin D and individual semen parameters such as semen volume, sperm concentration, motility and morphology as detailed in Chapter 6. The impact of vitamin D on overall “semen quality” has not been evaluated in studies so far.

“Optimal semen quality” however does not guarantee fertility success as the semen parameters are surrogate markers for sperm function which can only be demonstrated fully by the sperm’s ability to fertilise an egg and subsequent pregnancy and live birth. Therefore further study investigating the association between serum vitamin D and male fertility would explore the role of vitamin D in male reproduction in another potentially useful dimension.

Studies have shown that vitamin D deficiency in male rats adversely affects the reproductive potential of vitamin D replete female rats. Fertility (defined as successful pregnancies in sperm positive female rats) was significantly reduced by 73% in the litters from vitamin D deficient male rat inseminations compared to inseminations by vitamin D replete rats (Kwiecinski *et al.*, 1989a). Furthermore *in vitro* studies have shown that vitamin D enabled sperm capacitation, sperm survival and the acrosome reaction (Aquila *et al.*, 2008; Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011).

Although these processes may not be relevant to the same extent in standard assisted reproduction techniques such as IVF and ICSI as in natural conception, analysing the IVF treatment cycles allows us to consider fertilisation rates, embryo quality and ultimately conception rates (if not live birth rates) in relation to participant's vitamin D levels. We hypothesize that a variation in serum vitamin D in men may alter fertilisation rates and affect embryo quality in assisted reproduction with IVF.

7.2 Aim

To investigate if there is correlation between serum vitamin D and

1. Semen quality (defined by WHO 2010 criteria)
2. IVF treatment outcomes (as detailed in objectives).

7.3 Objectives

To investigate

1. If there is a difference in semen quality between men in various vitamin D groups.
2. The difference (improvement or deterioration) in semen quality with vitamin D supplementation in vitamin D deficient participants and with the seasonal rise in vitamin D levels in non-vitamin D deficient participants.
3. To compare the fertilisation rates, embryo quality, conception and cryopreservation rates between IVF treatment cycles in Winter/Spring and Summer/Autumn, with rise in serum vitamin D in the male partner.

7.4 Outcome measures

To determine if serum vitamin D levels in the male partner affect semen quality and the chances of conception in their female partner by assisted reproductive techniques.

7.5 Subjects and Methods

This section has been detailed in Chapter 6 (Section 6.5) and summarised here apart from detailing the additional relevant points.

7.5.1 Subjects

124 men attending NFC for fertility investigations / treatment are included in this section.

7.5.2 Study design

It is a prospective longitudinal observational study.

7.5.3 Investigations

7.5.3.1 Blood sample

All participants provided a blood sample to analyse their serum 25(OH)D levels.

Participants were stratified into four groups based on their 25(OH)D levels as follows

- < 25nmol/l - Deficiency
- 25-50nmol/l –Insufficiency
- 51-75nmol/l – Adequate
- >75nmol/l – Optimal

7.5.3.2 Semen sample

All participants produced a semen sample and were analysed as detailed in Chapter 6 (Section 6.5.8.2.3). Normal semen parameters as defined by WHO 2010 criteria are shown in Table 37. As described earlier in introduction if semen parameters met the WHO 2010 requirements, the sample was defined as being of “optimal semen quality” and if it failed to meet at least one of the above criteria it was defined as “sub-optimal semen quality”. However semen volume and vitality parameters were not included in defining the “semen quality” for this work as sample loss was not recorded at the recruitment phase and also sperm vitality was not assessed in the analysis.

7.5.4 Study method

At recruitment phase, the above investigations were obtained from the 124 participants and all of them were invited to return six months later for repeat investigations.

Participants seen in Winter and Spring were to be reviewed in Summer and Autumn respectively. Meanwhile vitamin D deficient participants were notified of the result and recommended treatment with vitamin D supplementation (detailed in Chapter 6 Section 6.5.11). 87 participants returned for follow up, however 84 participants could be included in the cohort study (1 participant’s blood sample was lost in transit; 1 participant excluded when he revealed to be on cod liver oil supplements and 1 vitamin D deficient participant did not receive vitamin D supplementation.

For further analysis, all the participants who returned for follow up were stratified into two groups based on their initial 25(OH)D level (detailed in Chapter 6, Section 6.7.4) as follows:

- Vitamin D deficient (< 25nmol/l)
- Vitamin D non-deficient (>25nmol/l)

The details of vitamin D deficient and non-deficient participants are described in Chapter 6 Sections 6.7.4.1 and 6.7.4.2 respectively.

This chapter investigates:

- The “semen quality” in the 124 recruits stratified by the four groups.
- The semen quality before and after supplementation in the 36 vitamin D deficient men who received vitamin D supplementation.
- The semen quality in Winter/Spring and Summer/Autumn in the 48 vitamin D non-deficient men exhibiting a seasonal rise in vitamin D levels.

The flow diagram detailing the recruitment for vitamin D and semen quality study is shown in the Figure 13.

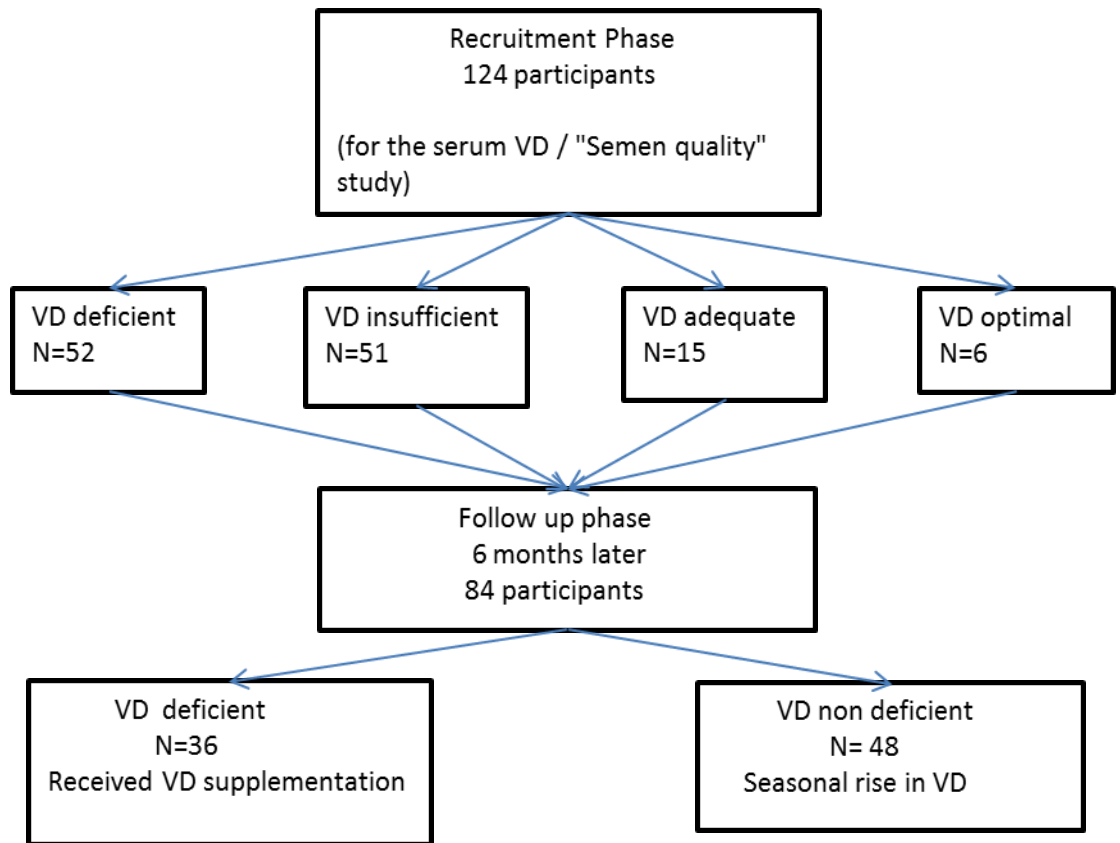


Figure 13: Participant's flow chart for Vitamin D and "Semen quality" study

7.5.4.1 Male fertility (IVF/ICSI treatment)

14/87 participants were going through IVF treatment cycle at follow up and were seen on their partner's egg collection day. Two of these participants (2/14) were initially recruited from clinic i.e. their partner was not going through an IVF treatment cycle at recruitment. One (1/14) was excluded as he later was revealed to have been on cod liver oil. Another (1/14) participant's partner had a poor ovarian response and was unsuitable for oocyte retrieval and therefore IUI treatment was done instead of IVF.

Finally there were 10 participants whose partners had egg collection at the initial recruitment stage and also at 6 month follow up. The phases were divided into two based on the season of treatment i.e. Winter/Spring and Summer/Autumn.

The procedure of IVF has been briefly explained in Chapter 3 (Section 3.5.3) in relation to D-IVF. A flow chart with sequence of events in IVF is shown in Chapter 6 (Section 6.5.3). In addition, fertilisation in IVF involves a few 100,000 prepared sperms inseminated with the oocytes in the culture medium in the laboratory, whilst ICSI involves injecting a sperm into each oocyte.

The variables compared were the number of mature oocytes retrieved at egg collection, the number of oocytes fertilised (by IVF / ICSI), the fertilisation rate (number of oocytes fertilised/number of oocytes $\times 100$), the number of normally fertilised embryos (embryos with two pronuclei) and the percentage of normal fertilisation (number of oocytes normally fertilised /number of oocytes $\times 100$). The quality of the embryos has also been compared. The grading of the embryos is based on the blastomere number, size and fragmentation and shown in Appendix K. The number of top, good, slow and other quality embryos was noted and their percentage (out of normally fertilised embryos) was calculated. Clinical pregnancy rates (including missed miscarriages and viable intrauterine pregnancies at 7 week scan) were compared. All the participants with two or more spare top and or good quality spare embryos after embryo transfer were eligible for cryopreservation of their embryos. Cryopreservation rates were also compared between Winter/Spring and Summer/Autumn.

7.6 Statistical analysis

Statistical analysis was performed using SPSS (version 19) and Minitab (version 16). Variables were expressed as percentages and median with centiles. Vitamin D levels showed non-normal distribution. Semen quality was compared between vitamin D groups by applying the chi square test. The change in the semen quality (improvement or deterioration) with vitamin D supplementation and seasonal rise in vitamin D was investigated by the McNemar test.

Vitamin D and IVF /ICSI outcome: The distributions of continuous variables were checked using the Anderson Darling normality test. Vitamin D, number of mature oocytes, total oocytes fertilised, number of normally fertilised oocytes, percentage of normally fertilised oocytes, percentage of good quality embryos and percentage of other quality embryos were normally distributed. The remaining variables fertilisation rate, the number of top quality embryos, percentage of top quality embryos, number of good quality embryos, number of slow quality embryos, percentage of slow quality embryos and other quality embryos were not normally distributed. Both parametric (paired tests) and non-parametric (Wilcoxon rank test) were applied to compare the fertilisation rates and embryo quality during the treatment cycles in Winter/Spring and Summer/Autumn yielded similar results. To ease the reading and retain uniformity in reporting, non-parametric test results are shown. The Independent sample t test was used to demonstrate the difference in vitamin D levels. The McNemar test was applied to examine the differences in conception results and numbers of embryos for freezing. A p value of <0.05 was considered significant.

7.7 Results

7.7.1 Semen quality in four vitamin D groups

The semen quality (Optimal or suboptimal) as per the WHO 2010 criteria was not significantly different among the men in the four vitamin D groups (Table 38).

Semen quality N=124	Vitamin D Deficiency <25nmol/l N=52	Vitamin D Insufficiency 26-50nmol/l N=51	Vitamin D Adequate 51-75nmol/l N= 15	Vitamin D Optimal >75nmol/l N=6	(Chi square test) P value
Sub optimal semen quality (%)	13 (25)	15 (29)	6 (40)	3 (50)	0.49
Optimal semen quality (%)	39 (75)	36 (71)	9 (60)	3 (50)	

Table 38: Semen quality by vitamin D group

7.7.2 Semen quality with vitamin D supplementation

Among the 36 vitamin D supplemented men, 29 had semen quality unchanged before and after vitamin D supplementation. Although this deteriorated in 6 participants to sub-optimal quality and improved to optimal quality in one participant, there was no statistically significant change in the semen quality with a rise in serum vitamin D levels on supplementation (McNemar $p=0.12$) as shown in Table 39.

Phase / vitamin D [nmol/l; Median (25 -75) percentile]	Semen quality		Total	Mc Nemar test P value
	Suboptimal Semen quality	Optimal semen quality		
At the diagnosis of vitamin D deficiency 14 (9.9-16.7)	11 (30.6%)	25 (69.4%)	36 (100.0%)	0.12
Following vitamin D supplementation 82 (63.2-114.2)	16 (44.4%)	20 (55.6%)	36 (100.0%)	

Table 39: Semen quality with vitamin D supplementation

The details of semen quality deterioration and improvement with vitamin D supplementation are discussed in the following sections.

7.7.2.1 Semen quality deterioration (optimal to suboptimal) with vitamin D supplementation

Among the six participants with semen quality deterioration following vitamin D supplementation one participant had a lowered count but partial sample loss was recorded; two participants had a decline in all semen parameters but one of them was known to have variable semen quality; three participants had worsened morphology and interestingly two of them had history of poor fertilisation with IVF. All the details are shown in the Table 40.

Participant	First Vitamin D	Following supplementation	Semen parameters / (Quality) before supplementation	Semen parameters / (Quality) After supplementation	Comments
46	9.9	32	80 million/ml (Optimal) 55% prog. motile 9% normal	8 million/ml (Sub-optimal) 19% prog. motile 2% normal	Known variable semen quality
65	13	47	137 million/ml (Optimal) 55% prog. motile 11% normal	57.4 million/ml (Sub-optimal) 41% prog. motile 2% normal	
66	9.9	17	34 million/ml (Optimal) 63% prog. motile 8% normal	10 million/ml (Sub-optimal) 45% prog. motile 10% normal	Partial sample loss
91	20	112	56 million/ml (Optimal) 77% prog. motile 6% normal	53 million/ml (Sub-optimal) 51% prog. motile 0% normal	Poor fertilisation with IVF
105	22	93	91 million/ml (Optimal) 77% prog. motile 10% normal	51.5 million/ml (Sub-optimal) 60% prog. Motile 3% normal	1 st cycle: good fert*, 2 nd cycle: poor fert* with IVF
108	16	104	134 million/ml (Optimal) 59% prog. motile 10% normal	9.5 million/ml (Sub-optimal) 18% prog. motile 0% normal	

Table 40: Serum vitamin D levels and semen parameters before and after supplementation in 6 participants with change in semen quality from Optimal to suboptimal

*(prog.motile – progressive motility; * fert- fertilisation)*

7.7.2.2 Semen quality improvement with vitamin D supplementation

In one participant, semen quality improved to optimal (sperm morphology 3 to 10%) with vitamin D supplementation.

7.7.3 Semen quality with seasonal rise in vitamin D

Among the 48 non vitamin D deficient participants, 45 had unchanged semen quality (optimal or suboptimal) in Winter/Spring and Summer/Autumn. In one participant the semen quality improved to optimal quality whilst in two participants this deteriorated to sub-optimal quality from Winter/Spring to Summer/Autumn. Overall there was no difference in the semen quality with seasonal rise in vitamin D levels (Table 41).

Season / vitamin D [nmol/l; Median (25 -75) percentile]	Semen quality		Total	Mc Nemar test P value
	Suboptimal Semen quality	Optimal semen quality		
Winter / Spring 41 (31-59)	16 (33.3%)	32 (66.7%)	48 (100.0%)	1.0
Summer / Autumn 62 (52-75.7)	17 (35.4%)	31 (64.6%)	48 (100.0%)	

Table 41: Semen quality with seasonal rise in vitamin D

The details of semen quality deterioration and improvement with seasonal rise in serum vitamin D are discussed in the following sections.

7.7.3.1 Semen quality improvement from Winter/Spring to Summer/Autumn

In one participant (Table 42) the semen sample in Spring was of sub-optimal quality when the 25(OH)D level was 101nmol/l. In Autumn the semen quality improved to optimal when the 25(OH)D level, (although appearing lower at 97nmol/l) was in the same range. It is notable that monthly median serum 25(OH)D levels (Chapter 6, section 6.7.5, Figure 12) in May were higher than November, although the levels were overall lower in Spring compared to Autumn (Chapter 6, Section 6.7.5, Table 34). Interestingly, although the semen sample for this participant appeared to be better in Autumn, it did not prepare well for treatment and the couple required ICSI. Furthermore whilst this treatment was ultimately unsuccessful the couple went on to conceive naturally.

Participant	Vitamin D Winter/Spring	Vitamin D Summer/Autumn	Semen parameters (Quality) Winter/Spring	Semen parameters (Quality) Summer/Autumn
7	101 (May)	97 (Nov)	7 million/ml 2% normal (Sub-optimal)	16 million/ml 6% normal (Optimal)

Table 42: Serum vitamin D levels and semen parameters in a participant with change in semen quality (suboptimal to optimal) from Winter/Spring to Summer/Autumn

7.7.3.2 Semen quality deterioration from Winter/Spring to Summer/Autumn

In one participant the deterioration in semen quality seems to be mainly because of sperm morphology. The partial sample loss recorded for this participant may not explain the decline in sperm morphology but interestingly the couple had a spontaneous conception during the course of the study. The other participant in this group had both sperm count and morphology which were markedly lower in association with improved serum vitamin D levels and interestingly there was history of poor fertilisation in their previous IVF cycles. The details of the semen quality are shown in Table 43.

Participant	Vitamin D Winter/Spring	Vitamin D Summer/Autumn	Semen parameters (Quality) Winter/Spring	Semen parameters (Quality) Summer/Autumn	Comments
56	29	73	200 million/ml 52% prog. motile 7% normal (Optimal)	137 million/ml 51% prog. motile 3% normal (Sub-optimal)	Partial sample loss in summer/autumn semen sample; Spontaneous conception prior to summer/autumn sample.
87	40	90	127 million/ml 65% tot. motile 5% normal (Optimal)	3.5 million/ml 41% tot. motile 3% normal (Sub-optimal)	History of poor fertilisation with IVF

Table 43: Serum vitamin D levels and semen parameters in two participants with change in semen quality (optimal to suboptimal) from Winter/Spring to Summer/Autumn

(prog motile – progressive motile; tot.motile – total motility)

7.7.4 Vitamin D and IVF/ICSI outcome

Ten participant couples undertook 2 cycles of treatment Winter/Spring and Summer/Autumn over the period of the study (Figure 14). 2 of these men were deficient in vitamin D and received vitamin D supplementation. 1 couple had a history of poor fertilisation with IVF in their first cycle and therefore had ICSI in the subsequent cycle. The remaining participants had similar treatment (i.e. IVF or ICSI) in both cycles. As has been shown, 25(OH)D levels [nmol/l; Median (25th -75th centiles)] increase significantly from Winter/Spring to Summer/Autumn [52 (28-61.5) vs.82 (42.7-96.5) p=0.03)].

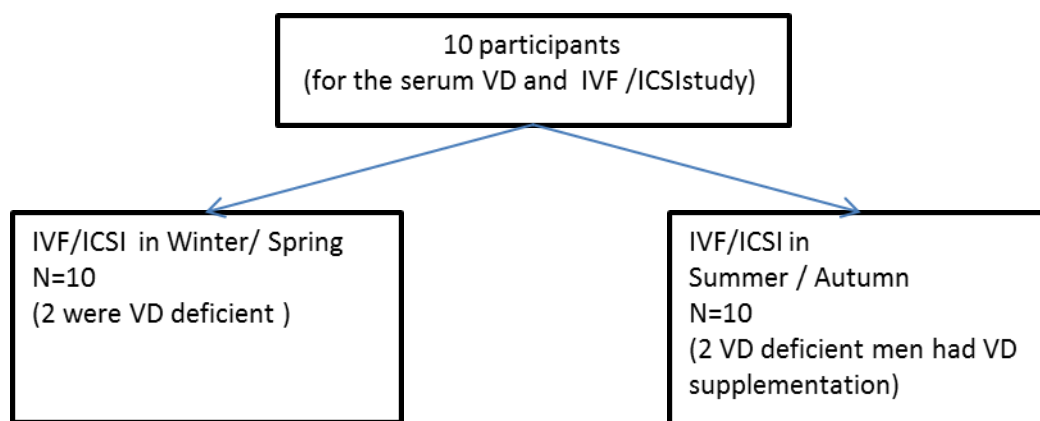


Figure 14: Participant's flow chart for vitamin D (VD) and IVF/ICSI study

There was no significant difference in the fertilisation rates and embryo quality with the rise of serum vitamin D levels in the male partners. These results were similar even after excluding the one participant where treatment changed from IVF to ICSI in the second cycle (results not shown).

There were 2 pregnancies (both missed miscarriages) in Winter/Spring treatment cycles, whilst the Summer/Autumn treatment cycles resulted in 1 viable pregnancy. One couple in each season were able to freeze spare embryos. Therefore, as expected there was no significant difference in the pregnancy rates and freeze rates between the two groups of treatments carried out in Winter/Spring and Summer/Autumn. These results are shown in
in
Table **44** and Table 45.

Variables N=10 Median (25 th -75 th centiles)	Winter / Spring	Summer / Autumn	Wilcoxon Signed Rank test P value
Vitamin D (nmol/l)	52.0 (28.0-61.5)	82.0 (42.7-96.5)	p=0.03 (Mann Whitney test)
No: of mature oocytes	5.0 (2.0-9.7)	6.0 (4.0-9.0)	0.9
Total no. oocytes fertilised	4.0 (1.7-9.2)	5.0 (3.0-7.5)	0.9
Fertilisation rate (%)	81.5 (73.0-100.0)	91.5 (65.7-100.0)	0.8
No. embryos normally fertilised	3.5 (1.5-4.7)	3.5 (2.0-5.5)	0.7
% normally fertilised	77.5 (50.0-100.0)	69.0 (41.2- 75.7)	0.4
No. top quality embryos	0 (0-0)	0 (0-1)	0.5
% of top quality embryos	0 (0-0)	0 (0-11)	0.3
No. of good quality embryos	1.0 (0.0-2.0)	1.0 (0.0-2.2)	0.8
% of good quality embryos	27.0 (0.0-45.5)	29.0 (0.0-52.5)	0.9
No. of slow quality embryos	0.5 (0.0-2.2)	0.0 (0.0-1.0)	0.2
% of slow quality embryos	16.5 (0.0-69.0)	0.0 (0.0-16.7)	0.1
No. of other quality embryos	1.0 (0.0-3.0)	2.0 (1.0-3.0)	0.4
% of other quality embryos	37.5 (0.0-81.2)	50.0 (28.0-76.2)	0.7

Table 44: Comparison of fertilisation and embryo quality between Winter/Spring and Summer/Autumn

Variables n=10	Winter/ Spring	Summer/ Autumn	McNemar test P value
Pregnancy (pregnant / non pregnant)	2/8	1/9	1.0
Embryos for freezing (yes / no)	1/9	1/9	1.0

Table 45: Comparison of pregnancy and embryo freezing rates between Winter/Spring and Summer/Autumn

7.8 Discussion

In this chapter, we have investigated the association between serum vitamin D and semen quality (not individual semen parameters), the variation in semen quality with vitamin D supplementation and also with the seasonal rise in serum vitamin D levels. The association between the male partner's serum vitamin D levels and IVF outcomes were also evaluated.

Semen quality was not significantly different among the vitamin D groups. This is similar to the finding in the previous chapter (Chapter 6, section 6.7.2.5) where there was no association between serum vitamin D and individual semen parameters. In cross sectional studies investigating the association between serum vitamin D and semen parameters, despite the varied potential associations such as a positive association (Blomberg Jensen *et al.*, 2011; Blomberg Jensen *et al.*, 2012; Yang *et al.*, 2012) or a 'U' shaped association (Hammoud *et al.*, 2012) or no association (Ramlau-Hansen *et al.*, 2011) suggested, semen parameters were generally normal (WHO 2010 criteria) in men whatever their vitamin D status. Thus it is debateable how clinically relevant such associations may be.

Although a significant negative association was seen between serum vitamin D levels and individual semen parameters with vitamin D supplementation and seasonal rise in vitamin D levels in Chapter 6, there was no significant change in the overall semen quality with the same.

In men with optimal semen quality, the rise or fall of individual semen parameters with vitamin D may not be of much clinical significance as long as the semen parameters are in normal range, however in men with borderline semen parameters, a rise or fall in semen parameters can be very significant as it can change the overall semen quality to optimal or suboptimal respectively and affecting their fertility potential. A double-blinded randomized clinical trial is underway by Blomberg *et al* to investigate the effect of vitamin D supplementation in infertile men, (as their previous cross sectional association study revealed a positive association between vitamin D and semen parameters) and we await the results with interest (Clinical Trials.gov Identifier: NCT01304927). An improvement in the overall semen quality to optimal with vitamin D could potentially be a useful and easy therapeutic option in the management of idiopathic oligospermia, however our results do not support the use of this intervention.

In individual participants where a variation in semen quality (optimal to suboptimal or vice versa) was seen, this seems mainly due to change in sperm morphology. In participants with semen quality deterioration in their subsequent samples, it was interesting to note that there was history of poor fertilisation in three participants and of semen samples not preparing well for IVF necessitating ICSI, in one participant. It is important to note that researcher MG was blinded to the results of first semen analysis results until the second semen analysis was performed.

There was no significant difference in the fertilisation rates, quality of embryos, pregnancy rates and embryos for freezing between the two treatments carried out in Winter/Spring and Summer/Autumn even though serum vitamin D levels increased significantly in the male partner. This could be because the processes involved in assisted reproduction, such as sperm washing and preparation may eliminate the effects of vitamin D on sperm function. However there are no human studies as yet investigating the association of male partner's vitamin D levels with natural conception rates or outcomes of assisted reproduction and our study will contribute to the literature regarding the same for future.

7.9 Limitations of the study

To investigate the association of serum vitamin D in men and outcome of IVF treatment (i.e. fertilisation rates, quality of embryos, pregnancy rates and rates of cryo preservation), female partner's demographics such as age and ovarian reserve are essential. Although this information is lacking in our study, we conducted paired test analysis where the woman acted as her own control and therefore eliminated the need to adjust for female variables. Whilst a similar seasonal variation of vitamin D in female partners is likely, possibly supporting the lack of any seasonal differences in treatment outcome, assessing their vitamin D status would have added to our understanding of the impact of vitamin D on reproduction.

The study size (124) was small and particularly the subset investigating the IVF outcomes (n=10) and therefore we advise caution in interpreting these results.

7.10 Conclusion

There was no association between serum vitamin D status and semen quality (as per the WHO 2010 criteria). Furthermore the semen quality remained unchanged with vitamin D supplementation or the seasonal rise in serum vitamin D levels. A rise in male

partner's serum vitamin D levels did not affect the IVF outcome in their female partner. Over all, this suggests vitamin D may not have a role in improving sperm function.

Chapter 8 Discussion

A national problem of sperm donor shortage leading to the cessation of donor sperm treatment services in some centres was recognised by the BFS in their Working Party report in 2008 (Hamilton *et al.*, 2008a). At NFC, over the past few years increasing numbers of patients have had to wait over a year for donor sperm treatments.

My study examining the donor programme at NFC from 2000 to 2010, to investigate if there was a true problem of donor sperm shortage, revealed a significant change over this time; with fewer donors recruited and a smaller pool of donors leading to fewer patients receiving treatment; a decline in the number of donor insemination cycles undertaken accompanied by rising donor IVF cycle numbers; increasing sperm imports and a longer waiting time for treatments (Chapter 3).

There is a lack of published evidence in the literature about the experience of individual fertility centres in UK over the past decade; therefore information has been retrieved from the web news (Bionews) (Adams *et al.*, 2006; Ahuja, 2008; Wardle, 2008) and surveys (BBC survey, 2006; British Fertility Society survey, 2006) to understand if there has been a similar problem with donor sperm shortages at other fertility centres. In addition national data is provided by the HFEA giving some insight into the issues although may in part be falsely reassuring.

In the 1990s, the decline in the number of donor sperm treatment cycles tallied with increasing number of ICSI cycles, however this decline continued even after ICSI was well established in 2000 (Pacey, 2010). The possibility that the loss of anonymity in 2005 contributed to the current shortage of donors has been extensively argued with contradicting views, but is confounded by the fact that the decline in donor recruitment was reported even before that came into effect (Pike and Pacey, 2005; BBC survey, 2006; British Fertility Society survey, 2006; Witjens, 2007; Blyth and Frith, 2008; Wardle, 2008; Tomlinson *et al.*, 2010). Interestingly however, HFEA data shows a rise in the number of sperm donors from 2005 and the numbers of patients treated / treatment cycles undertaken from 2007 (HFEA, 2011c; HFEA, 2011d).

It is estimated that approximately 4000 patients need donor sperm treatment per year in UK and that 500 sperm donors are needed to meet this demand (Hamilton *et al.*, 2008a). In 2010, 480 sperm donors were recruited, however the number of patients treated was

less in 2010 than in 2004 (2960 vs. 3712 patients) when there were only 239 donors, suggesting fewer patients per donor are being treated. Furthermore even though the number of sperm donors in 2010 (480) are near enough to the estimated need (500), a shortage of sperm donors nationally is evident by increasing waiting times for treatment, sperm import, and the numbers of patients travelling abroad for treatment (Hamilton, 2010; Pennings, 2010; Shenfield *et al.*, 2010; Culley *et al.*, 2011; Hudson *et al.*, 2011). It is suggested that the rise in donors is due to an increase in overseas and known donors (Hamilton, 2010; Tomlinson *et al.*, 2010) and is supported by HFEA data confirming the rise in overseas donors from 11% in 2005 to 24% in 2010 (HFEA, 2011e) and 16% of newly registered sperm donors in 2008 limiting their donation to one family (HFEA, 2011b). This improvement in sperm donor recruitment nationally is therefore an artefact since such donors benefit fewer patients overall.

My data shows a rise in the proportion of D-IVF treatments to total donor sperm treatments, similar to the national HFEA data (HFEA, 2011c). The rise at our centre can be explained not only by strategies to improve the efficiency of donor sperm treatment, as has been suggested previously in the literature (Pike and Pacey, 2005; British Fertility Society survey, 2006), but also the increase in NHS funding for IVF treatment from two to three cycles in the majority of primary care trusts in the North East region. Hence there is an actual and proportional rise in D-IVF.

Despite the absence of published evidence, it is felt that many fertility centres are struggling to recruit sufficient sperm donors across the UK to meet demand and certainly the encouraging HFEA data do not reflect the experience of many centres including our own (Wardle, 2008; Hamilton and Pacey, 2008b). However it is certainly not universal, as some centres continue to be successful in the current framework (Adams *et al.*, 2006; Ahuja, 2008; Tomlinson *et al.*, 2010).

In my opinion the increase in waiting times for treatment, sperm import and the request for known sperm donations seen at our centre reflects the sperm donor shortage following the abrupt loss of our large pool of anonymous sperm donors with removal of donor anonymity and the subsequent decline in sperm donor recruitment.

One solution to the problem would be to revoke the legislation relating to donor anonymity. However the experience from other countries that moved from anonymous

to open donation has shown that the change did not negatively affect donor recruitment (Van den Akker, 2006; Blyth and Frith, 2008) although some recent reports have been conflicting (Ernst *et al.*, 2007; Ekerhovd and Fauruskov, 2008). Furthermore a sperm donor shortage is also seen in countries where donor anonymity is protected (Blyth and Frith, 2008) suggesting that removal of anonymity may not be only reason for the shortage. Even in countries where anonymous donation is allowed, a shift towards increasing numbers of non-anonymous donors is seen (Scheib and Cushing, 2007) suggesting a change in trend towards open donation and increasingly more countries are now adopting open donation. Therefore I would not support this stance. A review in 2008 of the regulations regarding revoking the donor anonymity did not receive much support for change (Hamilton, 2010).

Despite the fact that arguments for and against donor anonymity are still contentious, the donor conceived children's rights to know the truth and their genetic origins have prevailed as it was deemed in their best interests. The long term psychosocial impact of removing donor anonymity on donor conceived children, donors and parents is unknown although, the initial research results reveal that majority of the contact experiences are positive (Freeman *et al.*, 2009; Jadvá *et al.*, 2011).

If the centre succeeds in reverting to donor recruitment levels similar to that seen prior to the removal of anonymity (40 in 10 years i.e. about four per year) (Paul *et al.*, 2006), it would still take at least ten years to build the donor bank to allow optimal choice and immediate treatment for patients. This is a problem which will take years to recover from unless other measures are taken to improve the sperm donor recruitment. This is vital so that the patients meanwhile do not suffer from the lack of treatment and an opportunity to create a family.

We have seen that only 3.6% of potentially interested men are finally released as sperm donors (Paul *et al.*, 2006). Whilst many default in the early stages, the commonest cause of rejection of sperm donors is sub-optimal semen quality (Paul *et al.*, 2006; Hamilton *et al.*, 2008a). However, the notion of sub-optimal semen quality is poorly understood. Some external factors that may influence semen quality such as season and vitamin D have been widely studied in the literature and have been considered further in this project. A better understanding of their impact on semen quality (if any) may be of

value in improving the both efficiency of the sperm donor programme and of donor recruitment.

Seasonal variation in semen parameters although variable and inconsistent has been demonstrated mostly in the Northern hemisphere (Levine, 1999) and generally in temperate climates rather than tropical climates (Chia *et al.*, 2001). Studies from the UK are sparse, however a study done nearest to our centre geographically, in Edinburgh revealed a pattern of improved sperm counts in Winter and Spring (though not statistically significant) (Mortimer *et al.*, 1983). The retrospective database review of all the semen analyses performed at NFC from men attending for infertility work-up between December 2006 and November 2009 to investigate the seasonal variation in semen parameters, revealed sperm morphology to be the only parameter significantly better in Winter and Spring compared with Summer and Autumn, but there was no significant seasonal variation in other semen parameters (Chapter 4). The actual improvement in sperm morphology however was low and of debateable clinical significance.

In the following Chapter (5) however, a longitudinal investigation of the seasonal variation in semen parameters of 32 sperm donors used for DI treatments between 2005 and 2010 revealed significant seasonal variations in semen parameters with semen volume being significantly higher in Spring compared to Winter. Sperm concentration parameters (sperm concentration, total motile and progressive motile) were all significantly higher in Winter and Spring compared with Summer and Autumn and the motility (progressive and total) parameters appeared to be lowest in Autumn.

Interestingly there are major differences in the results on seasonal variation in semen parameters between the retrospective patient and the longitudinal donor studies. Our longitudinal study showed greater seasonal variation in semen parameters compared to the retrospective study. The study group in the retrospective study comprised men undergoing preliminary fertility work-up whilst the cohort study consisted of donors with proven or likely fertility. Our retrospective study included sub-optimal semen samples (601 samples with count less than 15million/ml i.e. 14.7 %; 701 samples with total motility less than 40 i.e.17.2%) whilst in the cohort study despite some semen donors having had a few sub-optimal semen samples (3 samples with count less than 15million/ml i.e. 0.2 %; 12 samples with total motility of less than 40 i.e.1%), the

majority were normal. Although the cohort study was not as large as the retrospective study (4067 samples), it was still of considerable size with 1108 semen samples and had the advantage of accommodating for intra-individual variation (as the samples were from 32 donors only with an average of 34 semen samples from each donor). Of note, there was no semen analysis methodology related bias between the studies.

Most longitudinal studies of seasonal sperm parameters reveal a lack of seasonal variation (Mallidis *et al.*, 1991; Ombelet *et al.*, 1996; Carlsen *et al.*, 2004), but not all (Levine *et al.*, 1990; Levine *et al.*, 1992). Similar to our study, seasonal variation was noted more in sperm donors (sperm count, motility) than in men attending infertility clinics (only sperm morphology) suggesting that the possible pathology in the ‘suspected infertile’ male may not be affected by the season to the same extent (Centola and Eberly, 1999). Levitas *et al.* (2013) studied the seasonal variation in normozoospermic samples (≥ 20 million/ml) and oligozoospermic samples independently and by way of confirmation it has shown that seasonal variation was more marked in normozoospermic samples compared to oligozoospermic samples.

However other studies conducted on men attending fertility clinics did show seasonal variation (Andolz *et al.*, 2001; Chen *et al.*, 2003) and that seasonal changes in semen parameters were not restricted to semen donors (Saint Pol *et al.*, 1989; Gyllenborg *et al.*, 1999) or men attending infertility clinics, but were also seen in volunteers from the general population (Levine *et al.*, 1990; Levine *et al.*, 1992), men prior to vasectomy (Tjoa *et al.*, 1982; Sobreiro *et al.*, 2005) and subfertile men (Politoff *et al.*, 1989).

At NFC (in the Northern hemisphere with a temperate climate), the seasonal variation noted in donor semen with a deterioration of sperm parameters (sperm count and or motility) in Summer and/or Autumn is similar to the seasonal variation reported in several other temperate climates (Tjoa *et al.*, 1982; Levine *et al.*, 1988; Reinberg *et al.*, 1988; Politoff *et al.*, 1989; Saint Pol *et al.*, 1989; Levine *et al.*, 1990; Levine *et al.*, 1992; Gyllenborg *et al.*, 1999; Andolz *et al.*, 2001; Jørgensen *et al.*, 2001; Chen *et al.*, 2003; Chen *et al.*, 2004; Moskovtsev and Mullen, 2005; Calonge *et al.*, 2009; Levitas *et al.*). In the NFC study however, I also demonstrated seasonal variation in semen volume. The majority of the above studies did not report a seasonal change in semen volume whilst a couple of studies revealed similar findings to our cohort study of increased semen volume in Spring in semen donors (Zhang and Yao, 2010) and also in

men pre-vasectomy (Reinberg *et al.*, 1988). It has to be acknowledged however, that semen volume was missing in 71 of our samples which may have affected the results. The missing data could have led to the false positive finding of seasonal change in semen volume.

Improving the efficiency of the donor programme was one of the main aims of this project. As only thawed sperm is used for donor insemination treatments, it was vital to investigate if the process of freezing and thawing [associated with deterioration of semen parameters / sperm function (Mahadevan and Trounson, 1984; Yogev *et al.*, 2004)] eliminated the seasonality of semen parameters.

In my study, even after thawing, the sperm concentration continued to be significantly better in Winter and Spring (Chapter 5). Similar to our study Yogev *et al.* (2004), reported significantly higher post-thaw progressive motile sperm concentrations in Winter compared with Autumn and Zhang and Yao (2010) reported higher progressive motile sperm counts in Spring. Zhang *et al.* (2012) in their further study revealed that progressive motility recovery rate (defined as % post-thaw progressive motility / % pre-freeze progressive motility x 100%) was lowest in semen samples donated in Summer compared to other seasons.

In my study there was no seasonal variation in post-thaw sperm motility. In Yogev *et al.* (2004) study, there was no seasonal variation in the percentage of post-thaw sperm motility. In that study however, there was no seasonal variation in pre-freeze motility either. Although in our study there was a statistically significant seasonal variation in pre-freeze motility with lower parameters in Autumn, the difference clinically between the seasons was small. As cryopreservation does decrease sperm motility (Sharma *et al.*, 1997; Stanic *et al.*, 2000), this seasonality disappeared after thawing.

Cryopreservation does not adversely affect sperm concentration, therefore the post-thaw sperm concentration parameters continued to be better in Winter and Spring similar to pre-freeze counts and there lies an argument for the seasonal collection of semen samples from sperm donors.

Several studies have shown a correlation between seasonal variation in sperm counts and birth rates (Fisch *et al.*, 1997; Ossenbühn, 1998; Levine, 1999; Levitas *et al.*, 2013);

therefore I investigated the effect of seasonality of sperm production on the chances of conception, to strengthen the argument for seasonal recruitment of sperm donors.

There was no seasonal variation in the conceptions by donor insemination either by season of original sperm production or by season of treatment in 496 DI treatment cycles performed between 2005 and 2010 at NFC (Chapter 5). Few studies in the literature have investigated the seasonal variation in donor conceptions by artificial insemination which reinforces the fact that the seasonal changes in sperm parameters may not be clinically significant and weakens the argument that benefit is to be derived from seasonal donor collections.

A couple of studies have suggested a seasonal variation in donor conceptions in artificial insemination programmes (Paraskevaides *et al.*, 1988; Ronnberg, 1989). However Paraskevaides *et al.* (1988) study was limited by the fact that both fresh and frozen sperm were used and the results were not presented separately for each method. As the highest sperm counts occurred from February to May in their study (and not October to March when the donor conceptions were at highest), the authors suggested that female factors (egg quality / endometrial receptivity) may explain the seasonal variation. Ronnberg (1989), who found a similar increase in conceptions from October to March using only frozen sperm, considered frozen sperm as a constant factor and the contribution by seasonality of sperm quality to the conception was disregarded and it was suggested again that possible variation in female factors explained the seasonal variation in donor conceptions. In contrast to this (but similar to our study), Mayaux and Spira (1989), in a large French epidemiological survey (using only frozen sperm) did not find seasonal variation in donor conceptions. This is the only published study that investigated donor conceptions by the month or season of sperm collection as we have. In this study there was no seasonal variation in donor conceptions even by the season of treatment similar to our study, making us question the role of female factors in seasonal variation of donor conceptions.

As there is significant seasonal variation in donor semen parameters (both pre-freeze and post-thaw) in this study with improved parameters in Winter and Spring (Chapter 5), whilst there may be little clinical significance, it appears to be a reasonable strategy to aim to recruit applicants for sperm donation in these seasons to avoid potential rejection based on sub-optimal semen parameters. Particularly because even though

sperm donors overall had less than 1% of suboptimal semen parameters as per WHO 2010 criteria, approximately 11% of the samples were not suitable for freezing as they did not meet criteria for freezing (Chapter 5, Section 5.7.4). Zhang *et al.* (2012) recommended that semen donation should be encouraged in seasons other than Summer to obtain improved progressive motility recovery rate.

However, importantly there was no seasonal variation in the outcome of the donor conceptions in my study by the season of original sperm production despite Spring being the most common season of sperm donation and most of the treatment cycles carried out with sperm produced in Spring.

The reasons for significant seasonal variation in semen parameters not translating to improved pregnancy rates may be explained by the following couple of studies in addition to several other factors affecting conception. It has been shown that increasing sperm concentration improves the chance of conception but only up to 40 million/ml and a rise beyond that does not increase the likelihood of a pregnancy (Bonde *et al.*, 1998). Despite showing distinct differences in semen parameters between fertile and subfertile men in their study, Guzick *et al.* (2001) found an extensive overlap of fertile and sub-fertile ranges of semen parameters (count/motility/morphology) in both fertile and subfertile men and concluded that none of the semen parameters were powerful predictors of pregnancy.

Interestingly some of the studies which showed a correlation with semen parameters and natural conception and birth rates compared the seasonal variation in those parameters in a cohort of men and correlated this to regional birth rates (Fisch *et al.*, 1997; Ossenbühn, 1998; Levine, 1999; Levitas *et al.*, 2013). However in our study we investigated the seasonal variation in the artificial insemination conception rates and also the semen quality of the semen donors, whose sperm was used directly for those treatments, providing a potentially stronger link.

Even though there is a seasonal difference in donor semen parameters but not donor conceptions, I do not believe that it is worthwhile restricting recruitment of sperm donors to certain seasons, particularly during these times of sperm donor shortage. Clearly donor convenience also overrides seasonal collection of semen samples as we have no strong argument in favour the latter.

In humans, a wide expression of VDR and vitamin D metabolising enzymes in the male reproductive tract (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2010; Foresta *et al.*, 2011) along with *in vitro* studies revealing that vitamin D enables sperm capacitation, improves sperm motility, survival and induces the acrosome reaction (Aquila *et al.*, 2008; Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011) suggests a role of vitamin D in male reproduction.

We investigated the correlation between semen parameters and serum vitamin D in a cross sectional association study and also a cohort study (which is the first ever longitudinal study undertaken) to examine the change in semen parameters with a rise in serum vitamin D level, both with seasonal rise (in vitamin D non-deficient participants) and vitamin D supplementation (in vitamin D deficient participants).

There was a significant rate of vitamin D deficiency in men attending NFC. The prevalence of vitamin D deficiency in Winter and Spring was almost three times the UK national prevalence of 16% (Hyppönen and Power, 2007). Even though the prevalence of hypovitaminosis D increases as one moves north in the UK (Hyppönen and Power, 2007), the prevalence in our study is higher than that reported regionally (Hyppönen and Power, 2007) and persisted even after excluding our 12% high risk non-Caucasian participants. Whether men attending fertility clinic are also a specific risk group for hypovitaminosis D needs further study.

In the cross sectional association study there was no significant difference in the various semen parameters between vitamin D groups, although the numbers of men with adequate (15) and optimal levels (6) of vitamin D were low. These low numbers were unexpected as the high prevalence of hypovitaminosis D in the Winter/Spring period when participants were recruited was unanticipated.

Sperm counts and morphology were lower in the optimal (>75nmol/l) vitamin D group compared to other three groups but this did not reach statistical significance. These findings are similar to the cross sectional association study by Ramlau-Hansen *et al.* (2011), which showed no association between serum vitamin D levels and semen parameters. The authors, similar to our study suggested that serum vitamin D deficiency (<25nmol/l) is not associated with reduced semen quality. In another small study

recruiting 90 men attending an infertility clinic, there was no correlation between vitamin D and semen parameters (Knopf *et al.*, 2011)

Conversely, in another association study of Danish men, there was a weak positive but significant association between serum vitamin D level and sperm motility. However there was no association between vitamin D and semen volume, sperm count and sperm morphology (Blomberg Jensen *et al.*, 2011).

In our cohort study, sperm count, motility and morphology deteriorated significantly with increased serum vitamin D levels secondary to vitamin D supplementation and also seasonal rise. There is some evidence that higher serum vitamin D levels are associated with lower sperm parameters. In Ramlau-Hansen *et al.* (2011) study, it appeared that men with high vitamin D (i.e. between 94- 227nmol/l in their study) had lower sperm counts and a lower percentage of normal sperm morphology, but after transforming the data and adjusting for the confounders, as mentioned previously they found no association between serum vitamin D and semen parameters.

In another association study by Hammoud *et al.* (2012), sperm concentration, progressive sperm motility, sperm morphology and the total progressive motile sperm count were lower in men with high concentrations of vitamin D, but this group included only men with a serum vitamin D level above 125nmol/l. Unlike our study, they also found worse sperm parameters (total progressive motile sperm count and total sperm count) in men with a serum vitamin D of less than 50nmol/l. They suggested a 'U' shaped association between vitamin D levels and semen parameters, similar to the association seen between vitamin D and some non-skeletal conditions such as cardiovascular disease and pancreatic cancer (Ross *et al.*, 2011; Rosen *et al.*, 2012).

Male factor subfertility was seen in 43% of our study participants and may have had a significant impact on the results as it was shown that subfertile men have a lower expression of VDR and vitamin D metabolizing enzymes in testis and sperm compared to normal men (Aquila *et al.*, 2009; Foresta *et al.*, 2011; Blomberg Jensen *et al.*, 2012) and this may then contribute to the lack of improvement in the semen parameters with rising vitamin D levels. In *vitro* experiments conducted by Blomberg *et al* revealed that 1,25(OH)₂D increased sperm motility in young men but not in sub-fertile men (Blomberg Jensen *et al.*, 2012).

However cross sectional association studies including fertile and subfertile men surprisingly still revealed a positive association between vitamin D and semen parameters. Blomberg Jensen *et al.* (2012) in their study of 70 men (involving subfertile and young men), demonstrated that progressive sperm motility was significantly higher in the vitamin D non-deficient group (>25nmol/l) compared to the vitamin D deficient group. Similarly, in a recent large cross sectional association study by Yang *et al.* (2012) again a significant positive association between serum vitamin D and sperm motility and also sperm morphology was seen in the subfertile men group. Surprisingly, in fertile men where a more positive association is anticipated because of the abundance of receptor expression, this positive association was seen only between vitamin D and sperm motility and not sperm morphology.

Vitamin D inactivating enzyme (CYP24A1) expression in sperm has been shown to correlate positively with semen parameters and distinguish young men from subfertility clinic recruits, however as serum vitamin D was seen to be similar in men with low and high CYP24A1 expression, it was suggested that the local vitamin D metabolism may influence CYP24A1 expression in sperm and may be more significant than systemic vitamin D levels (Blomberg Jensen *et al.*, 2012). We did not check for this in our study. Furthermore cellular vitamin D responsiveness is complex and not as tightly regulated as systemic vitamin D metabolism (Blomberg Jensen, 2012).

Overall from the association studies published so far, the association between vitamin D and semen parameters appears to be inconsistent, varying from a positive association (Blomberg Jensen *et al.*, 2011; Blomberg Jensen *et al.*, 2012; Yang *et al.*, 2012) to a 'U' shaped association (Hammoud *et al.*, 2012) or to no association (Knopf *et al.*, 2011; Ramlau-Hansen *et al.*, 2011). Our study has contributed to this uncertainty showing an apparent negative correlation.

Variations in the study participants (healthy volunteers / men attending infertility clinic / fertile and subfertile men), serum vitamin D measurement techniques and vitamin D group stratification (2 /3 /4 groups with different cut offs) may have contributed to the variation in results seen between studies. As spermatogenesis starts about 72 days prior to semen sample collection, it has been suggested that serum vitamin D levels be checked a few months prior to the semen analysis to investigate the true association (Blomberg Jensen *et al.*, 2011).

The association between vitamin D and sperm function (acrosin activity or sperm motility) in *in vitro* studies appears to be dose dependent and importantly with higher concentrations of vitamin D these responses became either ineffective or inhibitory (Aquila *et al.*, 2008; Blomberg Jensen *et al.*, 2011). A possibility of receptor down-regulation with higher levels of vitamin D has been suggested (Aquila *et al.*, 2009). Such phenomena may have contributed to the marked deterioration seen in semen parameters with rising vitamin D in our study, but the effect of other seasonal factors independent of vitamin D cannot be ruled out.

Studies have so far investigated the association of serum vitamin D and individual semen parameters; I further investigated the association between serum vitamin D and semen quality (divided into optimal and sub-optimal based on WHO 2010 criteria) as opposed to individual semen parameters, since a change in the semen quality (from optimal to sub-optimal or vice versa) may be clinically more relevant than an increase or decrease in individual sperm parameters. In my study there was no significant difference in the number of participants with optimal and suboptimal semen quality in each of the four vitamin D groups (deficiency /insufficiency / adequate and optimal) (Chapter 7).

Although a significant negative association was seen between serum vitamin D levels and individual semen parameters with vitamin D supplementation and seasonal rise in vitamin D levels (Chapter 6), there was no significant change in the numbers of participants with optimal and sub-optimal semen quality following vitamin D supplementation and seasonal rise in vitamin D levels [88% (74/84) had unchanged semen quality] (Chapter 7). This means that the impact of vitamin D on individual semen parameters mostly does not change the overall semen quality. In other words for the majority of the participants there were no obvious clinical implications.

An improvement in the overall semen quality to optimal with vitamin D could potentially be a useful and easy therapeutic option in the management of idiopathic oligospermia, however our results do not support the use of this intervention, and we recommend caution, as it could cause a deterioration in semen parameters. A double-blinded randomized clinical trial is underway by Blomberg *et al* to investigate the effect of vitamin D supplementation in subfertile men and we await the results with interest (Clinical Trials.gov Identifier: NCT01304927).

Whilst the assessment of the effects of season and vitamin D on sperm parameters and overall semen quality does not appear to have a clinical impact these are surrogate markers for sperm function. To investigate the hypothesis that a variation in the serum vitamin D in men may alter fertilisation rates and also affect embryo quality, the association between the male partner's serum vitamin D levels and female partner IVF outcomes were evaluated (Chapter 7). Importantly there were no significant differences in the fertilisation rates, quality of embryos, pregnancy rates and numbers of embryos for freezing between the treatments carried out in Winter/Spring and Summer/Autumn even though serum vitamin D levels increased significantly in the male partner. This could be because the processes involved in assisted reproduction, such as sperm preparation, may eliminate the effects of vitamin D on sperm function. However there are no human studies as yet investigating the association of male partner's vitamin D levels with natural conception rates or outcomes of assisted reproduction and our study contributes to the literature regarding this.

Although vitamin D deficiency has been shown to be very common in men attending our centre, it is not associated with poor semen parameters. Based on the results from our preliminary study of the negative association between vitamin D and semen parameters, vitamin D supplementation may be harmful to semen parameters and I believe therefore that it should not be recommended. However vitamin D supplementation for other health reasons such as bone health is essential and should continue. The negative association seen in our study is intriguing and warrants further research to clarify the mechanism.

Interestingly the vitamin D study results (Chapter 6) are consistent with our seasonal variation in semen parameters (Chapter 5) which revealed improved parameters in Winter/Spring when serum vitamin D levels are lower than Summer/Autumn. Therefore I wonder if other effects of season (by the changes in temperature and photoperiod, although not proven; Chapter 1 Section 1.3) were more important to semen parameters, overwhelming any influence of vitamin D. This could be clarified by a study in which participants are recruited throughout the year and followed up throughout the following year as opposed to our study where participants were recruited in Winter/Spring and followed up in Summer/Autumn). This would have the potential advantage of more participants with adequate and optimal levels of vitamin D for comparison and eliminate the effect of season on an individual. Until further research clarifies, I

recommend vitamin D supplementation should not be given specifically for sperm quality. More studies are needed to confirm or refute the negative association seen in our study before such recommendations can be made.

In conclusion I have confirmed that there is a problem of sperm donor shortage at Newcastle Fertility Centre, but to address this I do not recommend the seasonal recruitment of sperm donors to improve the efficiency of the sperm donor programme, or the use of vitamin D supplementation to improve semen quality.

Meanwhile to improve sperm donor recruitment nationally, NGDT continues to raise the awareness of the problem of sperm donor shortages and the HFEA has introduced policies for improved donor compensation allowing a fixed sum of £35 per visit for sperm donors (HFEA, 2011a) from April 2012. The results of the pilot study of national sperm donation service programme are awaited which may provide valuable information to improve sperm donor recruitment and if considered successful, may be an effective solution for the national problem of sperm donor shortage with the most efficient use of the available limited resources. Locally at NFC, the donor selection and recruitment criteria have been addressed to improve sperm donor recruitment; strategies have been introduced to improve the efficiency of the donor sperm use by modifying the laboratory processing of semen samples to obtain more straws per ejaculate and introducing protocols which decrease the utilization of straws, whilst continuing to assist patients in their efforts for donor sperm treatment without compromise to success rates.

Academic Achievements so far from this project

International oral presentations

1. How has the Disclosure of Donor Information act affected the donor programme at a tertiary care fertility centre in the UK?

Presented at 27th Annual Meeting of the European Society of Human Reproduction and Embryology, Stockholm, Sweden; July 2011. *Human Reproduction*: 2011, volume 26, issue supplement 1, Ppi22-124 (doi:10.1093/humrep/26.s1.16). This presentation also attracted the media attention and was published in *Daily telegraph* on 7th July 2011 <http://www.telegraph.co.uk/health/healthnews/8623260/Law-leads-to-18-month-wait-for-fertility-treatment.html>.

2. 'Does a rise in vitamin D level improve semen quality?'

Presented at 28th Annual Meeting of the European Society of Human Reproduction and Embryology, Istanbul, Turkey; July 2012. *Human Reproduction*: 2012, volume 27, issue supplement 2, ii47-ii49.(doi: 10.1093/humrep/27.s2.32)

National oral presentations

1. Is there a correlation between serum levels of Vitamin D and male fertility?

Presented at Fertility 2012, 6th-7th January 2012, Leeds, UK, British Fertility Society Annual meeting. *Human Fertility*, 2012; 15(1): 43

2. Is there a seasonal variation in conceptions by donor insemination?

Presented at Fertility 2013 (8th biennial conference of the UK Fertility Societies), January 2013, Liverpool, UK. *Human Fertility*, 2013; 16(3): 221

Local presentations

1. How has the 'Removal of Donor Anonymity' affected the donor programme at Newcastle Fertility Centre at Life? Presented at Northern Deanery Trainee Prize competition in October 2010 and was awarded best prize.

2. 'Association of vitamin D and semen quality' – presented at North East Fertility Forum NEFF meeting in January 2012.

Poster presentations

1. How has the 'Removal of Anonymity' law affected the Donor Programme at Newcastle Fertility Centre? Presented at Fertility 2011, 5th–7th January 2011, Dublin, 7th biennial conference of the UK Fertility Societies. Posted online, Poster 128, *Human Fertility*, 2011; Early Online, 1–88

<http://informahealthcare.com/doi/abs/10.3109/14647273.2011.575665>

2. 'Seasonal variation of semen quality in a 3 year study' Presented at Fertility 2012, 6th-7th January 2012, Leeds, UK, British Fertility Society Annual meeting. *Human Fertility*, 2012; 15(1): 45

Publication

1. "The Sperm Donor programme over 11 years at Newcastle Fertility Centre" published in *Human Fertility*, December 2013, Vol 16, No: 4, Pages 258-265.

Appendix A: Patient information sheet

Seasonal Variation of vitamin D levels and sperm

parameters (Version number / Date – 02/ 221010)

Information for potential research patients

We would like to invite you to take part in research. Before you decide it is important that you understand why the research is being done and what it will involve. This information sheet is yours to keep. It tells you the purpose of the study and explains what will happen to you if you take part. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Your standard NHS clinical care will not be affected whether or not you decide to help in the study.

What is the purpose of the study?

It is known that vitamin D levels in the blood vary in different seasons due to different amount of sunlight exposure at different times of the year. We also know that there are seasonal variations in number and quality of a man's sperm. This research aims to consider the seasonal variation in Vitamin D levels and its possible effect on sperm characteristics.

What am I asked to do?

We will fill in a questionnaire about your health when you attend the clinic. We are asking you for a blood test for vitamin D levels and a semen sample on 2 occasions about 6 months apart, once in winter/spring and once in summer/autumn. Semen analysis will be done as a part of your standard investigations and this can be used for the study. The blood test (about a tablespoon of blood) can be taken when you attend the clinic to avoid extra visits where possible. Sometimes however you may be asked to attend the unit for a test specifically for research. If you are happy to participate in this research you will need to sign the consent form attached.

What are the possible benefits of taking part?

Occasionally we may find a man with very low vitamin D levels that would lead to softening of bone tissue. We would let you know the results as this would need treatment. There are no direct benefits to you with regards to fertility.

What will happen to me if I take part?

Taking part in the research will not alter your treatment. It will not directly increase or decrease your chances of having a baby. Your visits to the unit to submit tests for the research will be minimised and co-ordinated with your clinical management as much as possible.

What possible problems might occur?

Semen samples

There are no specific problems associated with producing a semen sample although you will be asked to produce it in the unit. You may well have already produced samples in this way. If you are unable to produce a sample at the appointment time don't worry we can rearrange the appointment if necessary or make other arrangements if appropriate.

Blood samples

There is rarely a problem with taking a small blood sample – usually from a vein in your arm. You may experience some discomfort or on occasion some bruising at the site of blood taking but you will be instructed on how best to avoid this at the time.

What will happen to my samples if I take part?

Your samples are a gift to the Newcastle Upon Tyne Hospitals NHS Foundation Trust and will be used solely for the purposes of this research project. Taking part in this research study has no financial benefit to you. Both blood and semen samples will be disposed of after analysis.

Will participating in research affect our treatment?

No. You can be assured that your treatment always comes first and we would not do anything that would compromise your chance of achieving a successful pregnancy.

Data and Results

Will my taking part in research be kept confidential?

All information collected about you during the course of the research will be kept confidential by those involved in the research. The collected data will be kept by the researchers for 6-12 months after the completion of the study.

What will happen to the results of the study?

Whenever possible we will publish the results of our studies in scientific journals. We will also present data at scientific conferences. You will not be identified personally in any way in any publication or presentation.

Will we get any results that we will need to know about?

If the level of the vitamin D is low enough to warrant treatment we will let you know as soon as possible. You can be notified about the final outcome of the research if you wish.

Management of this research

Who is funding the research?

Research is supported by Department of Reproductive Medicine, Newcastle Fertility Centre at Life.

How is the research overseen?

Research is overseen by the Newcastle upon Tyne NHS Hospitals Foundation trust.

What if something goes wrong?

It is highly unlikely anything could go wrong in this study but in the rare event that you are harmed during the research due to someone's negligence, then you may have the grounds for a legal action for compensation against Newcastle upon Tyne hospitals NHS Foundation Trust but you may have to pay your legal costs.

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of the study, the normal NHS complaints procedure are available to you. You could also discuss any issues with PALS (Patient Advice Liaison Service).

Address –

North of Tyne PALS,
The Old Stables,
Grey's Yard,
Morpeth,

NE61 1QD.
Phone – 0800 032 0202
Text – 01670 511260
E-mail – northoftynepals@nhct.nhs.uk

Contact for further information

For further information you can contact Dr.Madhavi Gudipati during 9am to 5pm,
Monday to Friday on 0191 2138213 .

Who is leading the research?

Dr. Madhavi Gudipati,

Clinical Research Fellow,
Newcastle Fertility Centre at Life,
Contact No: - 07771985883.

Dr. Jane Stewart,

Consultant in Reproductive Medicine,
Newcastle Fertility Centre at Life,
Contact No: 0191 2135011.

Mr. Kevin McEleny,

Consultant Andrologist,
Newcastle Fertility Centre at Life
Contact No: 0191 2138223.

Professor Simon Pearce,

Professor of Endocrinology,
Institute of Human Genetics,
International Centre for Life,
Contact No: 0191 2418674.

Appendix B: Questionnaire

CODE

Questionnaire (version number / Date: 02/ 201010)

1. Hosp.No:
2. Age
3. Ethnic Group
4. Occupation
5. Primary / Secondary subfertility
6. Medical problems affecting fertility- Yes / No
7. Febrile illness in the last 3 months- Yes /No
8. Surgical problems affecting fertility – Yes / No
9. STD- Yes / No
10. Alcohol - units/day
11. Smoking- per day
12. Regular medication- Yes / No
13. Recreational drugs/ Steroids – Yes /No
14. Vitamin Supplements containing vitaminD – Yes / No
15. Recent travel to hot countries / southern hemisphere or to tropical places in last 3 months Yes / No

16. Vegetarian – Yes / No

17. Margarine – daily / weekly / less than weekly

18. Oily fish – weekly / less than wkly / never
(salmon / trout / mackerel)

19. Use of sun protection – Usually / sometimes / rare or never

20. Time spent watching TV/ using PC - <2H / 2-3H / >3H per day

21. Outdoor activity in previous month - <1H/ 1-3H / >3H per day

22. BMI >30 – Yes / No

23. Results

Month	Vitamin D	Semen analysis

Comments

Appendix C: Consent form



Seasonal variation in Vitamin D levels and sperm parameters

CONSENT FORM

- I have read and understood the information sheet and the consent form about the study; and have had the opportunity to discuss it.
- I understand that I have to submit blood and semen samples on 2 occasions to take part in the research study.
- I understand that I am under no obligation to take part in this study and that a decision not to participate will not alter any treatment that I would normally receive.
- I am aware that I can withdraw my consent at any time.
- I understand that relevant sections of my medical notes and data collected during the study may be looked at by regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- I consent to be reminded of the test appointment by letter / phone / text message /mail.
- I consent to the storing and processing of data collected about me. I understand the data will be kept for 6 – 12 months after the completion of the study. I understand data will be kept secure and confidential. I am aware that electronic data base will not have any identifying information and is coded.
- I understand that an abnormally low Vitamin D level may be found and am happy for my GP to be informed of this result to arrange replacement treatment.
- I understand that I will not benefit financially from this research and its outcome.
- I want / do not want to be informed of the outcome of the study.

I consent to my participation in the study.

Patient's name -Signature.....Date.....

Clinician's name-.....Signature.....Date.....

Best contact-

CODE-.....

Appendix D: Invitation letter to the participants

a) For participant's with normal vitamin D levels

Head of Department: Professor Alison Murdoch
Consultant in Reproductive Medicine: Dr Jane Stewart
Consultant in Reproductive Medicine: Dr Alka Prakash
Consultant Andrologist: Mr Kevin McEleny
Scientific Director: Dr Mary Herbert
Senior Nurse: Eilis Moody/Jan Dutton

Reference MG

Typed: 24/8/2011

Private & Confidential

Mr. xxxxx
Address

Dear Mr.xxxxx,

Many thanks for participating in the research project 'Seasonal variation in vitamin D and semen parameters'. I am pleased to inform you that we have completed the first phase of the research. Your participation is greatly appreciated.

This is to remind you your next appointment which includes a blood and semen sample from you has been arranged for

Monday 17TH October 2011 at 16.00

I would be very grateful if you could attend. It would be helpful if you could acknowledge this letter to confirm your participation.

If you have routine appointment in this Unit, please let me know so that I can arrange to see you then instead. If you cannot attend please do not hesitate to contact me and I will be able to find a suitable alternate time for you. You may find a weekend or evening appointment more convenient.

You may remember abstinence for 2-7 days prior to producing the semen sample for optimal analysis. You can consider producing the semen sample at home if you live nearby and it suits you to do so. I would be able to arrange this for you.

Your failure to attend the appointment will unfortunately leave the research incomplete and we will be unable to draw any valid conclusions. So I am most grateful for your co-operation.

Kind regards
Madhavi Gudipati
Research registrar
Newcastle fertility centre
Phone – 0191 2138213
E-Mail – madhavi.gudipati@nuth.nhs.uk

b) For participant's with deficient vitamin D levels

Head of Department: Professor Alison Murdoch

Consultant in Reproductive Medicine: Dr Jane Stewart

Consultant in Reproductive Medicine: Dr Alka Prakash

Consultant Andrologist: Mr Kevin McEleny

Scientific Director: Dr Mary Herbert

Senior Nurse: Eilis Moody/Jan Dutton

Reference MG

Typed:

Private & Confidential

Mr.

Address

Dear Mr.

Many thanks for participating in the research project 'Seasonal variation in vitamin D and semen parameters'. I am pleased to inform you that we have completed the first phase of the research. Your participation is greatly appreciated.

This is to remind you your next appointment which includes a blood and semen sample from you has been arranged for

Friday 14th October 2011 at 14.30

I would be very grateful if you could attend. It would be helpful if you could acknowledge this letter to confirm your participation. The repeat blood test will help confirm that your vitamin D levels have returned to normal with treatment.

If you have routine appointment in this Unit, please let me know so that I can arrange to see you then instead. If you cannot attend please do not hesitate to contact me and I will be able to find a suitable alternate time for you. You may find a weekend or evening appointment more convenient.

You may remember abstinence for 2-7 days prior to producing the semen sample for optimal analysis. You can consider producing the semen sample at home if you live near by and it suits you to do so. I would be able to arrange this for you.

Your failure to attend the appointment will unfortunately leave the research incomplete and we will be unable to draw any valid conclusions. So I am most grateful for your co-operation.

Kind regards

Madhavi Gudipati

Research registrar

Newcastle fertility centre

Phone – 0191 2138213

E-Mail – madhavi.gudipati@nuth.nhs.uk

Appendix E: NEQAS calculations and results interpretation

Definitions from the NEQAS participants handbook

ALTM - All laboratory trimmed mean, this is the geometric mean of the entire set of trimmed results for a specimen.

MRTM - Method related trimmed mean is a geometric mean of 'trimmed' results from one methodology.

Bias - The difference between your result and the designated value expressed as a percentage.

BIS - The bias divided by a chosen co-efficient of variation (CCV) multiplied by 100.

CCVs are selected to give more leeway to lower designated values so as better to reflect clinical relevance. Values can either be positive or negative.

Calculations:

$$\text{Bias (\%)} = \left(\frac{\text{(Your result - NEQAS designated value)}}{\text{NEQAS designated value}} \right) \times 100$$

$$\text{Bias Index Score (BIS)} = \frac{\text{Bias (\%)}}{\text{CCV}} \times 100$$

Chosen Co-efficient of Variation (CCV)

Sperm Motility

NEQAS designated value	CV
<10	0.0
10<20	0.0
20<30	25.0
>30	0.0

Sperm Concentration

NEQAS designated value	CCV
<15	50.0
15<30	25.0
>30	20.0

Interpretation of results

Sperm Motility

If BIS is $\geq +100$ or ≤ -100 for at least 2 out of the 3 parameters in at least 3 out of the 4 samples = unsatisfactory performance.

Concentration

If BIS is $\geq +100$ or ≤ -100 for at least 3 out of the 4 samples = unsatisfactory performance.

Sample Morphology

If result is $>$ twice the NEQAS designated value for at least 3 out of the 4 samples = unsatisfactory performance.

Online Morphology

Not yet defined

For each assessment (motility, concentration, morphology), if this happens on three distributions within the last eight distributions, the laboratory will be contacted as a persistent unsatisfactory performer.

Reference: UK NEQAS, Participants' Handbook for Reproductive Science Schemes; www.cmft.nhs.uk/media/227268/ph%202012.pdf

Appendix F: Training check list

Training Checklist: Routine Semen Analysis

Name: _____

Note: Continue until all assessments completed accurately on 5 consecutive samples.

Date: ____/____/____

Sample Details: GP / NFC

Workstation set up correctly Y / N

Sample acceptance process followed Y / N

Liquefaction process assessed correctly Y / N

Sample volume measured accurately Y / N

pH test performed appropriately: Y / N

Slides loaded correctly:

Glass slide: Y / N

Microcell: Y / N

Concentration assessment (Neubauer):

Initial dilution determined correctly Y / N

Loading of C-Chip performed correctly Y / N

Number of squares counted determined correctly Y / N

Final total concentration determined correctly (<10% variation) Y / N

Trainee result: _____ M/ml Trainer result: _____M/ml

Comments (eg if greater than > 10% variation)

Motility / Morphology assessment:

Classification	Trainee result (%)	Trainer result (%)
Progressive sperm		
Non-Progressive sperm		
Immotile sperm		
Normal sperm		
Abnormal sperm		

Comments (eg main types of abnormalities / if greater than > 5% variation between operator results)

Other observations:

Accurate recognition of need for specialist processing Y / N
 (Log any tests performed on Lab-RF/Staff/44 - Specialist testing of semen samples training checklist)

Post analysis procedures followed correctly:
 Reporting procedure Y / N
 Disinfection procedure Y / N

Competency demonstrated throughout process Y / N

Full competency demonstrated (on 5 consecutive occasions) Y / N

(Concentration, motility and morphology results can be collated using Lab-RF/Staff/12 for future reference)

Trainer's signature _____

Date ____/____/____

Appendix G: NEQAS: My accreditation paper work

The details of my participation in the quality control process with the results are described below. The distribution (Semen samples) number with the month/ year of assessment is as follows

Distribution-68: Feb 2011

Distribution-69: May 2011

Distribution-70: Aug 2011

The detailed assessment with BIS and BIAS scores are shown in the table format, however the results are described before-

Internal QC:

In distributions 69+70 where internal QC was done, I did not have any answers which lie > 2 SD away from the mean (of all NFC staff that took part), this was the critical level, rather than 1 SD.

External QC:

Distribution 68: Assessment of DVD motility and morphology were acceptable but Sperm concentration revealed unsatisfactory performance.

Distribution 69: Assessment of all parameters was to acceptable standards.

Distribution 70: Assessment of DVD motility, concentration and morphology were acceptable; however the online motility assessment showed unsatisfactory performance.

Distribution 68 Feb 2011			EQA	Red fill = BIS > +100 or < -100	
DVD sperm motility			If BIS is out for 3+ out of 4 parameters in 3+ out of 4 samples = unsatisfactory performance		
Sample	Molality %	MG			
	MO245	a	23.3	NEQAS Designated Values	CCV
		b	28.2		
		c	9.7		
d		38.8			
Bias	a	-7.9	25.31	25.0	
	b	33.1	21.18	25.0	
	c	-12.7	11.11	30.0	
	d	-8.1	42.2	20.0	
BIS	a	-31.8			
	b	132.6			
	c	-42.3			
	d	-40.4			
Sample	Molality %	MG			
	MO246	a	33.3	NEQAS Designated Values	CCV
		b	41.2		
		c	7.8		
d		17.6			
Bias	a	-10.2	37.09	20.0	
	b	64.3	25.08	25.0	
	c	-42.9	13.66	30.0	
	d	-24.8	23.4	25.0	
BIS	a	-51.1			
	b	257.1			
	c	-143.0			
	d	-99.0			
Sample	Molality %	MG			
	MO247	a	25.5	NEQAS Designated Values	CCV
		b	35.5		
		c	13.1		
d		26.2			
Bias	a	5.1	24.27	25.0	
	b	3.6	34.27	20.0	
	c	-7.6	14.17	30.0	
	d	-1.1	26.5	25.0	
BIS	a	20.3			
	b	17.9			
	c	-25.2			
	d	-4.2			
Sample	Molality %	MG			
	MO248	a	64.3	NEQAS Designated Values	CCV
		b	12.1		
		c	3.8		
d		19.7			
Bias	a	6.0	60.66	20.0	
	b	32.2	9.15	40.0	
	c	-17.0	4.58	40.0	
	d	-22.9	25.6	25.0	
BIS	a	30.0			
	b	80.6			
	c	-42.6			
	d	-91.6			

Distribution 68 Feb 2011		EQA	Red fill = BIS > +100 or < -100		
			If BIS is out in 3+ out of 4 samples = unsatisfactory performance		
Sample sperm concentration					
Sample	MG	NEQAS Designated Values	CCV		
S269	29	24.58	25.0		
Bias	18.0				
BIS	71.9				
Sample	MG	NEQAS Designated Values	CCV		
S270	134.5	55.84	20.0		
Bias	140.9				
BIS	704.3				
Sample	MG	NEQAS Designated Values	CCV		
S271	91.25	74.03	20.0		
Bias	23.3				
BIS	116.3				
Sample	MG	NEQAS Designated Values	CCV		
S272	35.4	22.74	25.0		
Bias	55.7				
BIS	222.7				

Distribution 69 May 2011			QC	Grey text = Outside 1 SD		Blue fill = Outside 2 SD		
DVD sperm motility			EQA	Red fill = BIS > +100 or < -100				
If BIS is out for 2+ out of 3 parameters in 3+ out of 4 samples = unsatisfactory performance								
Sample	Motility %	MG			± 1 SD		± 2 SD	
			Mean	SD	Lower	Upper	Lower	Upper
MO249	PR	52.7						
	NP	5.5	45.7	5.0	40.7	50.8	35.7	55.8
	IM	41.8	12.5	4.6	7.9	17.1	3.3	21.7
MO250	PR	94	41.8	5.9	35.9	47.7	30.1	53.5
	NP	0.5	90.6	2.7	87.9	93.3	85.3	95.9
	IM	5.5	2.3	1.0	1.3	3.3	0.3	4.3
MO251	PR	47.4	7.1	2.0	5.2	9.1	3.2	11.1
	NP	8.3	55.0	7.9	47.0	62.9	39.1	70.9
	IM	44.3	10.9	4.4	6.6	15.3	2.2	19.6
MO252	PR	33.9	34.1	8.5	25.6	42.5	17.1	51.0
	NP	23.1	36.2	7.9	28.3	44.1	20.4	52.1
	IM	43	23.5	5.9	17.5	29.4	11.6	35.3
MO249	PR	52.7	40.3	9.9	30.4	50.2	20.6	60.0
	NP	5.5						
	IM	41.8						
Bias	PR	22.3	NEQAS Designated Values		CCV			
	NP	-41.8	43.08		20.0			
	IM	-12.1	9.45		40.0			
BIS	PR	111.7	47.57		20.0			
	NP	-104.5						
	IM	-60.6						
MO250	PR	94.0						
	NP	0.5						
	IM	5.5						
Bias	PR	7.1	NEQAS Designated Values		CCV			
	NP	-84.0	87.80		20.0			
	IM	-38.6	3.12		40.0			
BIS	PR	35.3	8.96		40.0			
	NP	-209.9						
	IM	-96.5						
MO251	PR	47.4						
	NP	8.3						
	IM	44.3						
Bias	PR	-6.7	NEQAS Designated Values		CCV			
	NP	-19.8	50.83		20.0			
	IM	14.7	10.35		30.0			
BIS	PR	-33.7	38.62		20.0			
	NP	-66.0						
	IM	73.5						
MO252	PR	33.9						
	NP	23.1						
	IM	43.0						
Bias	PR	-2.6	NEQAS Designated Values		CCV			
	NP	13.0	34.82		20.0			
	IM	-3.0	20.44		25.0			
BIS	PR	-13.2	44.34		20.0			
	NP	52.1						
	IM	-15.1						

Distribution 69 May 2011		IQC	Grey text = Outside 1 SD	Blue fill = Outside 2 SD				
		EQA	Red fill = BIS > +100 or < -100					
If BIS is out for 3+ out of 4 samples = unsatisfactory performance								
Sample sperm concentration								
					± 1 SD		± 2 SD	
Sample	MG	Mean	SD	Lower	Upper	Lower	Upper	
S273	69.5	82.4	20.3	62.1	102.7	41.8	122.9	
S274	6	8.5	2.8	5.7	11.4	2.9	14.2	
S275	36	46.9	14.1	32.8	61.0	18.7	75.1	
S276	31.5	32.2	3.2	29.0	35.3	25.8	38.5	
Sample	MG	NEQAS Designated Value	CCV					
S273	69.5	78.39	20.0					
Bias	-11.3							
BIS	-56.7							
Sample	MG	NEQAS Designated Value	CCV					
S274	6.0	9.52	50.0					
Bias	-37.0							
BIS	-73.9							
Sample	MG	NEQAS Designated Value	CCV					
S275	36.0	42.60	20.0					
Bias	-15.5							
BIS	-77.5							
Sample	MG	NEQAS Designated Value	CCV					
S276	31.5	33.59	20.0					
Bias	-6.2							
BIS	-31.1							

Distribution 69 May 2011		IQC	Grey text = Outside 1 SD	Blue fill = Outside 2 SD				
		EQA	Red fill = result > twice the designated value					
If result is > twice the designated value for 3 out of 4 samples = unsatisfactory performance								
Sample sperm morphology								
				± 1 SD		± 2 SD		
Sample	MG	Mean	SD	Lower	Upper	Lower	Upper	
S273	3	4.4	1.9	2.5	6.3	0.6	8.2	
S274	7	5.0	2.2	2.8	7.2	0.7	9.3	
S275	3	4.8	2.6	2.2	7.4	-0.4	10.0	
S276	6	6.2	2.5	3.7	8.7	1.1	11.3	
		NEQAS Designated Value		Designated Value * 2				
Sample	MG	7.07		14.14				
S273	3							
		NEQAS Designated Value		Designated Value * 2				
Sample	MG	4.85		9.7				
S274	7							
		NEQAS Designated Value		Designated Value * 2				
Sample	MG	6.40		12.8				
S275	3							
		NEQAS Designated Value		Designated Value * 2				
Sample	MG	6.08		12.16				
S276	6							

Distribution 70 Aug 2011			IQC	Grey text = Outside 1 SD	Blue fill = Outside 2 SD				
Online sperm motility			EQA	Red fill = BIS > +100 or < -100					
Sample	Motility %	MG	If BIS is out for 2+ out of 3 parameters in 3+ out of 4 samples = unsatisfactory performance						
1	PR	33.1							
	NP	2.2							
	IM	64.6							
2	PR	91.4							
	NP	1.4							
	IM	7.1							
3	PR	47.9							
	NP	2.8							
	IM	49.3							
4	PR	23.5							
	NP	15.2							
	IM	61.4							
Sample	Motility %	MG							
1	PR	33.1							
	NP	2.2							
	IM	64.6							
Bias	PR	-20.11							
	NP	-74.68							
	IM	29.72							
BIS	PR	-101							
	NP	-187							
	IM	149							
Sample	Motility %	MG							
2	PR	91.4							
	NP	1.4							
	IM	7.1							
Bias	PR	4.5							
	NP	-47.8							
	IM	-24.3							
BIS	PR	22.4							
	NP	-119.4							
	IM	-60.8							
Sample	Motility %	MG							
3	PR	47.9							
	NP	2.8							
	IM	49.3							
Bias	PR	-8.3							
	NP	-67.7							
	IM	28.0							
BIS	PR	-41.6							
	NP	-169.3							
	IM	139.8							
Sample	Motility %	MG							
4	PR	23.5							
	NP	15.2							
	IM	61.4							
Bias	PR	-35.6							
	NP	-10.9							
	IM	31.8							
BIS	PR	-178.0							
	NP	-36.2							
	IM	158.9							

Mean	SD	± 1 SD		± 2 SD	
		Lower	Upper	Lower	Upper
41.1	4.8	36.3	45.9	31.5	50.7
9.0	6.0	3.0	15.0	-3.0	21.0
49.9	8.9	41.0	58.7	32.1	67.6
89.7	2.1	87.6	91.8	85.4	94.0
2.3	1.6	0.7	4.0	-1.0	5.6
8.2	1.7	6.5	10.0	4.7	11.7
49.9	5.5	44.5	55.4	39.0	60.8
9.2	5.3	3.8	14.5	-1.5	19.9
40.9	7.8	33.1	48.7	25.4	56.5
31.7	4.9	26.9	36.6	22.0	41.4
21.2	5.7	15.5	26.9	9.8	32.6
47.0	7.7	39.2	54.7	31.5	62.4

NEQAS Designated Values	CCV
41.43	20.0
8.69	40.0
49.80	20.0

NEQAS Designated Values	CCV
87.48	20.0
2.68	40.0
9.38	40.0

NEQAS Designated Values	CCV
52.25	20.0
8.67	40.0
38.53	20.0

NEQAS Designated Values	CCV
36.49	20.0
17.05	30.0
46.59	20.0

Distribution 70 Aug 2011		IQC	Grey text = Outside 1 SD	Blue fill = Outside 2 SD				
		EQA	Red fill = BIS > +100 or < -100					
			If BIS is out for 3+ out of 4 samples = unsatisfactory performance					
Sample sperm concentration								
					± 1 SD		± 2 SD	
Sample	MG	Mean	SD	Lower	Upper	Lower	Upper	
S277	26	25.6	5.1	20.5	30.7	15.4	35.8	
S278	49	60.7	13.5	47.2	74.2	33.7	87.6	
S279	6	13.9	7.1	6.8	21.0	-0.3	28.1	
S280	28	37.0	7.9	29.1	44.9	21.2	52.8	
Sample	MG	NEQAS Designated Value	CCV					
S277	26.0	24.98	25.0					
Bias	4.1							
BIS	16.3							
Sample	MG	NEQAS Designated Value	CCV					
S278	49	60.59	20.0					
Bias	-19.13							
BIS	-96							
Sample	MG	NEQAS Designated Value	CCV					
S279	6.0	8.56	50.0					
Bias	-29.9							
BIS	-59.8							
Sample	MG	NEQAS Designated Value	CCV					
S280	28.0	39.4	20.0					
Bias	-28.9							
BIS	-144.7							

Distribution 70 Aug 2011		IQC	Grey text = Outside 1 SD Blue fill = Outside 2 SD					
		EQA	Red fill = result > twice the designated value					
			If result is > twice the designated value for 3 out of 4 samples = unsatisfactory performance					
Sample sperm morphology								
					± 1 SD		± 2 SD	
Sample	MG	Mean	SD	Lower	Upper	Lower	Upper	
S277	7	6.6	1.6	5.0	8.2	3.4	9.8	
S278	5	5.0	1.9	3.1	6.9	1.1	8.9	
S279	4	3.8	2.6	1.1	6.4	-1.5	9.0	
S280	4	4.8	1.2	3.6	5.9	2.4	7.1	
Sample	MG	NEQAS Designated Value	Designated Value * 2					
S277	7	6.18	12.36					
Sample	MG	NEQAS Designated Value	Designated Value * 2					
S278	5	6.81	13.62					
Sample	MG	NEQAS Designated Value	Designated Value * 2					
S279	4	5.03	10.06					
Sample	MG	NEQAS Designated Value	Designated Value * 2					
S280	4	6.87	13.74					

Appendix H: Letter to participants with vitamin D level deficiency

Head of Department: Professor Alison Murdoch
Consultant in Reproductive Medicine: Dr Jane Stewart
Consultant in Reproductive Medicine: Dr Alka Prakash
Consultant Andrologist: Mr Kevin McEleny
Scientific Director: Dr Mary Herbert
Senior Nurse: Eilis Moody/Jan Dutton

MG/0890852Y

15/03/11

Joe bloggs
Address

Private & Confidential

Dear Joe,

This is regarding the blood test done recently as part of the research study “Seasonal variation in Vitamin D and sperm parameters”. Your Vitamin D level is low (<25nmol/l). This is nothing to worry about, but we recommend Vitamin D supplementation therapy. I will write to your GP to organise treatment and perhaps you may wish to discuss this with your GP. Please do not hesitate to contact me if you want to discuss this further. We can also discuss it when you attend the unit next time (please ask for me at the reception). As you know we will repeat your Vitamin D blood test in 6 months time as part of the research study. This will also help to ensure the levels have returned to normal with treatment. I have enclosed an information sheet on vitamin D treatment which also has alternatives while you are waiting for your GP prescription.

Kind regards

Yours sincerely,

Dr Madhavi Gudipati

Appendix I: Letter to Participant's GP

Head of Department: Professor Alison Murdoch
Consultant in Reproductive Medicine: Dr Jane Stewart
Consultant in Reproductive Medicine: Dr Alka Prakash
Consultant Andrologist: Mr Kevin McEleny
Scientific Director: Dr Mary Herbert
Senior Nurse: Eilis Moody/Jan Dutton

MG/91063611

Typed: 24/03/11

Dr.M. Henderson,
Address

Private & Confidential

Dear Dr.Henderson,

Re: Hosp No:, DOB

The above patient had their serum Vitamin D level (Serum 25-Hydroxy Vitamin D) checked as a part of a research study being done at Newcastle Fertility Centre. The result shows that the Vitamin D level is low (20mol/l). I would be grateful if you could organise for Vitamin D supplementation therapy. The general recommendation is a 6-8 week course of Cholecalciferol 20,000 IU, 3 Capsules per week. Thereafter, patients can go on a Vitamin D regime of 1,000 IU per day, or a modified diet to include oily fish twice weekly. As per the research protocol, we will repeat the blood test for Vitamin D levels 6 months after the initial test. This will also help to ensure the levels have returned to normal with treatment. Many thanks for your help.

I am enclosing a Dekristol (Colecalciferol) information sheet which you may find helpful.

Kind regards

Yours sincerely,

Dr Madhavi Gudipati

Appendix J: Vitamin D information leaflet

Dekristol information sheet for primary care & community pharmacies

1. *Dekristol* = Colecalciferol (Vitamin D₃) 20,000 Unit capsules.
2. Prescribed for severe Vitamin D deficiency, where standard Calcium+Vitamin D will be inadequate and/or the Calcium component undesired (*eg.* due to dyspepsia, constipation, *etc.*).
3. Although *Dekristol* is not listed in the BNF, its use has been suggested by the *Society for Endocrinology* and it has been incorporated into the Newcastle, North Tyneside & Northumbria Formulary by the Area Medicines Committee, because the relevant licensed products (Colecalciferol 10,000 IU and Ergocalciferol [D₂] 50,000 IU) listed in the BNF are no longer being imported into the UK.
4. Repeat prescriptions in primary care may need to be handwritten if it does not appear on the electronic drop-down prescribing system.
5. Are there any alternatives?
 - a. Patient buys Vitamin D tablets (25mcg =1000 Units) over the counter (*eg.* from *Holland & Barrett's* at around £7.99 for 100; 2-for-1 offers sometimes available). Advice to take 2 tablets per day when vitamin D level is below 25nmol/l.
6. Is it expensive?
 - a. No. The wholesale price for a bottle of 50 *Dekristol* capsules is only around £15 if ordered directly from IDIS, the sole importer.
 - b. But the cost can escalate if the community Chemist tries to order smaller quantities and/or insists on obtaining it via his/her usual wholesaler, rather than directly from IDIS.
 - c. As patients rarely need to take more than one capsule every 1-2 weeks, prescribing a 3-4 month course of treatment will be more cost-effective than a monthly script.
7. My chemist says that he/she is unable to source *Dekristol* from the usual suppliers.....

- a. Ask them to order it from:

IDIS World Medicines
IDIS house
Churchfield Road
Weybridge
Surrey KT13 8DB
Tel 01932 824000

Dr Richard Quinton
Consultant Endocrinologist
Dr Glyn Trueman
Formulary Pharmacist
The Newcastle-upon-Tyne Hospitals NHS Foundation Trust
August 2009

Appendix K: Embryo grading

Blastomere number	
Blastomere size	4 = regular (<10% difference)
	3 = 10-20%
	2 = 20-50%
	1 = >50%
Fragmentation	4 = <10% fragmentation by volume
	3 = 10-20%
	2 = 20-50%
	1 = >50%
Other features	Multinucleation
	Granular cytoplasm
	Vacuoles
	Compacting

Table 46: Embryo grading Day 2 / day 3

Grades	Scoring	
Top Quality	Day 2: 4-6 cells	4/4
	Day 3 \geq 7 cells	
Good quality	Day 2: 4-6 cells	4/3
	Day 3 \geq 7 cells	3/4
Other quality	Any other combination	
Slow	Day 2: All 2 cells; Day 3 All \leq 4 cells	

Table 47: Embryo grading

Recorded as blastomere number (cells) (size / fragmentation)

References

- Adamopoulos, D.A., Pappa, A., Nicopoulou, S., Andreou, E., Karamertzanis, M., Michopoulos, J., Deligianni, V. and Simou, M. (1996) 'Andrology: Seminal volume and total sperm number trends in men attending subfertility clinics in the Greater Athens area during the period 1977–1993', *Human Reproduction*, 11(9), pp. 1936-1941.
- Adams, J., Pease, E. and Lieberman, B. (2006) *Donor recruitment: Change attitudes, not the law*. Available at: http://www.bionews.org.uk/page_37904.asp (Accessed: July 2011).
- Agic, A., Hong, X., Altgassen, C., Noack, F., Wolfler, M.M., Diedrich, K., Friedrich, M., Taylor, R.N. and Hornung, D. (2007) 'Relative Expression of 1,25-Dihydroxyvitamin D₃ Receptor, Vitamin D 1 α -Hydroxylase, Vitamin D 24-Hydroxylase, and Vitamin D 25-Hydroxylase in Endometriosis and Gynecologic Cancers', *Reproductive Sciences*, 14(5), pp. 486-497.
- Ahuja, K. (2008) *Donor insemination in the post anonymity world : one clinic's experiences*. Available at: http://www.bionews.org.uk/page_37968.asp (Accessed: July 2011).
- Aloia, J.F. (2011) 'The 2011 Report on Dietary Reference Intake for Vitamin D: Where Do We Go From Here?', *Journal of Clinical Endocrinology and Metabolism*, 96(10), pp. 2987-2996.
- Andersen, R., Molgaard, C., Skovgaard, L.T., Brot, C., Cashman, K.D., Jakobsen, J., Lamberg-Allardt, C. and Ovesen, L. (2007) 'Pakistani immigrant children and adults in Denmark have severely low vitamin D status', *European Journal Clinical Nutrition*, 62(5), pp. 625-634.
- Andolz, P., Angeles Bielsa, M. and Andolz, A. (2001) 'Circannual variation in human semen parameters', *International Journal of Andrology*, 24(5), pp. 266-271.

Anifandis, G., Dafopoulos, K., Messini, C., Chalvatzas, N., Liakos, N., Pournaras, S. and Messinis, I. (2010) 'Prognostic value of follicular fluid 25-OH vitamin D and glucose levels in the IVF outcome.', *Reproductive Biology Endocrinology*, 8, p. 91.

Appell, R.A., Evans, P.R. and Blandy, J.P. (1977) 'The Effect of Temperature on the Motility and Viability of Sperm', *British Journal of Urology*, 49(7), pp. 751-756.

Aquila, S., Guido, C., Middea, E., Perrotta, I., Bruno, R., Pellegrino, M. and Andò, S. (2009) 'Human male gamete endocrinology: 1 α , 25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) regulates different aspects of human sperm biology and metabolism.', *Reproductive Biology Endocrinology*, 7, p. 140.

Aquila, S., Guido, C., Perrotta, I., Tripepi, S., Nastro, A. and Andò, S. (2008) 'Human sperm anatomy: ultrastructural localization of 1 α ,25-dihydroxyvitamin D₃ receptor and its possible role in the human male gamete', *Journal of Anatomy*, 213(5), pp. 555-564.

Ardabili, H.R., Gargari, B.P. and Farzadi, L. (2012) 'Vitamin D supplementation has no effect on insulin resistance assessment in women with polycystic ovary syndrome and vitamin D deficiency', *Nutrition Research*, 32(3), pp. 195-201.

Armas, L.A.G., Dowell, S., Akhter, M., Duthuluru, S., Huerter, C., Hollis, B.W., Lund, R. and Heaney, R.P. (2007) 'Ultraviolet-B radiation increases serum 25-hydroxyvitamin D levels: The effect of UVB dose and skin color', *Journal of the American Academy of Dermatology*, 57(4), pp. 588-593.

Ashwell, M., Stone, E.M., Stolte, H., Cashman, K.D., Macdonald, H., Lanham-New, S., Hiom, S., Webb, A. and Fraser, D. (2010) 'UK Food Standards Agency Workshop Report: an investigation of the relative contributions of diet and sunlight to vitamin D status', *British Journal of Nutrition*, 104(4), pp. 603-611.

Association of Biomedical Andrologists, Association of Clinical Embryologists, British Andrology Society, British Fertility Society and Gynaecologists, R.C.o.O. (2008) 'UK guidelines for the medical and laboratory screening of sperm, egg and embryo donors (2008)', *Human Fertility*, 11(4), pp. 201-210.

Auger, J., Eustache, F., Ducot, B., Blandin, T., Daudin, M., Diaz, I., Matribi, S.E., Gony, B., Keskes, L., Kolbezen, M., Lamarte, A., Lornage, J., Nomal, N., Pitaval, G., Simon, O., Virant-Klun, I., Spira, A. and Jouannet, P. (2000) 'Intra- and inter-individual variability in human sperm concentration, motility and vitality assessment during a workshop involving ten laboratories', *Human Reproduction*, 15(11), pp. 2360-2368.

Axelsson, J., Rylander, L., Rignell-Hydbom, A. and Giwercman, A. (2011) 'No secular trend over the last decade in sperm counts among Swedish men from the general population', *Human Reproduction*, 26(5), pp. 1012-1016.

Barrera, D., Avila, E., Hernández, G., Halhali, A., Biruete, B., Larrea, F. and Díaz, L. (2007) 'Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol', *The Journal of Steroid Biochemistry and Molecular Biology*, 103(3–5), pp. 529-532.

Barrera, D., Avila, E., Hernandez, G., Mendez, I., Gonzalez, L., Halhali, A., Larrea, F., Morales, A. and Diaz, L. (2008) 'Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts', *Reproductive Biology and Endocrinology*, 6, p. 3.

Barrett, H. and McElduff, A. (2010) 'Vitamin D and pregnancy: An old problem revisited.', *Best Practice Research Clinical Endocrinology and Metabolism*, 24(4), pp. 527-39.

Barton, M. (1945) 'Artificial Insemination', *British Medical Journal*, 4384(13th january), pp. 40-43.

BBC survey (2006) *Clinics in 'crisis' and having 'great difficulty' getting sperm donors*
Available at:
http://www.bbc.co.uk/pressoffice/pressreleases/stories/2006/09_september/14/sperm.shtml
(Accessed: July 2011).

Benoff, S., Cooper, G.W., Hurley, I., Mandel, F.S., Rosenfeld, D.L., Scholl, G.M., Gilbert, B.R. and Hershlag, A. (1994) 'The effect of calcium ion channel blockers on sperm fertilization potential', *Fertility and Sterility*, 62(3), pp. 606-17.

Berman, N.G., Wang, C. and Paulsen, C.A. (1996) 'Methodological issues in the analysis of human sperm concentration data', *Journal of Andrology*, 17(1), pp. 68-73.

Besselink, D., Farquhar, C., Kremer, J., Marjoribanks, J. and O'Brien, P. (2008) 'Cervical insemination versus intra-uterine insemination of donor sperm for subfertility.', *Cochrane Database of Systematic Reviews*, (2), p. DOI: 10.1002/14651858.CD000317.pub3. .

Blackburn-Starza, A. (2009) *Banking crisis- what should be done about the sperm donor shortage?* Available at: http://www.bionews.org.uk/page_45488.asp (Accessed: September 2012).

Blomberg Jensen, M. (2012) 'Vitamin D metabolism, sex hormones, and male reproductive function', *Reproduction*, 144(2), pp. 135-52.

Blomberg Jensen, M., Bjerrum, P.J., Jessen, T.E., Nielsen, J.E., Joensen, U.N., Olesen, I.A., Petersen, J.H., Juul, A., Dissing, S. and Jørgensen, N. (2011) 'Vitamin D is positively associated with sperm motility and increases intracellular calcium in human spermatozoa', *Human Reproduction*, 26(6), pp. 1307-1317.

Blomberg Jensen, M. and Dissing, S. (2012) 'Non-genomic effects of vitamin D in human spermatozoa', *Steroids*, 77(10), pp. 903-909.

Blomberg Jensen, M., Jørgensen, A., Nielsen, J.E., Bjerrum, P.J., Skalkam, M., Petersen, J.H., Egeberg, D.L., Bangsbøll, S., Andersen, A.N., Skakkebaek, N.E., Juul, A., Rajpert-De Meyts, E., Dissing, S., Leffers, H. and Jørgensen, N. (2012) 'Expression of the vitamin D metabolizing enzyme CYP24A1 at the annulus of human spermatozoa may serve as a novel marker of semen quality', *International Journal of Andrology*, 35(4), pp. 499-510.

Blomberg Jensen, M., Nielsen, J.E., Jørgensen, A., Rajpert-De Meyts, E., Kristensen, D.M., Jørgensen, N., Skakkebaek, N.E., Juul, A. and Leffers, H. (2010) 'Vitamin D receptor and vitamin D metabolizing enzymes are expressed in the human male reproductive tract', *Human Reproduction*, 25(5), pp. 1303-1311.

Blyth, E. and Frith, L. (2008) 'The UK's gamete donor `crisis' - a critical analysis', *Critical Social Policy*, 28(1), pp. 74-95.

Bonde, J.P.E., Ernst, E., Jensen, T.K., Hjollund, N.H.I., Kolstad, H., Scheike, T., Giwercman, A., Skakkebaek, N.E., Henriksen, T.B. and Olsen, J. (1998) 'Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners', *The Lancet*, 352(9135), pp. 1172-1177.

Borkowski, J., Gmyrek, G.B., Madej, J.P., Nowacki, W., Goluda, M., Gabrys, M., Stefaniak, T. and Chelmonska-Soyta, A. (2008) 'Serum and peritoneal evaluation of vitamin D-binding protein in women with endometriosis', *Postepy higieny i medycyny doswiadczonej (Online)*, 62, pp. 103-9.

Bosomworth, N.J. (2011) 'Mitigating epidemic vitamin D deficiency: The agony of evidence', *Canadian Family Physician*, 57(1), pp. 16-20.

Bouillon, R., Carmeliet, G., Verlinden, L., van Etten, E., Verstuyf, A., Luderer, H.F., Lieben, L., Mathieu, C. and Demay, M. (2008) 'Vitamin D and Human Health: Lessons from Vitamin D Receptor Null Mice', *Endocrine Reviews*, 29(6), pp. 726-776.

Breitbart, H. (2002) 'Intracellular calcium regulation in sperm capacitation and acrosomal reaction', *Molecular and Cellular Endocrinology*, 187(1-2), pp. 139-44.

Bremmer, F., Thelen, P., Pottek, T., Behnes, C.L., Radzun, H.J. and Schweyer, S. (2012) 'Expression and Function of the Vitamin D Receptor in Malignant Germ Cell Tumour of the Testis', *Anticancer Research*, 32(1), pp. 341-349.

British Andrology Society (1999) 'British Andrology Society guidelines for the screening of semen donors for donor insemination (1999)', *Human Reproduction*, 14(7), pp. 1823-1826.

British Fertility Society survey (2006) *BFS Donor Survey 2005/6*. Available at: http://www.fertility.org.uk/news/documents/2006_09-BFS%20Donor%20Sperm%20Survey.pdf (Accessed: August 2011).

Bronson, F.H. (1995) 'Seasonal Variation in Human Reproduction: Environmental Factors', *The Quarterly Review of Biology*, 70(2), pp. 141-164.

Bronson, F.H. (2004) 'Are Humans Seasonally Photoperiodic?', *Journal of Biological Rhythms*, 19(3), pp. 180-192.

Calle, C., Maestro, B. and Garcia-Arencibia, M. (2008) 'Genomic actions of 1,25-dihydroxyvitamin D3 on insulin receptor gene expression, insulin receptor number and insulin activity in the kidney, liver and adipose tissue of streptozotocin-induced diabetic rats', *BMC Molecular Biology*, 9, p. 65.

Calonge, R.N., Olaya, E., Guijarro, A., Cortés, S., Gago, M. and Caballero, P. (2009) 'Temporal trends and seasonality in semen quality over a period of 12 years in Madrid; 9th International Congress of Andrology, Barcelona, Spain, 7-10 March, 2009.', www.clinicatambre.com.

Camargo, C.A., Rifas-Shiman, S.L., Litonjua, A.A., Rich-Edwards, J.W., Weiss, S.T., Gold, D.R., Kleinman, K. and M.W., G. (2007) 'Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age.', *American Journal of Clinical Nutrition*, 85 (3), pp. 788-795.

Cancho-Candela, R., Andrés-de Llano, J.M. and Ardura-Fernández, J. (2007) 'Decline and loss of birth seasonality in Spain: analysis of 33 421 731 births over 60 years', *Journal of Epidemiology and Community Health*, 61(8), pp. 713-718.

Carlsen, E., Giwercman, A., Keiding, N. and Skakkebaek, N.E. (1992) 'Evidence for decreasing quality of semen during past 50 years', *British Medical Journal*, 305(6854), pp. 609-613.

Carlsen, E., Petersen, J.H., Andersson, A.-M. and Skakkebaek, N.E. (2004) 'Effects of ejaculatory frequency and season on variations in semen quality', *Fertility and Sterility*, 82(2), pp. 358-366.

Carroll, N. and Palmer, J.R. (2001) 'A comparison of intrauterine versus intracervical insemination in fertile single women', *Fertility and Sterility*, 75(4), pp. 656-660.

Carter, G., Carter, R., Jones, J. and Berry, J. (2004) 'How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme.', *Clinical Chemistry*, 50(11), pp. 2195-7.

Casado-Diaz, A., Cuenca-Acevedo, R., Navarro-Valverde, C., Díaz-Molina, C., Caballero-Villarraso, J., Santiago Mora, R., Dorado, G. and Quesada-Gómez, J.M. (2013) 'D status and the Cdx-2 polymorphism of the vitamin D receptor gene are determining factors of bone mineral density in young healthy postmenopausal women', *The Journal of Steroid Biochemistry and Molecular Biology*, 136, pp. 187-189.

Centola, G.M. (1996) 'Comparison of Manual Microscopic and Computer-Assisted Methods for Analysis of Sperm Count and Motility', *Systems Biology in Reproductive Medicine*, 36(1), pp. 1-7.

Centola, G.M. and Eberly, S. (1999) 'Seasonal variations and age-related changes in human sperm count, motility, motion parameters, morphology, and white blood cell concentration', *Fertility and Sterility*, 72(5), pp. 803-808.

Chemineau, P., Guillaume, D., Migaud, M., Thiéry, J.C., Pellicer-Rubio, M.T. and Malpoux, B. (2008) 'Seasonality of Reproduction in Mammals: Intimate Regulatory Mechanisms and Practical Implications', *Reproduction in Domestic Animals*, 43, pp. 40-47.

Chen, Z., Godfrey-Bailey, L., Schiff, I. and Hauser, R. (2004) 'Impact of seasonal variation, age and smoking status on human semen parameters: The Massachusetts General Hospital', *Journal of Experimental and clinical Assisted Reproduction*, 1(2).

Chen, Z., Toth, T., Godfrey-Bailey, L., Mercedat, N., Schiff, I. and Hauser, R. (2003) 'Seasonal Variation and Age-Related Changes in Human Semen Parameters', *Journal of Andrology*, 24(2), pp. 226-231.

Chia, S.-E., Lim, S.-T.A., Ho, L.-M. and Tay, S.-K. (2001) 'Monthly variation in human semen quality in male partners of infertile women in the tropics', *Human Reproduction*, 16(2), pp. 277-281.

Chia, S.E., Ong, C.N., Lee, S.T. and Tsakok, F.H. (1994) 'Study of the effects of occupation and industry on sperm quality', *Annals of the Academy of Medicine, Singapore*, 23(5), pp. 645-9.

Chick, H., Dolyell, E. and Hume, E. (1923) 'Studies of rickets in Vienna 1919–1922.', *Med Res Counc (GB) Spec Rep Ser*, 77.

Chlebowski, R.T., Johnson, K.C., Kooperberg, C., Pettinger, M., Wactawski-Wende, J., Rohan, T., Rossouw, J., Lane, D., O'Sullivan, M.J., Yasmeen, S., Hiatt, R.A., Shikany, J.M., Vitolins, M., Khandekar, J. and Hubbell, F.A. (2008) 'Calcium Plus Vitamin D Supplementation and the Risk of Breast Cancer', *Journal of the National Cancer Institute*, 100(22), pp. 1581-1591.

Cook, R. and Golombok, S. (1995) 'Ethics and society: A survey of semen donation: phase II — the view of the donors', *Human Reproduction*, 10(4), pp. 951-959.

Cooke, I.D. (1993) 'Secrecy, Openness, and DI in the UK', *Politics and the Life Sciences*, 12(2), pp. 176-177.

Cooper, T.G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H.W.G., Behre, H.M., Haugen, T.B., Kruger, T., Wang, C., Mbizvo, M.T. and Vogelsong, K.M. (2010) 'World Health Organization reference values for human semen characteristics', *Human Reproduction Update*, 16(3), pp. 231-245.

Corbett, S.T., Hill, O. and Nangia, A.K. (2006) 'Vitamin D receptor found in human sperm', *Urology*, 68(6), pp. 1345-1349.

Couse, J., Mahato, D., Eddy, E. and Korach, K. (2001) 'Molecular mechanism of estrogen action in the male: insights from the estrogen receptor null mice.', *Reproduction, Fertility and development*, 13(4), pp. 211-219.

Culley, L., Hudson, N., Rapport, F., Blyth, E., Norton, W. and Pacey, A.A. (2011) 'Crossing borders for fertility treatment: motivations, destinations and outcomes of UK fertility travellers', *Human Reproduction*, 26(9), pp. 2373-2381.

Daniels, K. (1997) 'The controversies regarding privacy versus disclosure among patients using donor gametes in Assisted reproductive technology', *Journal of Assisted Reproduction and Genetics*, 14(7), pp. 373-375.

Daniels, K., Blyth, E., Crawshaw, M. and Curson, R. (2005) 'Short Communication: Previous semen donors and their views regarding the sharing of information with offspring', *Human Reproduction*, 20(6), pp. 1670-1675.

Daniels, K., Feyles, V., Nisker, J., Perez-y-Perez, M., Newton, C., Parker, J., Tekpetey, F. and Haase, J. (2006) 'Sperm donation: implications of Canada's Assisted Human Reproduction Act 2004 for recipients, donors, health professionals, and institutions.', *Journal of Obstetrics and Gynaecology Canada*, 28(7), pp. 608-615.

Daniels, K. and Lalos, O. (1995) 'Ethics and Society: The Swedish insemination act and the availability of donors', *Human Reproduction*, 10(7), pp. 1871-1874.

Daniels, K.R. and Taylor, K. (1993) 'Secrecy and Openness in Donor Insemination', *Politics and the Life Sciences*, 12(2), pp. 155-170.

Dawson-Hughes, B., Heaney, R.P., Holick, M.F., Lips, P., Meunier, P.J. and Vieth, R. (2005) 'Estimates of optimal vitamin D status', *Osteoporosis International*, 16(7), pp. 713-716.

Deary, A.J., Seaton, J.E., Prentice, A., Morton, N.C., Booth, A.K. and Smith, S.K. (1997) 'Single versus double insemination: a retrospective audit of pregnancy rates with two treatment protocols in donor insemination', *Human Reproduction*, 12(7), pp. 1494-1496.

Del Valle, A., Bradley, L. and Said, T. (2008) 'Anonymous semen donor recruitment without reimbursement in Canada.', *Reproductive BioMedicine Online*, 17 (supplement 1), pp. 15-20.

DeLuca, H.F. (2004) 'Overview of general physiologic features and functions of vitamin D', *American Journal of Clinical Nutrition*, 80(6), pp. 1689S-1696S.

Department of Health (1998) 'Nutrition and bone health with particular reference to calcium and vitamin D report of the Working Group on the Nutritional Status of the Population of the Committee on Medical Aspects of Food and Nutrition Policy ', *The Stationery Office, London*, p. 128.

Department of Health (2000) *Margarine regulations. Statutory Instrument no. 1867 as amended. 1967. London, United Kingdom: H.M. Stationery Office, 1967.* Available at: http://archive.food.gov.uk/dept_health/pdf/evmpdf/evm9925.pdf (Accessed: January 2013).

Department of Health (2001) 'Providing Information About Sperm, Egg and Embryo Donors.', *Department of Health*, London.

Department of Health (2012) *Vitamin D-advice on supplements for at risk groups.* Available at: <http://www.dh.gov.uk/health/2012/02/advice-vitamin-d/> (Accessed: December 2012).

Deti, L., Paine, T., Yelian, F.D., Puscheck, E.E., Kruger, M. and Diamond, M.P. (2006) 'Seasonal variation of semen parameters in a five year study', *Fertility and Sterility*, 86(3 supplement), p. S370.

Dicken, C.L., Israel, D.D., Davis, J.B., Sun, Y., Shu, J., Hardin, J. and Neal-Perry, G. (2012) 'Peripubertal vitamin D(3) deficiency delays puberty and disrupts the estrous cycle in adult female mice', *Biology of Reproduction*, 87(2), p. 51.

Dindyal, S. (2004) 'The sperm count has been decreasing steadily for many years in Western industrialised countries: Is there an endocrine basis for this decrease?', *The Internet Journal of Urology* 2(1).

Directive 2004/23/EC (2004) 'Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells ', *Official Journal of the European Union*, L 102, pp. 0048-0058.

Du, H., Daftary, G.S., Lalwani, S.I. and Taylor, H.S. (2005) 'Direct Regulation of HOXA10 by 1,25-(OH)₂D₃ in Human Myelomonocytic Cells and Human Endometrial Stromal Cells', *Molecular Endocrinology*, 19(9), pp. 2222-2233.

Dyer, C. (2004) 'Shortage of sperm donors predicted when anonymity goes', *British Medical Journal*, 328(7434), p. 244.

Dyer, C. (2010) 'HFEA investigates websites matching sperm donors to would be mothers', *British Medical Journal*, 341(c5353), p. 693.

Ekerhovd, E. and Faurskov, A. (2008) 'Swedish sperm donors are driven by altruism, but shortage of sperm donors leads to reproductive travelling', *Upsala Journal of Medical Sciences*, 113(3), pp. 305-314.

Engel, L.S., Orlow, I., Sima, C.S., Satagopan, J., Mujumdar, U., Roy, P., Yoo, S., Sandler, D.P. and Alavanja, M.C. (2012) 'Vitamin D Receptor Gene Haplotypes and Polymorphisms and Risk of Breast Cancer: A Nested Case–Control Study', *Cancer Epidemiology Biomarkers & Prevention*, 21(10), pp. 1856-1867.

Engelsen, O., Brustad, M., Aksnes, L. and Lund, E. (2005) 'Daily duration of vitamin D synthesis in human skin with relation to latitude, total ozone, altitude, ground cover, aerosols and cloud thickness', *Photochemistry and Photobiology*, 81(6), pp. 1287-1290.

Ernst, E., Jakob Ingerslev, H., Schou, O. and Stoltenberg, M. (2007) 'Attitudes among sperm donors in 1992 and 2002: a Danish questionnaire survey', *Acta Obstetrica et Gynecologica Scandinavica*, 86(3), pp. 327-333.

Faserl, K., Golderer, G., Kremser, L., Lindner, H., Sarg, B., Wildt, L. and Seeber, B. (2011) 'Polymorphism in vitamin D-binding protein as a genetic risk factor in the pathogenesis of endometriosis', *Journal of Clinical Endocrinology and Metabolism*, 96(1), pp. E233-241.

Fisch, H., Andrews, H., Hendricks, J., Goluboff, E.T., Olson, J.H. and Olsson, C.A. (1997) 'The Relationship of Sperm Counts to Birth Rates: A Population Based Study', *The Journal of Urology*, 157(3), pp. 840-843.

Fisch, H., Andrews, H.F., Fisch, K.S., Golden, R., Liberson, G. and Olsson, C.A. (2003) 'The relationship of long term global temperature change and human fertility', *Medical Hypotheses*, 61(1), pp. 21-28.

Fleet, J.C. (2008) 'Molecular actions of vitamin D contributing to cancer prevention', *Molecular Aspects of Medicine*, 29(6), pp. 388-396.

Flierman, P.A., Hogerzeil, H.V. and Hemrika, D.J. (1997) 'A prospective, randomized, cross-over comparison of two methods of artificial insemination by donor on the incidence of conception: intracervical insemination by straw versus cervical cap', *Human Reproduction*, 12(9), pp. 1945-1948.

Foresta, C., Strapazzon, G., De Toni, L., Perilli, L., Di Mambro, A., Muciaccia, B., Sartori, L. and Selice, R. (2011) 'Bone Mineral Density and Testicular Failure: Evidence for a Role of Vitamin D 25-Hydroxylase in Human Testis', *Journal of Clinical Endocrinology and Metabolism*, 96(4), pp. E646-E652.

Foss, Y.J. (2009) 'Vitamin D deficiency is the cause of common obesity', *Medical Hypotheses*, 72(3), pp. 314-321.

Fraile, B., Paniagua, R., Rodríguez, M.C., Sáez, F.J. and Jimenez, A. (1989) 'Annual changes in the number, testosterone content and ultrastructure of glandular tissue cells of the testis in the marbled newt *Triturus marmoratus*.', *Journal of Anatomy*, 167, pp. 85-94.

Freedman, D.M., Chang, S.-C., Falk, R.T., Purdue, M.P., Huang, W.-Y., McCarty, C.A., Hollis, B.W., Graubard, B.I., Berg, C.D. and Ziegler, R.G. (2008) 'Serum Levels of Vitamin D Metabolites and Breast Cancer Risk in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial', *Cancer Epidemiology Biomarkers & Prevention*, 17(4), pp. 889-894.

Freeman, T., Jadvá, V., Kramer, W. and Golombok, S. (2009) 'Gamete donation: parents' experiences of searching for their child's donor siblings and donor', *Hum Reproduction*, 24(3), pp. 505-16.

Frith, L. (2001) 'Gamete donation and anonymity', *Human Reproduction*, 16(5), pp. 818-824.

Frith, L., Blyth, E. and Farrand, A. (2007) 'UK gamete donors' reflections on the removal of anonymity: implications for recruitment', *Human Reproduction*, 22(6), pp. 1675-1680.

Gagnon, C., Baillargeon, J.-P., Desmarais, G. and Fink, G.D. (2010) 'Prevalence and predictors of vitamin D insufficiency in women of reproductive age living in northern latitude', *European Journal of Endocrinology*, 163(5), pp. 819-824.

Gazvani, M., Wood, S., Thomson, S., Kingsland, C. and Lewis-Jones, D. (1997) 'Payment or altruism? The motivation behind gamete donation.', *Human Reproduction*, 12(9), pp. 1845-1846.

Glerup, H., Mikkelsen, K., Poulsen, L., Hass, E., Overbeck, S., Thomsen, J., Charles, P. and Eriksen, E.F. (2000) 'Commonly recommended daily intake of vitamin D is not sufficient if sunlight exposure is limited', *Journal of Internal Medicine*, 247(2), pp. 260-268.

Goldberg, J., Mascha, E., Falcone, T. and Attaran, M. (1999) 'Comparison of intrauterine and intracervical insemination with frozen donor sperm: a meta-analysis.', *Fertility and Sterility*, 72(5), pp. 792-795.

Gollancz, D. (2001) 'Donor insemination: A question of rights*', *Human Fertility*, 4(3), pp. 164-167.

Golombok, S. (1997) 'Parenting and secrecy issues related to children of assisted reproduction', *Journal of Assisted Reproduction and Genetics*, 14(7), pp. 375-378.

Golombok, S. and Cook, R. (1994) 'A survey of semen donation: phase I—the view of UK licensed centres', *Human Reproduction*, 9(5), pp. 882-888.

Gosch, B. and Fischer, K. (1989) 'Seasonal changes of testis volume and sperm quality in adult fallow deer (*Dama dama*) and their relationship to the antler cycle', *Journal of Reproduction and Fertility*, 85(1), pp. 7-17.

Graafmans, W.C., Lips, P., Ooms, M.E., Van Leeuwen, J.P.T.M., Pols, H.A.P. and Uitterlinden, A.G. (1997) 'The Effect of Vitamin D Supplementation on the Bone Mineral Density of the Femoral Neck Is Associated with Vitamin D Receptor Genotype', *Journal of Bone and Mineral Research*, 12(8), pp. 1241-1245.

Greene-Finestone, L.S., Berger, C., Groh, M., Hanley, D.A., Hidiroglou, N., Sarafin, K., Poliquin, S., Krieger, J., Richards, J.B. and Goltzman, D. (2011) '25-Hydroxyvitamin D in Canadian adults: biological, environmental, and behavioral correlates', *Osteoporosis International*, 22(5), pp. 1389-1399.

Grundmann, M. and von Versen-Hoyneck, F. (2011) 'Vitamin D - roles in women's reproductive health?', *Reproductive Biology and Endocrinology*, 9, p. 146.

Guerin, J. (1998) 'The donation of gametes is possible without paying donors: experience of the French CECOS Federation. Centre for the cryopreservation of eggs and semen', *Human Reproduction*, 13(5), pp. 1129-1130.

Gündüz, M., Cacina, C., Toptaş, B., Yaylım-Eraltan, I., Tekand, Y. and T, I. (2012) 'Association of vitamin d receptor gene polymorphisms with colon cancer', *Genetic testing and molecular biomarkers*, 16(9), pp. 1058-1061.

Guzick, D.S., Overstreet, J.W., Factor-Litvak, P., Brazil, C.K., Nakajima, S.T., Coutifaris, C., Carson, S.A., Cisneros, P., Steinkampf, M.P., Hill, J.A., Xu, D. and Vogel, D.L. (2001) 'Sperm morphology, motility, and concentration in fertile and infertile men', *New England Journal of Medicine*, 345(19), pp. 1388-1393.

Gyllenborg, Skakkebaek, Nielsen, Keiding and Giwercman (1999) 'Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977–1995', *International Journal of Andrology*, 22(1), pp. 28-36.

Haimes, E.V. (1993) 'Ethics and Society: Do clinicians benefit from gamete donor anonymity?', *Human Reproduction*, 8(9), pp. 1518-1520.

- Halhali, A., Acker, G.M. and Garabédian, M. (1991) '1,25-Dihydroxyvitamin D₃ induces in vivo the decidualization of rat endometrial cells', *Journal of Reproduction and Fertility*, 91(1), pp. 59-64.
- Hamilton, M. (2010) 'Sperm donation in the United Kingdom in 2010', *Human Fertility*, 13(4), pp. 257-262.
- Hamilton, M. and Pacey, A. (2008b) 'Sperm donation in the UK', *British Medical Journal*, 337, pp. 1124-1125.
- Hamilton, M., Pacey, A., Tomlinson, M., Brison, D., Shaw, L., Turner, C., Witjens, L., Morris, P., Brown, C., Montuschi, O., Adams, J., Lieberman, B. and Speirs, J. (2008a) 'Working Party on Sperm Donation Services in the UK', *Human Fertility*, 11(3), pp. 147-158.
- Hammoud, A.O., Wayne Meikle, A., Mathew Peterson, C., Stanford, J., Gibson, M. and Carrell, D. (2012) 'Association of 25-hydroxy-vitamin D levels with semen and hormonal parameters', *Asian Journal of Andrology*, 14(6), pp. 855-859.
- Handelsman, D.J. (2002) 'Optimal Power Transformations for Analysis of Sperm Concentration and Other Semen Variables', *Journal of Andrology*, 23(5), pp. 629-634.
- Harris, S.M. (2000) 'Establishing an intrauterine insemination programme from a nursing perspective', *Human Fertility*, 3(2), pp. 121-123.
- Haussler, M.R., Jurutka, P.W., Mizwicki, M. and Norman, A.W. (2011) 'Vitamin D receptor (VDR)-mediated actions of 1 α ,25(OH)₂vitamin D₃: Genomic and non-genomic mechanisms', *Best Practice & Research Clinical Endocrinology & Metabolism*, 25(4), pp. 543-559.
- Heaney, R.P. (2008) 'Vitamin D in health and disease', *Clinical Journal of the American Society of Nephrology*, 3(5), pp. 1535-41.
- Heine, G., Hoefler, N., Franke, A., Nöthling, U., Schumann, R.R., Hamann, L. and Worm, M. (2013) 'Association of vitamin D receptor gene polymorphisms with severe atopic dermatitis in adults', *British Journal of Dermatology*, 168(4), pp. 855-858.

Helzlsouer, K.J. and Vdpp Steering Committee (2010) 'Overview of the Cohort Consortium Vitamin D Pooling Project of Rarer Cancers', *American Journal of Epidemiology*, 172(1), pp. 4-9.

Heng, B. (2007) 'Legal and ethical issues in the international transaction of donor sperm and eggs', *Journal of Assisted Reproduction and Genetics*, 24(4), pp. 107-109.

HFEA (1996) *Directions given under the Human Fertilisation and Embryology Act 1990*. Available at: www.hfea.gov.uk/docs/D1996_1.pdf (Accessed: December 2010).

HFEA (1998) *Consultation on the implementation of withdrawal of payments to donors*. Available at: <http://www.hfea.gov.uk/537.html> (Accessed: July 2012).

HFEA (2011) *Facts and figures - Donor conception births*. Available at: <http://www.hfea.gov.uk/donor-conception-births.html> (Accessed: July 2012).

HFEA (2011a) *Payment for donors*. Available at: <http://www.hfea.gov.uk/500.html> (Accessed: October 2011).

HFEA (2011b) *Family limit for donated sperm and eggs*. Available at: <http://www.hfea.gov.uk/6192.html> (Accessed: July 2012).

HFEA (2011c) *Facts and figures - Donor conception treatments*. Available at: <http://www.hfea.gov.uk/donor-conception-treatments.html> (Accessed: July 2012).

HFEA (2011d) *Facts and figures New donor registrations*. Available at: <http://www.hfea.gov.uk/3411.html> (Accessed: July 2012).

HFEA (2011e) *Facts and figures UK and overseas donors*. Available at: <http://www.hfea.gov.uk/3413.html> (Accessed: July 2012).

HFEA (2011f) *HFEA agrees new policies to improve sperm and egg donation services*. Available at: <http://www.hfea.gov.uk/6700.html> (Accessed: July 2012).

HFEAct (2008) *Human Fertilisation and Embryology Act 2008*. Available at: http://www.dh.gov.uk/en/Publicationsandstatistics/Legislation/Actsandbills/DH_080211 (Accessed: July 2011).

Hiltrud, M., Hajo, Z. and Maria, B. (2010) 'Decreasing sperm quality: a global problem?', *BMC Public Health*, 10, p. 24.

Hirai, T., Tsujimura, A., Ueda, T., Fujita, K., Matsuoka, Y., Takao, T., Miyagawa, Y., Koike, N. and Okuyama, A. (2009) 'Effect of 1,25-dihydroxyvitamin d on testicular morphology and gene expression in experimental cryptorchid mouse: testis specific cDNA microarray analysis and potential implication in male infertility.', *Journal of Urology*, 181(3), pp. 1487-92.

HMSO (1990) *Human Fertilisation and Embryology ACT*. Available at: <http://www.legislation.gov.uk/ukpga/1990/37/contents> (Accessed: July 2010).

HMSO (2004) *Human Fertilisation and Embryology Authority Disclosure of Donor Information Regulations (2004) Statutory Instrument 2004 No:1511*. Available at: <http://www.legislation.gov.uk/uksi/2004/1511/contents/made> (Accessed: June 2011).

Holick, M.F. (2007) 'Medical Progress: Vitamin D Deficiency', *New England Journal of Medicine*, 357(3), pp. 266-281.

Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H. and Weaver, C.M. (2011) 'Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline', *Journal of Clinical Endocrinology and Metabolism*, 96(7), pp. 1911-1930.

Holick, M.F. and Chen, T.C. (2008) 'Vitamin D deficiency: a worldwide problem with health consequences', *American Journal of Clinical Nutrition*, 87(4), pp. 1080S-1086S.

Hollis, B.W., Johnson, D., Hulsey, T.C., Ebeling, M. and Wagner, C.L. (2011) 'Vitamin D supplementation during pregnancy: Double-blind, randomized clinical trial of safety and effectiveness', *Journal of Bone and Mineral Research*, 26(10), pp. 2341-2357.

Holvik, K., Meyer, H.E., Haug, E. and Brunvand, L. (2004) 'Prevalence and predictors of vitamin D deficiency in five immigrant groups living in Oslo, Norway: the Oslo Immigrant Health Study', *European Journal Clinical Nutrition*, 59(1), pp. 57-63.

Horii, I., Takizawa, S. and Fujii, T. (1992) 'Effect of 1,25-dihydroxyvitaminD on the female reproductive system in rats', *The Journal of Toxicological Sciences*, 17(3), pp. 91-105.

Hosmer, D. and Lemeshow, S. (2000) *Applied logistic regression*. Second edn. John Wiley & Sons.

Hudson, N., Culley, L., Blyth, E., Norton, W., Rapport, F. and Pacey, A. (2011) 'Cross-border reproductive care: a review of the literature', *Reproductive BioMedicine Online*, 22(7), pp. 673-685.

Huldshinsky, K. (1919) 'Heilung von Rachitis durch kunstlich Hohen-sonne. (The healing of rickets with artificial high altitude sun.)', *Deutsche Medizinische Wochenschrift*, 45, pp. 712-713.

Hunt, J. and Fleming, R. (2002) 'Department of health donor information consultation: Providing information about gamete or embryo donors', *Human Fertility*, 5(3), pp. 97-98.

Hyppönen, E. and Power, C. (2007) 'Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors', *American Journal of Clinical Nutrition*, 85(3), pp. 860-868.

Inpanbutr, N. and Taylor, A. (1992) 'Expression of calbindin-D28k in developing and growing chick testes', *Histochemistry*, 97(4), pp. 335-339.

Irvine, S., Cawood, E., Richardson, D., MacDonald, E. and Aitken, J. (1996) 'Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years', *British Medical Journal*, 312(7029), pp. 467-471.

Isachenko, V., Maettner, R., Petrunkina, A.M., Mallmann, P., Rahimi, G., Sterzik, K., Sanchez, R., Risopatron, J., Damjanoski, I. and Isachenko, E. (2011) 'Cryoprotectant-

free vitrification of human spermatozoa in large (up to 0.5 mL) volume: a novel technology', *Clinical Laboratory*, 57(9-10), pp. 643-650.

Jadva, V., Badger, S., Morrisette, M. and Golombok, S. (2009) 'Mom by choice, single by life's circumstance...' Findings from a large scale survey of the experiences of single mothers by choice', *Human Fertility*, 12(4), pp. 175-184.

Jadva, V., Freeman, T., Kramer, W. and Golombok, S. (2011) 'Sperm and oocyte donors' experiences of anonymous donation and subsequent contact with their donor offspring', *Human Reproduction*, 26(3), pp. 638-645.

Jain, V., Gupta, N., Kalaiyani, M., Jain, A., Sinha, A. and Agarwal, R. (2011) 'Vitamin D deficiency in healthy breastfed term infants at 3 months & their mothers in India: seasonal variation & determinants.', *Indian Journal of Medical Research.*, 133(3), pp. 267-73.

James, W.H. (1990) 'Seasonal variation in human births', *Journal of Biosocial Science*, 22(01), pp. 113-119.

Janssens, P.M.W. (2003) 'No reason for a reduction in the number of offspring per sperm donor because of possible transmission of autosomal dominant diseases', *Human Reproduction*, 18(4), pp. 669-671.

Janssens, P.M.W., Nap, A.W. and Bancsi, L.F.J.M.M. (2011) 'Reconsidering the number of offspring per gamete donor in the Dutch open-identity system', *Human Fertility*, 14(2), pp. 106-114.

Janssens, P.M.W., Simons, A.H.M., van Kooij, R.J., Blokzijl, E. and Dunselman, G.A.J. (2006) 'A new Dutch Law regulating provision of identifying information of donors to offspring: background, content and impact', *Human Reproduction*, 21(4), pp. 852-856.

Javaid, M., Crozier, S., Harvey, N., Gale, C., Dennison, E., Boucher, B., Arden, N., Godfrey, K. and Cooper, C. (2006) 'Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study.', *Lancet.*, 367(9504), pp. 36-43.

- Johnson, J.A., Grande, J.P., Roche, P.C. and Kumar, R. (1996) 'Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D₃ receptor in rat reproductive tissues', *Histochemistry and Cell Biology*, 105(1), pp. 7-15.
- Johnson, L.E. and DeLuca, H.F. (2001) 'Vitamin D Receptor Null Mutant Mice Fed High Levels of Calcium Are Fertile', *The Journal of Nutrition*, 131(6), pp. 1787-1791.
- Johnson, M. (1997) 'Payments to gamete donors: position of the human fertilisation and embryology authority.', *Human Reproduction*, 12(9), pp. 1839-1842.
- Johnston, R.C., Kovacs, G.T., Lording, D.H. and Baker, H.W. (1994) 'Correlation of semen variables and pregnancy rates for donor insemination: a 15-year retrospective', *Fertility and Sterility*, 61(2), pp. 355-9.
- Jørgensen, N., Andersen, A.-G., Eustache, F., Irvine, D.S., Suominen, J., Petersen, J.H., Andersen, A.N., Auger, J., Cawood, E.H.H., Horte, A., Jensen, T.K., Jouannet, P., Keiding, N., Vierula, M., Toppari, J. and Skakkebaek, N.E. (2001) 'Regional differences in semen quality in Europe', *Human Reproduction*, 16(5), pp. 1012-1019.
- Kamycheva, E., Joakimsen, R.M. and Jorde, R. (2003) 'Intakes of calcium and vitamin d predict body mass index in the population of Northern Norway', *Journal of Nutrition*, 133(1), pp. 102-106.
- Keel, B., Stembridge, T., Pineda, G. and Serafy, N.S. (2002) 'Lack of standardization in performance of the semen analysis among laboratories in the United States.', *Fertility and Sterility*, 78(3), pp. 603-608.
- Kinuta, K., Tanaka, H., Moriwake, T., Aya, K., Kato, S. and Seino, Y. (2000) 'Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads.', *Endocrinology*, 141(4), pp. 1317-1324.
- Knight, J.A., Wong, J., Blackmore, K.M., Raboud, J.M. and Vieth, R. (2010) 'Vitamin D association with estradiol and progesterone in young women', *Cancer Causes and Control*, 21(3), pp. 479-483.

Knopf, J., Gordetsky, J., van Wijngaarden, E. and O'Brien, J. (2011) 'The relationship between vitamin D and testosterone values in men referred for infertility', *The Journal of Urology*, 185(4, Supplement), p. e801.

Kovacs, C.S., Woodland, M.L., Fudge, N.J. and Friel, J.K. (2005) 'The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice', *American Journal of Physiology Endocrinology and Metabolism*, 289(1), pp. E133-144.

Krause, A. and Krause, W. (2002) 'Seasonal variations in human seminal parameters', *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 101(2), pp. 175-178.

Kwiecek, G.G., Petrie, G.I. and DeLuca, H.F. (1989b) '1,25-Dihydroxyvitamin D₃ restores fertility of vitamin D-deficient female rats', *American Journal of Physiology*, 256(4 Pt 1), pp. E483-487.

Kwiecek, G., Petrie, G. and DeLuca, H. (1989a) 'Vitamin D is necessary for reproductive functions of the male rat', *Journal of Nutrition*, 119(5), pp. 741-744.

Labopin, M. and Iacobelli, S. (2003) *Statistical guidelines for EBMT*. Available at: https://docs.google.com/viewer?a=v&q=cache:MRDsoSn35LoJ:portal.ebmt.org/sites/cli nt2/clint/Documents/StatGuidelines_oct2003.pdf+EBMT+statistical+guidelines&hl=en &gl=uk&pid=bl&srcid=ADGEESh6YpxuEBOU3GoN7btKJnTxwlfi4Gg4AlTKkT9GK IDb_VrHLyHYd300o-oxsIB7HeLQv148tpOD5YQKrXkkW8rIuWq19vQcp2ZLeysj63JW_6br5_M6YoYz mzOrvRgMoMo8Gi98&sig=AHIEtbQToMCgc3u0XLR2Q05L4-PUgNM1rw (Accessed: November 2012).

Larsen, L., Scheike, T., Jensen, T.K., Bonde, J.P., Ernst, E., Hjollund, N.H., Zhou, Y., Skakkebaek, N.E., Giwercman, A. and The Danish First Pregnancy Planner Study, T. (2000) 'Computer-assisted semen analysis parameters as predictors for fertility of men from the general population', *Human Reproduction*, 15(7), pp. 1562-1567.

Lee, D.M., Tajar, A., Pye, S.R., Boonen, S., Vanderschueren, D., Bouillon, R., O'Neill, T.W., Bartfai, G., Casanueva, F.F., Finn, J.D., Forti, G., Giwercman, A., Han, T.S.,

Huhtaniemi, I.T., Kula, K., Lean, M.E.J., Pendleton, N., Punab, M., Wu, F.C.W. and the, E.s.g. (2012) 'Association of hypogonadism with vitamin D status: the European Male Ageing Study', *European Journal of Endocrinology / European Federation of Endocrine Societies*, 166(1), pp. 77-85.

Lerchbaum, E. and Obermayer-Pietsch, B. (2012) 'Mechanisms in Endocrinology: Vitamin D and fertility: a systematic review', *European Journal of Endocrinology*, 166(5), pp. 765-778.

Leushuis, E., van der Steeg, J.W., Steures, P., Repping, S., Bossuyt, P.M., Blankenstein, M.A., Mol, B.W., van der Veen, F. and Hompes, P.G. (2010) 'Reproducibility and reliability of repeated semen analyses in male partners of subfertile couples', *Fertility and Sterility*, 94(7), pp. 2631-2635.

Levine, R. (1999) 'Seasonal variation of semen quality and fertility', *Scandinavian Journal of Work, Environment and Health*, 25(supplement 1), pp. 34-37.

Levine, R., Borsdon, B., Mathew, R., Brown, M., Stanley, B. and Starr, T. (1988) 'Deterioration of semen quality during summer in New Orleans', *Fertility and Sterility*, 49, pp. 900-907.

Levine, R., Brown, M., Bell, M., Shue, F., Greenberg, G. and Borsdon, B. (1992) 'Air-conditioned environments do not prevent deterioration of human semen quality during the summer', *Fertility and Sterility*, 57, pp. 1075-1083.

Levine, R., Mathew, R., Chenault, C., Brown, M., Hurtt, M., Bentley, K., Mohr, K. and Working, P. (1990) 'Differences in the quality of semen in outdoor workers during summer and winter', *New England Journal of Medicine*, 323, pp. 12-16.

Levitas, E., Lunenfeld, E., Weisz, N., Friger, M. and Har-Vardi, I. (2013) 'Seasonal variations of human sperm cells among 6455 semen samples: a plausible explanation of a seasonal birth pattern', *American Journal of Obstetrics and Gynecology*, 208(5), pp. 406.e1-406.e6.

Li, H.W., Brereton, R.E., Anderson, R.A., Wallace, A.M. and Ho, C.K. (2011) 'Vitamin D deficiency is common and associated with metabolic risk factors in patients with

polycystic ovary syndrome', *Metabolism: Clinical and Experimental*, 60(10), pp. 1475-1481.

Lips, B. (2006) 'Vitamin D physiology', *Progress in Biophysics and Molecular Biology*, 92, pp. 4-8.

Lips, P. (2001) 'Vitamin D Deficiency and Secondary Hyperparathyroidism in the Elderly: Consequences for Bone Loss and Fractures and Therapeutic Implications', *Endocrine Reviews*, 22(4), pp. 477-501.

Lips, P., Duong, T., Oleksik, A., Black, D., Cummings, S., Cox, D. and Nickelsen, T. (2001) 'A global study of vitamin D status and parathyroid function in postmenopausal women with osteoporosis: baseline data from the multiple outcomes of raloxifene evaluation clinical trial', *Journal of Clinical Endocrinology and Metabolism*, 86(3), pp. 1212-1221.

Luk, J., Torrealday, S., Neal Perry, G. and Pal, L. (2012) 'Relevance of vitamin D in reproduction', *Hum Reproduction*, 27(10), pp. 3015-3027.

Macdonald, H.M. (2013) 'Contributions of Sunlight and Diet to Vitamin D Status', *Calcified Tissue International*, 92(2), pp. 163-176.

Macleod, I.C., Irvine, D.S., Masterton, A., Taylor, A. and Templeton, A.A. (1994) 'Assessment of the conventional criteria of semen quality by computer-assisted image analysis: evaluation of the Hamilton–Thorn motility analyser in the context of a service andrology laboratory', *Human Reproduction*, 9(2), pp. 310-319.

Maestro, B., Davila, N., Carranza, M.C. and Calle, C. (2003) 'Identification of a Vitamin D response element in the human insulin receptor gene promoter', *Journal of Steroid Biochemistry and Molecular Biology*, 84(2-3), pp. 223-230.

Mahadevan, M.M. and Trounson, A.O. (1984) 'Relationship of fine structure of sperm head to fertility of frozen human semen', *Fertility and Sterility*, 41(2), pp. 287-293.

Mahato, D., Goulding, E.H., Korach, K.S. and Eddy, E.M. (2000) 'Spermatogenic Cells Do Not Require Estrogen Receptor- α for Development or Function', *Endocrinology*, 141(3), pp. 1273-1276.

Mallidis, C., Howard, E.J. and Baker, H.W. (1991) 'Variation of semen quality in normal men', *International Journal of Andrology*, 14(2), pp. 99-107.

Malloy, P.J., Peng, L., Wang, J. and Feldman, D. (2009) 'Interaction of the Vitamin D Receptor with a Vitamin D Response Element in the Müllerian-Inhibiting Substance (MIS) Promoter: Regulation of MIS Expression by Calcitriol in Prostate Cancer Cells', *Endocrinology*, 150(4), pp. 1580-1587.

Malm, G., Haugen, T.B., Henrichsen, T., Bjørsvik, C., Grotmol, T., Sæther, T., Malm, J., Figenschau, Y., Hagmar, L., Rylander, L., Levine, R.J. and Giwercman, A. (2004) 'Reproductive Function during Summer and Winter in Norwegian Men Living North and South of the Arctic Circle', *Journal of Clinical Endocrinology and Metabolism*, 89(9), pp. 4397-4402.

Marcus-Braun, N., Braun, G., Potashnik, G. and Har-Vardi, I. (2004) 'Effect of cryopreservation on quality and fertilization capacity of human sperm', *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 116(1), pp. 63-66.

Matsuoka, L.Y., Ide, L., Wortsman, J., MacLaughlin, J. and Holick, M.F. (1987) 'Sunscreens suppress cutaneous vitamin D₃ synthesis.', *Journal of Clinical Endocrinology and Metabolism*, 64(6), pp. 1165-1168.

Mayaux, M. and Spira, A. (1989) 'Seasonal distribution in conceptions achieved by artificial insemination by donor.', *British Medical Journal*, 298, p. 187.

McKinney, K., Breitkopf, C.R. and Berenson, A.B. (2008) 'Association of race, body fat and season with vitamin D status among young women: a cross-sectional study', *Clinical Endocrinology*, 69(4), pp. 535-541.

McWhinnie, A. (2001) 'Gamete donation and anonymity', *Human Reproduction*, 16(5), pp. 807-817.

- Meeker, J.D., Godfrey-Bailey, L. and Hauser, R. (2006) 'Relationships between serum hormone levels and semen quality among men from an infertility clinic', *Journal of Andrology*, 28(3), pp. 397-406.
- Mendoza, F.J., Perez-Marin, C.C., Garcia-Marin, L., Madueno, J.A., Henley, C., Aguilera-Tejero, E. and Rodriguez, M. (2012) 'Localization, distribution, and function of the calcium-sensing receptor in sperm', *Journal of Andrology*, 33(1), pp. 96-104.
- Menegaz, D., Barrientos-Duran, A., Kline, A., Silva, F.R.M.B., Norman, A.W., Mizwicki, M.T. and Zanello, L.P. (2010) ' $1\alpha,25(\text{OH})_2$ -Vitamin D₃ stimulation of secretion via chloride channel activation in Sertoli cells', *The Journal of Steroid Biochemistry and Molecular Biology*, 119(3-5), pp. 127-134.
- Menegaz, D., Rosso, A., Royer, C., Leite, L.D., Santos, A.R. and Silva, F.R. (2009) 'Role of $1\alpha,25(\text{OH})_2$ vitamin D₃ on alpha-[1-(14)C]MeAIB accumulation in immature rat testis', *Steroids*, 74(2), pp. 264-269.
- Menge, A.C. and Beitner, O. (1989) 'Interrelationships among semen characteristics, antisperm antibodies, and cervical mucus penetration assays in infertile human couples', *Fertility and Sterility*, 51(3), pp. 486-492.
- Merke, J., Hugel, U. and Ritz, E. (1985) 'Nuclear testicular $1,25$ -dihydroxyvitamin D₃ receptors in Sertoli cells and seminiferous tubules of adult rodents', *Biochemical and Biophysical Research Communications*, 127(1), pp. 303-309.
- Mieusset, R. and Bujan, L. (1994) 'The potential of mild testicular heating as a safe, effective and reversible contraceptive method for men', *International Journal of Andrology*, 17(4), pp. 186-191.
- Monticcielo, O., Teixeira, T., Chies, J., Brenol, J. and Xavier, R. (2012) 'Vitamin D and polymorphisms of VDR gene in patients with systemic lupus erythematosus', *Clinical Rheumatology*, 31(10), pp. 1411-1421.
- Mordan-McCombs, S., Brown, T., Wang, W.L., Gaupel, A.C., Welsh, J. and Tenniswood, M. (2010) 'Tumor progression in the LPB-Tag transgenic model of

prostate cancer is altered by vitamin D receptor and serum testosterone status', *Journal of Steroid Biochemistry and Molecular Biology*, 121(1-2), pp. 368-371.

Mortimer, D., Templeton, A.A., Lenton, E.A. and Coleman, R.A. (1982) 'Influence of Abstinence and Ejaculation-to-Analysis Delay on Semen Analysis Parameters of Suspected Infertile Men', *Systems Biology in Reproductive Medicine*, 8(4), pp. 251-256.

Mortimer, D., Templeton, A.A., Lenton, E.A. and Coleman, R.A. (1983) 'Annual Patterns of Human Sperm Production and Semen Quality', *Systems Biology in Reproductive Medicine*, 10(1), pp. 1-5.

Moskovtsev, S.I. and Mullen, B.M. (2005) 'The influence of seasonal variation on semen parameters and DNA integrity', *Fertility and Sterility*, 84(Supplement 1), pp. S218-S219.

Nangia, A., Hill, O., Waterman, M., Schwender, C. and Memoli, V. (2007) 'Testicular maturation arrest to testis cancer: spectrum of expression of the vitamin D receptor and vitamin D treatment in vitro', *Journal of Urology*, 178, pp. 1092-1096.

Navarrete, T., Johnson, A., Mixon, B. and Wolf, D. (2000) 'The relationship between fertility potential measurements on cryobanked semen and fecundity of sperm donors', *Human Reproduction*, 15(2), pp. 344-350.

Nejentsev, S., Godfrey, L., Snook, H., Rance, H., Nutland, S., Walker, N.M., Lam, A.C., Guja, C., Ionescu-Tirgoviste, C., Undlien, D.E., Rønningen, K.S., Tuomilehto-Wolf, E., Tuomilehto, J., Newport, M.J., Clayton, D.G. and Todd, J.A. (2004) 'Comparative high-resolution analysis of linkage disequilibrium and tag single nucleotide polymorphisms between populations in the vitamin D receptor gene', *Human Molecular Genetics*, 13(15), pp. 1633-1639.

NICE (2008) *CG62 Antenatal care NICE guideline*. Available at: <http://guidance.nice.org.uk/CG62/NICEGuidance/pdf/English> (Accessed: June 2010).

NICE (2013) *Fertility: assessment and treatment for people with fertility problems*. Available at: <http://www.nice.org.uk/nicemedia/live/14078/62770/62770.pdf> (Accessed: May 2013).

Nimptsch, K., Platz, E.A., Willett, W.C. and Giovannucci, E. (2012) 'Association between plasma 25-OH vitamin D and testosterone levels in men', *Clinical Endocrinology*, 77(1), pp. 106-112.

Olsen, J., Zhu, J.L. and Ramlau-Hansen, C.H. (2011) 'Has fertility declined in recent decades?', *Acta Obstetrica et Gynecologica Scandinavica*, 90(2), pp. 129-135.

Ombelet, W., Maes, M., Vandeput, H., Cox, A., Janssen, M., Pollet, H., Fourie, F.L.R., Steeno, O. and Bosmans, E. (1996) 'Chronobiological Fluctuations in Semen Parameters with a Constant Abstinence Period', *Systems Biology in Reproductive Medicine*, 37(2), pp. 91-96.

Ombelet, W., Pollet, H., Bosmans, E. and Vereecken, A. (1997) 'Results of a questionnaire on sperm morphology assessment', *Human Reproduction*, 12(5), pp. 1015-1020.

Osmundsen, B.C., Huang, H.F., Anderson, M.B., Christakos, S. and Walters, M.R. (1989) 'Multiple sites of action of the vitamin D endocrine system: FSH stimulation of testis 1,25-dihydroxyvitamin D₃ receptors', *Journal of Steroid Biochemistry*, 34(1-6), pp. 339-343.

Ossenbühn, S. (1998) 'Exogenous influences on human fertility: fluctuations in sperm parameters and results of in-vitro fertilization coincide with conceptions in the normal population', *Human Reproduction*, 13(8), pp. 2165-2171.

Ozkan, S., Jindal, S., Greenseid, K., Shu, J., Zeitlian, G., Hickmon, C. and Pal, L. (2010) 'Replete vitamin D stores predict reproductive success following in vitro fertilization', *Fertility and Sterility*, 94(4), pp. 1314-1319.

Pacey, A. (2010) 'Sperm donor recruitment in the UK', *The Obstetrician & Gynaecologist*, 12(1), pp. 43-48.

Pacey, A.A. (2006) 'Is quality assurance in semen analysis still really necessary? A view from the andrology laboratory', *Human Reproduction*, 21(5), pp. 1105-1109.

Panda, D.K., Miao, D., Tremblay, M.L., Sirois, J., Farookhi, R., Hendy, G.N. and Goltzman, D. (2001) 'Targeted ablation of the 25-hydroxyvitamin D 1alpha - hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction', *Proceedings of the National Academy of Sciences of the United States of America*, 98(13), pp. 7498-7503.

Paraskevaides, E.C., Pennington, G.W. and Naik, S. (1988) 'Seasonal distribution in conceptions achieved by artificial insemination by donor.', *British Medical Journal*, 1988 (297 (6659)), pp. 1309–1310.

Paraskevaides, E.C., Pennington, G.W., Naik, S. and Gibbs, A.A. (1991) 'Pre-freeze/post-freeze semen motility ratio', *The Lancet*, 337(8737), pp. 366-367.

Parikh, G., Varadinova, M., Suwandhi, P., Araki, T., Rosenwaks, Z., Poretsky, L. and Seto-Young, D. (2010) 'Vitamin D regulates steroidogenesis and insulin-like growth factor binding protein-1 (IGFBP-1) production in human ovarian cells.', *Hormone and Metabolic Research*, 42(10), pp. 754-757.

Paul, S., Harbottle, S. and Stewart, J.A. (2006) 'Recruitment of sperm donors: the Newcastle-upon-Tyne experience 1994–2003', *Human Reproduction*, 21(1), pp. 150-158.

Pearce, S.H.S. and Cheetam, T.D. (2010) 'Diagnosis and Management of vitamin D deficiency', *British Medical Journal*, 340(b5664), pp. 142-147.

Pellestor, F., Girardet, A. and Andreo, B. (1994) 'Effect of long abstinence periods on human sperm quality.', *International Journal of Fertility and Menopausal Studies*, 39(5), pp. 278-282.

Pennings, G. (1997) 'The internal coherence of donor insemination practice: attracting the right type of donor without paying.', *Human Reproduction*, 12(9), pp. 1842-1844.

Pennings, G. (2010) 'The rough guide to insemination : Cross border travelling for donor semen due to different regulations ', *Facts, Views & Vision in OBGYN*, pp. 55-60.

- Pérez-Fernandez, R., Alonso, M., Segura, C., Muñoz, I., Garcia-Caballero, T. and Diéguez, C. (1996) 'Vitamin D receptor gene expression in human pituitary gland', *Life Sciences*, 60(1), pp. 35-42.
- Pike, S. and Pacey, A. (2005) 'Sperm donation: Coping with change', *Human Fertility*, 8(3), pp. 173-174.
- Pilz, S., Frisch, S., Koertke, H., Kuhn, J., Dreier, J., Obermayer-Pietsch, B., Wehr, E. and Zittermann, A. (2011) 'Effect of vitamin D supplementation on testosterone levels in men', *Hormone and Metabolic Research*, 43(3), pp. 223-225.
- Pilz, S., März, W., Wellnitz, B., Seelhorst, U., Fahrleitner-Pammer, A., Dimai, H.P., Boehm, B.O. and Dobnig, H. (2008) 'Association of Vitamin D Deficiency with Heart Failure and Sudden Cardiac Death in a Large Cross-Sectional Study of Patients Referred for Coronary Angiography', *Journal of Clinical Endocrinology and Metabolism*, 93(10), pp. 3927-3935.
- Politoff, L., Birkhauser, M., Almendral, A. and Zorn, A. (1989) 'New data confirming a circannual rhythm in spermatogenesis.', *Fertility and Sterility*, 52(3), pp. 486-489.
- Pols, H.A.P., Uitterlinden, A.G. and van Leeuwen, J.P.T.M. (1998) 'How About Vitamin D Receptor Polymorphisms?', *Osteoporosis International*, 8(8), pp. S020-S023.
- Povey, A.C., Clyma, J.A., McNamee, R., Moore, H.D., Baillie, H., Pacey, A.A. and Cherry, N.M. (2012) 'Modifiable and non-modifiable risk factors for poor semen quality: a case-referent study', *Human Reproduction*, 27(9), pp. 2799-2806.
- Prisant, N., Cohen-Bacrie, P., Amar, E., Belaisch-Allart, J., Cohen-Bacrie, M., Olivennes, F., Aubriot, F. and Belloc, S. (2011) 'Teratozoospermia, myth or reality? A 10-years retrospective study on 101404 consecutive sperm samples (Article in French)', *Gynaecologie,obstetrique & fertilité*, 39(3), pp. 136-140.
- Priskorn, L., Holmboe, S.A., Jacobsen, R., Jensen, T.K., Lassen, T.H. and Skakkebaek, N.E. (2012) 'Increasing trends in childlessness in recent birth cohorts – a registry-based study of the total Danish male population born from 1945 to 1980', *International Journal of Andrology*, 35(3), pp. 449-455.

- Prosser, D.E. and Jones, G. (2004) 'Enzymes involved in the activation and inactivation of vitamin D', *Trends in Biochemical Sciences*, 29(12), pp. 664-673.
- Ramlau-Hansen, C., Moeller, U., Bonde, J., Olsen, J. and Thulstrup, A. (2011) 'Are serum levels of vitamin D associated with semen quality? Results from a cross-sectional study in young healthy men.', *Fertility and Sterility*, 95(3), pp. 1000-1004.
- Reichrath, J., Lehmann, B., Carlberg, C., Varani, J. and Zouboulis, C. (2007) 'Vitamins as hormones', *Hormone and Metabolic Research*, 39(2), pp. 71-84.
- Reinberg, A., Smolensky, M., Hallek, M., Smith, K. and Steinberger, E. (1988) 'Annual variation in semen characteristics and plasma hormone levels in men undergoing vasectomy.', *Fertility and Sterility*, 49(2), pp. 309-315.
- Riddell, D., Pacey, A. and Whittington, K. (2005) 'Lack of compliance by UK andrology laboratories with World Health Organization recommendations for sperm morphology assessment', *Human Reproduction*, 20(12), pp. 3441-3445.
- Riggs, D.W. and Russell, L. (2011) 'Characteristics of men willing to act as sperm donors in the context of identity-release legislation', *Human Reproduction*, 26(1), pp. 266-272.
- Rojansky, N., Brzezinski, A. and Schenker, J.G. (1992) 'Seasonality in human reproduction: an update', *Human Reproduction*, 7(6), pp. 735-745.
- Ronnberg, L. (1989) 'Seasonal distribution in conceptions achieved by artificial insemination by donor', *British Medical Journal*, 298, p. 187.
- Rosen, C.J., Adams, J.S., Bikle, D.D., Black, D.M., Demay, M.B., Manson, J.E., Murad, M.H. and Kovacs, C.S. (2012) 'The Nonskeletal Effects of Vitamin D: An Endocrine Society Scientific Statement', *Endocrine Reviews*, 33(3), pp. 456-492.
- Ross, A.C., Manson, J.E., Abrams, S.A., Aloia, J.F., Brannon, P.M., Clinton, S.K., Durazo-Arvizu, R.A., Gallagher, J.C., Gallo, R.L., Jones, G., Kovacs, C.S., Mayne, S.T., Rosen, C.J. and Shapses, S.A. (2011) 'The 2011 Report on Dietary Reference

Intakes for Calcium and Vitamin D from the Institute of Medicine: What Clinicians Need to Know', *Journal of Clinical Endocrinology and Metabolism*, 96(1), pp. 53-58.

Roth, H., Schmidt-Gayk, H., Weber, H. and Niederau, C. (2008) 'Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography-tandem mass spectrometry as a reference.', *Annals of Clinical Biochemistry*, 45 (Pt 2), pp. 153-159.

Russell, D., Douglas, A.S. and Allan, T.M. (1993) 'Changing seasonality of birth--a possible environmental effect', *Journal of Epidemiology and Community Health*, 47(5), pp. 362-367.

Sahin, S., Cetinkalp, S., Erdogan, M., Yilmaz, C. and Berdeli, A. (2012) 'Fas, Fas Ligand, and Vitamin D Receptor FokI Gene Polymorphisms in Patients with Type 1 Diabetes Mellitus in the Aegean Region of Turkey', *Genetic testing and molecular biomarkers*, 16(10), pp. 1179-1183.

Saint Pol, P., Beuscart, R., Leroy-Martin, B., Hermand, E. and Jablonski, W. (1989) 'Circannual rhythms of sperm parameters of fertile men.', *Fertility and Sterility*, 51(6), pp. 1030-1033.

Sánchez-Pozo, M.C., Mendiola, J., Serrano, M., Mozas, J., Björndahl, L., Menkveld, R., Lewis, S.E.M., Mortimer, D., Jørgensen, N., Barratt, C.L.R., Fernández, M.F. and Castilla, J.A. (2013) 'Proposal of guidelines for the appraisal of SEMen QUALity studies (SEMQUA)', *Human Reproduction*, 28(1), pp. 10-21.

Sanders, K., Stuart, A., Williamson, E., Simpson, J., Kotowicz, M., Young, D. and Nicholson, G. (2010) 'Annual high-dose oral vitamin d and falls and fractures in older women: A randomized controlled trial', *JAMA: The Journal of the American Medical Association*, 303(18), pp. 1815-1822.

Scheib, J. and Cushing, R. (2007) 'Open-identity donor insemination in the United States: is it on the rise?', *Fertility and Sterility*, 88(1), pp. 231-232.

SEED Report (2005) *A report on the Human Fertilisation & Embryology Authority's review of sperm, egg and embryo donation in the United Kingdom*. Available at: <http://www.hfea.gov.uk/534.html> (Accessed: June 2010).

Selimoglu, H., Duran, C., Kiyici, S., Ersoy, C., Guclu, M., Ozkaya, G., Tuncel, E., Erturk, E. and Imamoglu, S. (2010) 'The effect of vitamin D replacement therapy on insulin resistance and androgen levels in women with polycystic ovary syndrome', *Journal of Endocrinological Investigation*, 33(4), pp. 234-238.

Selva, D.M., Hirsch-Reinshagen, V., Burgess, B., Zhou, S., Chan, J., McIsaac, S., Hayden, M.R., Hammond, G.L., Vogl, A.W. and Wellington, C.L. (2004) 'The ATP-binding cassette transporter 1 mediates lipid efflux from Sertoli cells and influences male fertility', *Journal of Lipid Research*, 45(6), pp. 1040-1050.

Sharma, R.K., Vemulapalli, S., Kohn, S. and Agarwal, A. (1997) 'Effect of Centrifuge Speed, Refrigeration Medium, and Sperm Washing Medium on Cryopreserved Sperm Quality After Thawing', *Systems Biology in Reproductive Medicine*, 39(1), pp. 33-38.

Shenfield, F. (1994) 'Ethics and Society: Filiation in assisted reproduction: potential conflicts and legal implications', *Human Reproduction*, 9(7), pp. 1348-1354.

Shenfield, F., de Mouzon, J., Pennings, G., Ferraretti, A.P., Nyboe Andersen, A., de Wert, G., Goossens, V. and the, E.T.o.C.B.R.C. (2010) 'Cross border reproductive care in six European countries', *Human Reproduction*, 25(6), pp. 1361-1368.

Siegal, S. and John jr, N. (1988) *Non Parametric statistics for the behavioural sciences*. 2nd edn. New York: McGraw-Hill.

Sinclair, K. (2009) *NHS Grampian appeals for donated sperm and eggs on staff payslips*
Available at: http://www.bionews.org.uk/page_13655.asp (Accessed: August 2011).

Snowden, R. (1993) 'Sharing Information about DI in the UK', *Politics and the Life Sciences*, 12(2), pp. 194-195.

Snyder, P. (1990) 'Fewer sperm in the summer--it's not the heat, it's...!', *New England Journal of Medicine*, 323(1), pp. 54-56.

Sobreiro, B.P., Lucon, A.M., Pasqualotto, F.F., Hallak, J., Athayde, K.S. and Arap, S. (2005) 'Semen analysis in fertile patients undergoing vasectomy: Reference values and variations according to age, length of sexual abstinence, seasonality, smoking habits and caffeine intake', *Sao Paulo Medical Journal*, 123(4), pp. 161-166.

Somigliana, E., Panina-Bordignon, P., Murone, S., Di Lucia, P., Vercellini, P. and Vigano, P. (2007) 'Vitamin D reserve is higher in women with endometriosis', *Human Reproduction*, 22(8), pp. 2273-2278.

Somjen, D., Katzburg, S., Stern, N., Kohen, F., Sharon, O., Limor, R., Jaccard, N., Hendel, D. and Weisman, Y. (2007) '25 hydroxy-vitamin D3-1 α hydroxylase expression and activity in cultured human osteoblasts and their modulation by parathyroid hormone, estrogenic compounds and dihydrotestosterone', *The Journal of Steroid Biochemistry and Molecular Biology*, 107(3-5), pp. 238-244.

Sood, S., Marya, R.K., Reghunandanan, R., Singh, G.P.T.S., Jaswal, T.S. and Gopinathan, K. (1992) 'Effect of Vitamin D Deficiency on Testicular Function in the Rat', *Annals of Nutrition and Metabolism*, 36(4), pp. 203-208.

Sood, S., Reghunandanan, R., Reghunandanan, V., Marya, R. and Singh, P. (1995) 'Effect of vitamin D repletion on testicular function in vitamin D-deficient rats', *Annals of Nutrition and Metabolism*, 39(2), pp. 95-98.

Souberbielle, J.-C., Friedlander, G., Kahan, A. and Cormier, C. (2006) 'Evaluating vitamin D status. Implications for preventing and managing osteoporosis and other chronic diseases', *Joint, Bone, Spine: Revue du Rhumatisme*, 7(3), pp. 249-253.

Spallone, P. (1986) 'The Warnock report: The politics of reproductive technology', *Women's Studies International Forum*, 9(5-6), pp. 543-550.

Spiropoulos, J. (2001) 'Computerized Semen Analysis (CASA): Effect of semen concentration and chamber depth on measurements ', *Systems Biology in Reproductive Medicine*, 46(1), pp. 37-42.

Splingart, C., Frapsauce, C., Veau, S., Barthélémy, C., Royère, D. and Guérif, F. (2012) 'Semen variation in a population of fertile donors: evaluation in a French centre over a 34-year period', *International Journal of Andrology*, 35(3), pp. 467-474.

Sripada, S., Fonseca, S., Lee, A., Harrild, K., Giannaris, D., Mathers, E. and Bhattacharya, S. (2007) 'Trends in Semen Parameters in the Northeast of Scotland', *Journal of Andrology*, 28(2), pp. 313-319.

Stanic, P., Tandara, M., Sonicki, Z., Simunic, V., Radakovic, B. and Suchanek, E. (2000) 'Comparison of protective media and freezing techniques for cryopreservation of human semen', *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 91(1), pp. 65-70.

Stephanou, A., Ross, R. and Handwerger, S. (1994) 'Regulation of human placental lactogen expression by 1,25-dihydroxyvitamin D₃', *Endocrinology*, 135(6), pp. 2651-2656.

Sun, W., Xie, H., Ji, J., Zhou, X., Goltzman, D. and Miao, D. (2010) 'Defective female reproductive function in 1,25(OH)₂D-deficient mice results from indirect effect mediated by extracellular calcium and/or phosphorus', *American Journal of Physiology - Endocrinology and Metabolism*, 299(6), pp. 928-935.

Swatowski, D., Robak-Cholubek, D., Bakalczuk, S., Jakiel, G., Osinska-Stepien, J. and Przytula-Pilat, M. (1994) '[Seasonal changes in results of semen analysis from male members of an infertile married couple]', *Ginekologia Polska*, 65(1), pp. 29-34.

Tanamura, A., Nomura, S., Kurauchi, O., Furui, T., Mizutani, S. and Tomoda, Y. (1995) 'Purification and characterization of 1,25(OH)₂D₃ receptor from human placenta', *Journal of Obstetrics and Gynaecology (Tokyo)* 1995, 21(6), pp. 631-639.

Tangpricha, V., Turner, A., Spina, C., Decastro, S., Chen, T.C. and Holick, M.F. (2004) 'Tanning is associated with optimal vitamin D status (serum 25-hydroxyvitamin D concentration) and higher bone mineral density', *The American Journal of Clinical Nutrition*, 80(6), pp. 1645-1649.

Thienpont, L.M., Stepman, H.C.M. and Vesper, H.W. (2012) 'Standardization of measurements of 25-Hydroxyvitamin D3 and D2', *Scandinavian Journal of Clinical and Laboratory Investigation*, 72(S243), pp. 41-49.

Thomas, S., Fudge, A., Whiting, M. and Coates, P. (2011) 'The correlation between third-trimester maternal and newborn-serum 25-hydroxy-vitamin D in a selected South Australian group of newborn samples', *British Medical Journal Open*, 1(2).

Thyer, A.C., Patton, P.E., Burry, K.A., Mixon, B.A. and Wolf, D.P. (1999) 'Fecundability trends among sperm donors as a measure of donor performance', *Fertility and Sterility*, 71(5), pp. 891-895.

Tiwari, A. (2009) 'Elocalcitol, a vitamin D3 analog for the potential treatment of benign prostatic hyperplasia, overactive bladder and male infertility.', *IDrugs*, 12(6), pp. 381-393.

Tjoa, W., Smolensky, M., Hsi, B., Steinberger, E. and Smith, K. (1982) 'Circannual rhythm in human sperm count revealed by serially independent sampling.', *Fertility and Sterility*, 38(4), pp. 454-459.

Tomlinson, M., Lewis, S. and Morroll, D. (2013) 'Sperm quality and its relationship to natural and assisted conception: British Fertility Society Guidelines for practice', *Human Fertility*, 16(3), pp. 175-193.

Tomlinson, M.J., Pooley, K., Pierce, A. and Hopkisson, J.F. (2010) 'Sperm donor recruitment within an NHS fertility service since the removal of anonymity', *Human Fertility*, 13(3), pp. 159-167.

Toner, C.D., Davis, C.D. and Milner, J.A. (2010) 'The Vitamin D and Cancer Conundrum: Aiming at a Moving Target', *Journal of the American Dietetic Association*, 110(10), pp. 1492-1500.

Turner, A.J. and Coyle, A. (2000) 'What does it mean to be a donor offspring? The identity experiences of adults conceived by donor insemination and the implications for counselling and therapy', *Human Reproduction*, 15(9), pp. 2041-2051.

Uhland, A., Kwiecinski, G. and DeLuca, H. (1992) 'Normalization of serum calcium restores fertility in vitamin D-deficient male rats', *Journal of Nutrition*, 122(6), pp. 1338-44.

Van den Akker, O. (2006) 'A review of family donor constructs: Current research and future directions', *Human Reproduction Update*, 12(2), pp. 91-101.

Verstuyf, A., Carmeliet, G., Bouillon, R. and Mathieu, C. (2010) 'Vitamin D: a pleiotropic hormone', *Kidney International*, 78(2), pp. 140-145.

Vilarino, F.L., Bianco, B., Lerner, T.G., Teles, J.S., Mafra, F.A., Christofolini, D.M. and Barbosa, C.P. (2011) 'Analysis of vitamin D receptor gene polymorphisms in women with and without endometriosis', *Human Immunology*, 72(4), pp. 359-363.

Vutyavanich, T., Piromlertamorn, W. and Nunta, S. (2010) 'Rapid freezing versus slow programmable freezing of human spermatozoa', *Fertility and Sterility*, 93(6), pp. 1921-1928.

Wactawski-Wende, J., Kotchen, J.M., Anderson, G.L., Assaf, A.R., Brunner, R.L., O'Sullivan, M.J., Margolis, K.L., Ockene, J.K., Phillips, L., Pottern, L., Prentice, R.L., Robbins, J., Rohan, T.E., Sarto, G.E., Sharma, S., Stefanick, M.L., Van Horn, L., Wallace, R.B., Whitlock, E., Bassford, T., Beresford, S.A.A., Black, H.R., Bonds, D.E., Brzyski, R.G., Caan, B., Chlebowski, R.T., Cochrane, B., Garland, C., Gass, M., Hays, J., Heiss, G., Hendrix, S.L., Howard, B.V., Hsia, J., Hubbell, F.A., Jackson, R.D., Johnson, K.C., Judd, H., Kooperberg, C.L., Kuller, L.H., LaCroix, A.Z., Lane, D.S., Langer, R.D., Lasser, N.L., Lewis, C.E., Limacher, M.C. and Manson, J.E. (2006) 'Calcium plus vitamin D supplementation and the risk of colorectal cancer', *New England Journal of Medicine*, 354(7), pp. 684-696.

Wang, T.J., Zhang, F., Richards, J.B., Kestenbaum, B., van Meurs, J.B., Berry, D., Kiel, D.P., Streeten, E.A., Ohlsson, C., Koller, D.L., Peltonen, L., Cooper, J.D., O'Reilly, P.F., Houston, D.K., Glazer, N.L., Vandenput, L., Peacock, M., Shi, J., Rivadeneira, F., McCarthy, M.I., Anneli, P., de Boer, I.H., Mangino, M., Kato, B., Smyth, D.J., Booth, S.L., Jacques, P.F., Burke, G.L., Goodarzi, M., Cheung, C.-L., Wolf, M., Rice, K., Goltzman, D., Hidioglou, N., Ladouceur, M., Wareham, N.J., Hocking, L.J., Hart, D., Arden, N.K., Cooper, C., Malik, S., Fraser, W.D., Hartikainen, A.-L., Zhai, G.,

Macdonald, H.M., Forouhi, N.G., Loos, R.J.F., Reid, D.M., Hakim, A., Dennison, E., Liu, Y., Power, C., Stevens, H.E., Jaana, L., Vasan, R.S., Soranzo, N., Bojunga, J., Psaty, B.M., Lorentzon, M., Foroud, T., Harris, T.B., Hofman, A., Jansson, J.-O., Cauley, J.A., Uitterlinden, A.G., Gibson, Q., Järvelin, M.-R., Karasik, D., Siscovick, D.S., Econs, M.J., Kritchevsky, S.B., Florez, J.C., Todd, J.A., Dupuis, J., Hyppönen, E. and Spector, T.D. (2010) 'Common genetic determinants of vitamin D insufficiency: a genome-wide association study', *The Lancet*, 376(9736), pp. 180-188.

Wardle, P. (2008) *The real impact of removal of donor anonymity*. Available at: http://www.bionews.org.uk/page_37975.asp (Accessed: August 2011).

Webb, A.R., Kline, L. and Holick, M.F. (1988) 'Influence of Season and Latitude on the Cutaneous Synthesis of Vitamin D₃: Exposure to Winter Sunlight in Boston and Edmonton Will Not Promote Vitamin D₃ Synthesis in Human Skin', *Journal of Clinical Endocrinology and Metabolism*, 67(2), pp. 373-378.

Wehr, E., Pilz, S., Boehm, B., März, W. and Obermayer-Pietsch, B. (2010a) 'Association of vitamin D status with serum androgen levels in men.', *Clinical Endocrinology*, 73(2), pp. 243-248.

Wehr, E., Pilz, S., Boehm, B.O., Marz, W., Grammer, T.B. and Obermayer-Pietsch, B. (2010b) 'Sex steroids and mortality in men referred for coronary angiography', *Clinical Endocrinology*, 73(5), pp. 613-621.

Wehr, E., Pilz, S., Schweighofer, N., Giuliani, A., Kopera, D., Pieber, T.R. and Obermayer-Pietsch, B. (2009) 'Association of hypovitaminosis D with metabolic disturbances in polycystic ovary syndrome', *European Journal of Endocrinology of the European Federation of Endocrine Societies*, 161(4), pp. 575-582.

Wehr, E., Trummer, O., Giuliani, A., Gruber, H.J., Pieber, T.R. and Obermayer-Pietsch, B. (2011) 'Vitamin D-associated polymorphisms are related to insulin resistance and vitamin D deficiency in polycystic ovary syndrome', *European Journal of Endocrinology of the European Federation of Endocrine Societies*, 164(5), pp. 741-749.

Wehr, T.A. (2001) 'Photoperiodism in Humans and Other Primates: Evidence and Implications', *Journal of Biological Rhythms*, 16(4), pp. 348-364.

Wehr, T.A., Giesen, H.A., Moul, D.E., Turner, E.H. and Schwartz, P.J. (1995) 'Suppression of men's responses to seasonal changes in day length by modern artificial lighting', *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 269(1), pp. R173-R178.

Wellings, K., Macdowall, W., Catchpole, M. and Goodrich, J. (1999) 'Seasonal variations in sexual activity and their implications for sexual health promotion', *Journal of the Royal Society of Medicine*, 92(2), pp. 60-64.

Witjens, L. (2007) *Recruiting sperm donors: why it's time for a new approach*
Available at: http://www.bionews.org.uk/page_37926.asp (Accessed: August 2011).

World Health Organisation (1999) *WHO laboratory manual for the examination of human semen and sperm - cervical mucus interaction*. Fourth edn. Cambridge University Press.

World Health Organisation (2010) *WHO laboratory manual for the Examination and processing of human semen*. Fifth edn.

Yang, B., Sun, H., Wan, Y., Wang, H., Qin, W., Yang, L., Zhao, H., Yuan, J. and Yao, B. (2012) 'Associations between testosterone, bone mineral density, vitamin D and semen quality in fertile and infertile Chinese men', *International Journal of Andrology*, 35(6), pp. 783-792.

Yee, S. (2009) 'Gift without a price tag': altruism in anonymous semen donation', *Human Reproduction*, 24(1), pp. 3-13.

Yildizhan, R., Kurdoglu, M., Adali, E., Kolusari, A., Yildizhan, B., Sahin, H.G. and Kamaci, M. (2009) 'Serum 25-hydroxyvitamin D concentrations in obese and non-obese women with polycystic ovary syndrome', *Archives of Gynecology and Obstetrics*, 280(4), pp. 559-63.

Yogev, L., Kleiman, S., Shabtai, E., Botchan, A., Gamzu, R., Paz, G., Yavetz, H. and Hauser, R. (2004) 'Seasonal variations in pre - and post - thaw donor sperm quality', *Human Reproduction*, 19(4), pp. 880-885.

Yogev, L., Kleiman, S.E., Shabtai, E., Botchan, A., Paz, G., Hauser, R., Lehavi, O., Yavetz, H. and Gamzu, R. (2010) 'Long-term cryostorage of sperm in a human sperm bank does not damage progressive motility concentration', *Human Reproduction*, 25(5), pp. 1097-1103.

Yogev, L., Paz, G., Kleiman, S.E., Shabtai, E., Gamzu, R., Botchan, A., Lehavi, O., Yavetz, H. and Hauser, R. (2012) 'Freezability and Semen Parameters in Candidates of Sperm Bank Donors: 1992–2010', *Journal of Andrology*, 33(5), pp. 999-1006.

Yoshida, M., Kawano, N. and Yoshida, K. (2008) 'Control of sperm motility and fertility: diverse factors and common mechanisms', *Cellular and Molecular Life Sciences*, 65(21), pp. 3446-3457.

Yoshizawa, T., Handa, Y., Uematsu, Y., Takeda, S., Sekine, K., Yoshihara, Y., Kawakami, T., Arioka, K., Sato, H., Uchiyama, Y., Masushige, S., Fukamizu, A., Matsumoto, T. and Kato, S. (1997) 'Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning', *Nature Genetics*, 16(4), pp. 391-396.

Yuen, A.W.C. and Jablonski, N.G. (2010) 'Vitamin D: In the evolution of human skin colour', *Medical Hypotheses*, 74(1), pp. 39-44.

Zanatta, L., Zamoner, A., Zanatta, A.P., Bouraïma-Lelong, H., Delalande, C., Bois, C., Carreau, S. and Silva, F.R.M.B. (2011) 'Nongenomic and genomic effects of $1\alpha,25(\text{OH})_2$ vitamin D₃ in rat testis', *Life Sciences*, 89(15–16), pp. 515-523.

Zhang, X. and Yao, K. (2010) 'Impact of seasonal variation on pre- and post-thaw donor semen parameters', *Zhonghua Nan Ke Xue*, 16(7), pp. 619-622.

Zhang, X., Zhou, Y., Xia, W., Wu, H., Yao, K., Liu, H. and Xiong, C. (2012) 'Effect of pre-freezing conditions on the progressive motility recovery rate of human frozen spermatozoa', *Andrologia*, 44(5), pp. 343-348.

Zhu, G.D. and Okamura, W.H. (1995) 'Synthesis of Vitamin D (Calciferol)', *Chemical Reviews*, 95(6), pp. 1877-1952.

