An integrated approach to the analysis of environmental factors that influence male reproductive health

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……

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Abstract

At least 30 million men are infertile around the world, identifying male factor infertility as a global health issue. In the past 70 years, evidence of a significant general decline in sperm quality has been reported, prompting concerns about the implications for reproductive health. Over the same period, there have been substantial changes in human lifestyles. New technologies, such as mobile phones and wi-fi, have been proposed to have a negative impact on a range of health outcomes, from an increased risk of cancer to a decrease in fertility. However, these links remain controversial. Over the last 30 years, the introduction of assisted reproductive technologies (ART) has offered infertile patients, particularly men with severe male factor infertility, a successful treatment option. However, miscarriage rates associated with fertility treatment can be as high as 30% and how this risk had changed over time was unclear. In addition, there are natural fluctuations in human health, including seasonal changes to birth rates. However, the clinical implications of these fluctuations need to be established. In this thesis, using an integrated approach that combined epidemiological research with laboratory investigations, I show that sperm quality is negatively affected by exposure to RF-EMR from mobile phones and wi-fi. I also identified a seasonal summer increase in sperm motility and morphology that followed patterns of seasonality in birth rates and in the success of assisted conception cycles. I showed that although the number of successful conceptions from ART has increased over time, there has been an equal increase in miscarriage rates. Male reproductive health continues to be under-researched when compared with the female, this inequality needs to be addressed in order to understand the causes of the decline in male fertility and the relationship this has with subsequent reproductive success.

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Abbreviations

ACTH – Adrenocorticotropic hormone

AR – Androgen receptor

ART – assisted reproductive technologies

BTB – Blood testes barrier

CASA – Computer aided sperm assessment

Chlamydia trachomatis – Chlamydia

CI – Confidence interval

CYP – Cytochrome P450

DBCP – 1,2-dibromo-3-chloropropane

DSB – Double strand break

DTT – Dithiothreitoll

EEG - Electroencephalography

EHS – Electrohypersensitivity

EMR – Electromagnetic radiation

EPL – Early pregnancy loss

FEM – Fixed effect model

GAM – Generalised additive model

GAMM – Generalised additive mixed model

GLM – Generalised linear model

GLMM – Generalised linear mixed model

GPX – Glutathione peroxidase

GR – Glutathione reductase

GST – Glutathione S-transferases

HFEA – Human fertilisation and embryology authority

HPLC – High performance liquid chromatography

HSD – Honest significant differences

ICNIRP – International commission on non-ionising radiation protection

ICSI – Intracytoplasmic sperm injection

ICSI – Intra-cytoplasmic sperm injection

IUI – Intrauterine insemination

IVF – *In vitro* fertilisation

KO – Knockout

LH – Luteinising hormone

LIS – Lithium diiodosalicyclate

MMS – Methyl methanesulfonate

MRM – Multiple reaction monitoring

PUFA – Polyunsaturated fatty acids

REM – Random effect model

RF-EMR – Radiofrequency electromagnetic radiation

ROS – Reactive oxygen species

SAR – Specific absorption rate

SCA – Sperm class analyser

SCN – Suprachiasmatic nucleus

SIM – Selective ion monitoring

SOD – Superoxide dismutase

TFR – Total fertility rates

TTP – Time to pregnancy

WHO – World Health Organisation

1. INTRODUCTION

Infertility is a widespread issue across the world. In the UK, 14 percent of heterosexual couples will have some difficulty with conception (Wilkes et al., 2009, Oakley et al., 2008, Hull et al., 1985, Templeton et al., 1996). Increasingly, couples are turning to fertility treatment (HFEA, 2014). In part, this is due to a socioeconomic shift in the age at which couples first attempt conception, as well as improved treatment, diagnosis, and reporting (HFEA, 2014). However, there is clear evidence to suggest other factors are leading to a true increase in the condition and a delay in time to pregnancy (TTP) (Rolland et al., 2013, Axmon et al., 2006, Juul et al., 1999). In 2010, there were 48.5 million couples worldwide that had not had a child after five years of trying (Mascarenhas et al., 2012). Infertility is defined as a failure to conceive after one to two years of regular unprotected intercourse (Zegers-Hochschild et al., 2009).

In July 1978, the first child was born following *in vitro* fertilisation (IVF)(Steptoe and Edwards, 1978). Subsequently, an inquiry was formed to consider 'the social, ethical and legal implications of these developments' and the Warnock Report was published in 1984 (Warnock, 1984). This report formed the basis of the HFE Act 1990 and recommendations for a statutory licensing body resulted in the Human Fertilisation and Embryology Authority (HFEA) beginning in 1991, licensing all fertility treatment in the UK (HFEA, 2014). Since this time, the number of treatment cycles carried out annually has increased dramatically, from 18, 338 cycles in 1992 (HFEA, 2008) to 64, 600 cycles in 2013 (HFEA, 2014).

Conventional IVF, (whereby ~100,000 spermatozoa are incubated with oocytes and left to fertilise naturally in culture media (Sutcliffe et al., 2001)), is not an efficient method of treatment in cases of severe male factor infertility. Compared with the use of IVF to treat other causes of infertility, the use of IVF in cases of severe male factor infertility results in lower fertilisation and pregnancy rates per cycle (Tournaye et al., 1992). For men with severe oligospermia, intra-cytoplasmic sperm injection (ICSI) provides an effective alternative. In this procedure, a single spermatozoon is directly injected into a mature oocyte, with the first successful pregnancies reported in 1992 (Palermo et al., 1992).

Since ICSI bypasses the barriers of natural sperm selection there were initial concerns that sperm selected for treatment that otherwise would have been unable to fertilise an oocyte, would lead to developmental issues in children born from ICSI (Sutcliffe et al., 2001). Early follow up studies on the development of children born from ICSI were hindered by low sample size, poorly matched controls (Bowen et al., 1998) or no controls at all (Bonduelle et al., 1999). However, subsequent studies have provided reassurance that there are no significant differences in physical health and cognitive development, when compared with ICSI and naturally conceived children (Sutcliffe et al., 2001, Ponjaert-Kristoffersen et al., 2005, Williams et al., 2013). However, one meta-analysis has suggested that the risk of birth defects, including defects in the nervous and genitourinary system, are significantly higher in children born from both IVF and ICSI, although there was no difference between the two methods of insemination (Wen et al., 2012). It is likely that this risk is due to the couples' underlying infertility, rather than the treatments themselves. A better control for these studies would be infertile couples who conceived without the use of ART (Wen et al., 2012).

1.1 Miscarriage

Even under optimal conditions, only 30% of conceptions will result in a livebirth per natural cycle (Macklon et al., 2002, Slama et al., 2002). From the rest, 30% will not achieve implantation, 30% will have an early pregnancy loss (EPL)(before 7 weeks gestation) and 10% will suffer from a spontaneous miscarriage (between 7-24 weeks gestation)(Chard, 1991). In cases of ART, where the woman is under greater surveillance, the incidence of spontaneous miscarriage is up to 30% (Wang et al., 2004). Between 1970-2000, self-reported miscarriage rates in the US rose steadily by ~1% per year in the general population. In part this may be due to earlier diagnosis of pregnancy but may also be caused by negative environmental exposures (Lang and Nuevo-Chiquero, 2012). It is possible that subfertile women are at greater risk of pregnancy loss, with EPL rates at 70% compared with just 21% in women without known fertility issues. This may suggest that EPL is a large cause of subfertility, regardless of fertility treatment (Hakim et al., 1995).

Maternal age is a known risk factor for miscarriage (Maconochie et al., 2007, Feodor Nilsson et al., 2014, Templeton et al., 1996, Bhattacharya et al., 2013, Nybo Andersen et al., 2000, de la Rochebrochard and Thonneau, 2002). Advanced maternal age has been associated with many adverse reproductive outcomes, including an increased risk of infertility, pregnancy complications, such as pre-term birth, and congenital abnormalities, including heart defects, when compared with younger women (Miller et al., 2011, Reefhuis and Honein, 2004, Cnattingius et al., 1992, Dunson et al., 2004). From 1978 to1998, the number of children born per 1000 women, has increased from 19 to 37.4 in women ages 35-39 years (Ventura et al., 1988, Guyer et al., 1999, de la

Rochebrochard and Thonneau, 2002). This demonstrates the social changes in reproductive choices, and increases the number of women at risk of poor reproductive outcomes.

Maternal age is associated with decrease in oocyte quality, in part due to an increase in chromosomal abnormalities, which have been linked with 35-75% of pregnancy loss in older mothers (te Velde and Pearson, 2002, Rai and Regan 2006, Baird et al., 2005, Ljunger et al., 2005). Alongside maternal age, stress, high BMI and alcohol consumption have been associated with increased risk of miscarriage (Nybo Andersen et al., 2000, Maconochie et al., 2007, de la Rochebrochard and Thonneau, 2002, Feodor Nilsson et al., 2014, Veleva et al., 2008). In couples with an older women and male factor infertility, the risk for miscarriage was further increased (Bahceci and Ulug, 2005). In addition, pregnancy loss was associated with uterine factors (including congenital abnormalities or fibroids), diminished ovarian reserve and ovulatory dysfunction (Hipp et al., 2015).

Paternal factors such as age (de la Rochebrochard and Thonneau, 2002) and DNA damage in sperm, have also been implicated in pregnancy loss following ART (Zini et al., 2008, Leach et al., 2015, Robinson et al., 2012). In animal studies, whereby spermatozoa were subject to agents to increase DNA damage, abnormal embryo development and decreased implantation rates were seen (Perez-Crespo et al., 2008, Fatehi et al., 2006). This was also seen in humans, with poor embryo development in cases of increased sperm DNA damage (Morris et al., 2002, Zini et al., 2005). Whilst attempts have been made to elucidate the risk factors for miscarriage, information on trends in miscarriage

across populations are lacking and require further investigation (Annan et al., 2013).

1.2 Male fertility

At least 30 million men are infertile around the world, identifying male factor infertility as a global health issue (Agarwal et al., 2015). Thirty percent of infertility is attributed to male factors (NICE, 2013). There is a stigma associated with the disorder and a lack of research on the aetiology of male infertility (Agarwal et al., 2015, Skakkebaek et al., 2016). In the past 70 years, evidence of a significant general decline in sperm quality has been reported (Rolland et al., 2013, Carlsen et al., 1992, Swan et al., 2000, Centola et al., 2016), equating to an annual decline estimated at 1.5% in USA and 3% in Europe (Swan et al., 2000). Up to 20% of men, aged 18-25 years, are defined as oligospermic (sperm concentration of less than <20 x 10^6/ml) (Jorgensen et al., 2006). There has been a suggestion that a decline may be due to oestrogen exposure (Sharpe and Skakkebaek, 1993, Aitken et al., 2004). In animal models, environmental exposure to xeno-oestrogens has been associated with higher levels of genito-urinary defects (Gray et al., 2001, Aravindakshan et al., 2004a, Aravindakshan et al., 2004b). However, there is controversy over whether male fertility is in decline (Fisch et al., 1996, Saidi et al., 1999, Pacey, 2013). There is a suggestion that poor methodology, samples that are not representative of the general population and an inability to control for confounding factors, such as smoking and geographic location, has resulted in the variation between the findings (Fisch et al., 1996, Saidi et al., 1999, Pacey, 2013).

Throughout the world, semen analysis is used as an approach for assessing sperm quality and determining the most appropriate method of treatment.

Standard reference values for sperm motility, concentration and morphology are provided by the World Health Organisation (WHO) (Cooper et al., 2010). However, the value of these parameters in assessing fertility, is debated (Grow et al., 1994, Ernst et al., 1998, Slama et al., 2003, Zinaman et al., 2000). Motility is critical for penetration of cervical mucus, movement to the oocyte and penetration for fertilisation (Nallella et al., 2006). In intrauterine insemination (IUI), a non *in vitro* method of insemination, motile sperm count was predictive of the chance of success for pregnancy, from a cohort of 939 couples (Huang et al., 1996). The percentage of motile sperm was lower in those that did not achieve a pregnancy, compared with couples that were able to conceive (Zinaman et al., 2000). However, studies have suggested that motility is not useful for predicting the chance of pregnancy (Ernst et al., 1998, Slama et al., 2002). But when used together, sperm motility and concentration, were significantly different to allow the more accurate classification of fertile and subfertile groups (Nallella et al., 2006).

In a prospective study, probability of conception was positively correlated with sperm concentration up to 40 x 10^6/ml in couples from the general population planning a first pregnancy (Bonde et al., 1998). However, a threshold below which pregnancy is unachievable has not been identified. The WHO classifies a lower reference limit for sperm concentration as <15 x 10^6/ml (WHO, 2010), at which point a natural pregnancy is unlikely.

The proportion of sperm with normal morphology has been positively correlated with fertilisation rates (Grow et al., 1994, Obara et al., 2001, De Vos et al., 2003). When normal morphology was less than 4%, lower implantation rates and an increased risk of pregnancy loss were reported (Grow et al., 1994).

Increased proportions of normal morphology has also been associated with time to pregnancy (TTP)(Slama et al., 2002). However, a significant proportion of fertile men had sperm morphology values lower than the WHO reference values, demonstrating its limitation as a characteristic to determine between fertile and infertile men (Nallella et al., 2006).

Despite the concerns about falling sperm quality, it is difficult to ascertain whether this has led to changes in clinical infertility due to the near universal use of contraceptives and societal choices about family size and age at reproduction. Nonetheless, in Denmark observed declines in the conception rate have been linked, in part, with poor sperm quality (Jensen et al., 2008, Priskorn et al., 2012). In addition, total fertility rates (TFR) have fallen below replacement levels of 2.1 children per woman (Skakkebaek et al., 2016). TTP increases as sperm counts fall below $40x10^6$/ml (Slama et al., 2002, Bonde et al., 1998), if trends for decreasing sperm counts continue, it is likely that the incidence of infertility will increase in the future (Andersson et al., 2008).

There are a number of other factors involved in male reproductive health that have seen a recent increase worldwide, including testicular germ cell cancer, hypospadias (birth defect whereby the urethra opening is not at the head of the penis) and cryptorchidism (undescended testis) (Skakkebaek et al., 2016). Cryptorchidism occurs in 2-9% of boys born at full term (Boisen et al., 2004) and in the UK, the incidence rose from 2.7% in the 1950s (Scorer, 1964) to 5.9% in 2008 (Acerini et al., 2009). In untreated cases, 90% of men with bilateral cryptorchidism, and 14% of men with unilateral cryptorchidism, have azoospermia, compared with just 0.5% of the general population (Hadziselimovic and Herzog, 2001). The prevalence of this disorder may be

caused by environmental influences, with as much as 80% of the variation in sperm quality attributable to environmental factors (Storgaard et al., 2006).

There is also evidence that boys are entering puberty at an earlier age, suggesting changes in the early stages of reproductive development. Appearance of pubic hair in boys commenced at 11.4 years in a large British cohort monitored in 1999-2005, compared with 13.4 years in a similar dataset collected between 1949 and 1969 (Monteilh et al., 2011). In the most recent cohort, boys with increased BMI developed secondary sexual characteristics significantly earlier (Monteilh et al., 2011). The age at onset of male puberty was also found to be significantly lower in 2006-2008 than 15 years previously (1991-1993), although this effect was largely attributed to an increase in BMI over the two study periods (Sorensen et al., 2010). In the 1970's a hypothesis was proposed that suggested there was a critical body weight needed to trigger puberty (Frisch and Revelle, 1970, Frisch and Revelle, 1971). The decreasing age of puberty onset (Sorensen et al., 2010, Monteilh et al., 2011, Euling et al., 2008), coincides with trends for increasing obesity (WHO, 2000). In a large study of over 150,000 children of both sexes, increased weight at age 7 resulted in earlier puberty. The study suggested that factors such as environmental chemicals are also involved in the decline in age at start of puberty (Aksglaede et al., 2009).

Male reproductive health is sensitive to many chemicals and occupational exposure to industrial chemicals has been well-explored in relation to male fertility (Cherry et al., 2008, Martenies and Perry, 2013, Recio-Vega et al., 2008). In 1977, the negative effects of occupational exposure to the pesticide 1,2-dibromo-3-chloropropane (DBCP) were highlighted. Fourteen out of twenty-

five exposed men were diagnosed with azoospermia or oligospermia (Whorton et al., 1977). It is possible that spermatozoa may be differentially vulnerable to environmental stressors compared with other cells (Sharpe, 2010). This may be due to a susceptibility of sperm cells to oxidative stress, due to the high content of polyunsaturated fatty acids (PUFA) in their membranes, alongside a limited store of antioxidants (Agarwal et al., 2011). This can result in a loss of DNA repair capacity and an inability to undergo apoptosis (Aitken et al., 2005, De Iuliis et al., 2009). DNA is in a compact and condensed state within the mature spermatozoa, more compact than that found in somatic cells. Therefore, the sperm DNA is protected for its journey through the male and female reproductive tract. Consequently, if damaged, integrity of the sperm DNA is lost and this could impair fertility (Collins et al., 2008, Pasgualotto et al., 2001, Wright et al., 2014).

Reactive oxygen species (ROS), highly oxidative radicals, such as hydrogen peroxide (Wright et al., 2014), or a depleted antioxidant capacity, can cause oxidative stress, resulting in DNA damage (Song et al., 2006). A small amount of ROS are required for sperm capacitation, the acrosome reaction and binding to the oocyte (Garrido et al., 2004, de Lamirande and O'Flaherty, 2008, Rivlin et al., 2004). However, higher levels may cause damage: in a meta-analysis, ROS levels in spermatozoa were significantly negatively correlated with fertilisation rates after IVF (Agarwal et al., 2005). High levels of oxidative stress can be caused by mobile phones (De Iuliis et al., 2009) and smoking (Agarwal and Said, 2005), resulting in impaired sperm motility (Agarwal and Said, 2005) and DNA fragmentation (De Iuliis et al., 2009). In cases of infertile normospermic men, DNA fragmentation is much higher, which may help to explain some cases of idiopathic infertility (Pasgualotto et al., 2001).

To protect against increased levels of ROS, seminal plasma has an antioxidant system, which includes oxidative defence enzymes such as glutathione peroxidises (GPXs), glutathione reductase (GR) and superoxide dismutase (SOD) (Garrido et al., 2004) and non-enzymic antioxidants such as ascorbic acid and α-tocopherol (Omu et al., 1999, Song et al., 2006). Concentration of antioxidants in serum and seminal fluid, has been found to be significantly lower in infertile men when compared to healthy controls (Benedetti et al., 2012, Shamsi et al., 2010, Omu et al., 1999). In addition, sperm quality parameters, including progressive motility and morphology, are positively correlated with serum and seminal antioxidant concentration (Omu et al., 1999, Benedetti et al., 2012, Foresta et al., 2002, Shiva et al., 2011, Kao et al., 2008).

Ascorbic acid, α- tocopherol and urate, have all been found to provide protection against sperm DNA damage after exposure to irradiation (Hughes et al., 1998). In patients with low seminal ascorbic acid levels (<5 mg/dl), there was an increased risk of having an abnormal DNA fragmentation index (≥ 30%)(Song et al., 2006). Antioxidant treatment is able to reduce DNA fragmentation, but also increase DNA decondensation, increasing the risk of future DNA damage (Menezo et al., 2007). However, a systematic review of studies including more than 2,800 couples, found male antioxidant supplementation significantly increased the pregnancy rate and live births in couples who undertook ART procedures (Showell et al., 2011). None of the studies included reported any harmful side effects of antioxidant therapy, suggesting it is a safe intervention to apply on a widespread basis.

It may be possible to protect against some oxidative stress induced DNA damage in sperm through antioxidant supplementation. With the evidence

highlighted here regarding decreasing male reproductive health (Carlsen et al., 1992, Skakkebaek et al., 2016) and the susceptibility of sperm cells to oxidative stress (Agarwal et al., 2011), an improved understanding of which environmental factors are influencing this decrease is needed. The declines in sperm quality are offset by the 'excess' in sperm production, but may lead to a longer TTP (Skakkebaek et al., 2016). However, in cases of borderline fertility, environmental exposures may impact on the fertility potential of the individual (Oliva et al., 2001). Here I will look at some key modern day environmental factors and their relationship with male infertility.

1.3 Environmental Influences

1.3.1 *Cigarette Smoke*

In the UK, 25% of men of reproductive age (16-49 years) smoke (HSCIC, 2014). It is well established that tobacco use increases the incidence of death from cancer, stroke and ischemic heart disease (Eriksen et al., 2015). Smoking has also been linked with negative effects on male reproductive health. In smokers of >20 cigarettes a day, an association with early pregnancy loss (<6 weeks) has been demonstrated (Venners et al., 2004). This suggests an early negative effect on conceptuses, as clinical spontaneous miscarriage (6-20 weeks) is not associated with paternal smoking (Windham et al., 1992, Maconochie et al., 2007). Overall, couples in which the male smokes are more likely to suffer from infertility (Yang et al., 2016) and lower clinical pregnancy rates (CPR) following both IVF and ICSI (Zitzmann et al., 2003).

A potential mechanism for this effect is oxidative stress. In the sperm of smokers there were increased level of oxidants in sperm and decreased antioxidants in seminal plasma, such as α-tocopherol (Perrin et al., 2011, Fraga

et al., 1996), with studies identifying a significant increase in oxidative damage in smokers (Fraga et al., 1996, Fraga et al., 1991). DNA adducts (covalent binding to the DNA of carcinogens) from Benzo[a]pyrene, a cigarette smoke carcinogen, have been found in spermatozoa and transmitted paternally to the embryo (Zenzes et al., 1999, Perrin et al., 2011). Interestingly, paternal smoking has been associated with a 46% decrease in sperm concentration of their sons. In addition, there are suggestions that some childhood cancers are linked with heavier paternal smoking at the time of conception and during pregnancy but the available evidence is mixed (Chang et al., 2006, Farioli et al., 2014, Milne et al., 2012, Metayer et al., 2013, Ramlau-Hansen et al., 2007b).

Studies on the potential effects of cigarette smoke on sperm quality are conflicting. A meta-analysis in 1994 found sperm concentration was 13-17% lower than that of non-smokers. When infertility clinic patients were excluded, this effect increased, to a ~24% lower sperm concentration in smokers (Vine et al., 1994). Whilst some more recent studies have supported this finding (Kunzle et al., 2003, Joo et al., 2012, Ramlau-Hansen et al., 2007a), others have not (Martini et al., 2004, de Jong et al., 2014, Jensen et al., 2004, De Bantel et al., 2015). However, in an attempt to clarify if tobacco smoke has a negative impact on sperm quality, a new meta-analysis has been carried out which has suggested that smoking is able to reduce sperm concentration and motility. The effect size was higher in moderate to heavy smokers and in infertile men when compared with the general population (Sharma et al., 2016).

1.3.2 *Psychological Stress*

There is considerable evidence that psychological stress can adversely affect spermatogenesis (*op cit* (Nargund, 2015)). Hormones linked with hypothalamic-

pituitary axis' disruption are likely to be involved in the mechanism (Nargund, 2015). Decreased testosterone and luteinising hormone (LH) levels can affect spermatogenesis (King et al., 2005, Rose et al., 1972, Theorell et al., 1990, Kreuz et al., 1972, Klimek et al., 2005) (Figure 1). When stress hormones including adrenocorticotropic hormone (ACTH) and cortisol, increase, testosterone production can be disrupted. This can lead to an increase in the precursor, androstendion, lowering testosterone levels and decreasing sperm quality, including volume, concentration, motility and morphology (Bhongade et al., 2015, Klimek et al., 2005).

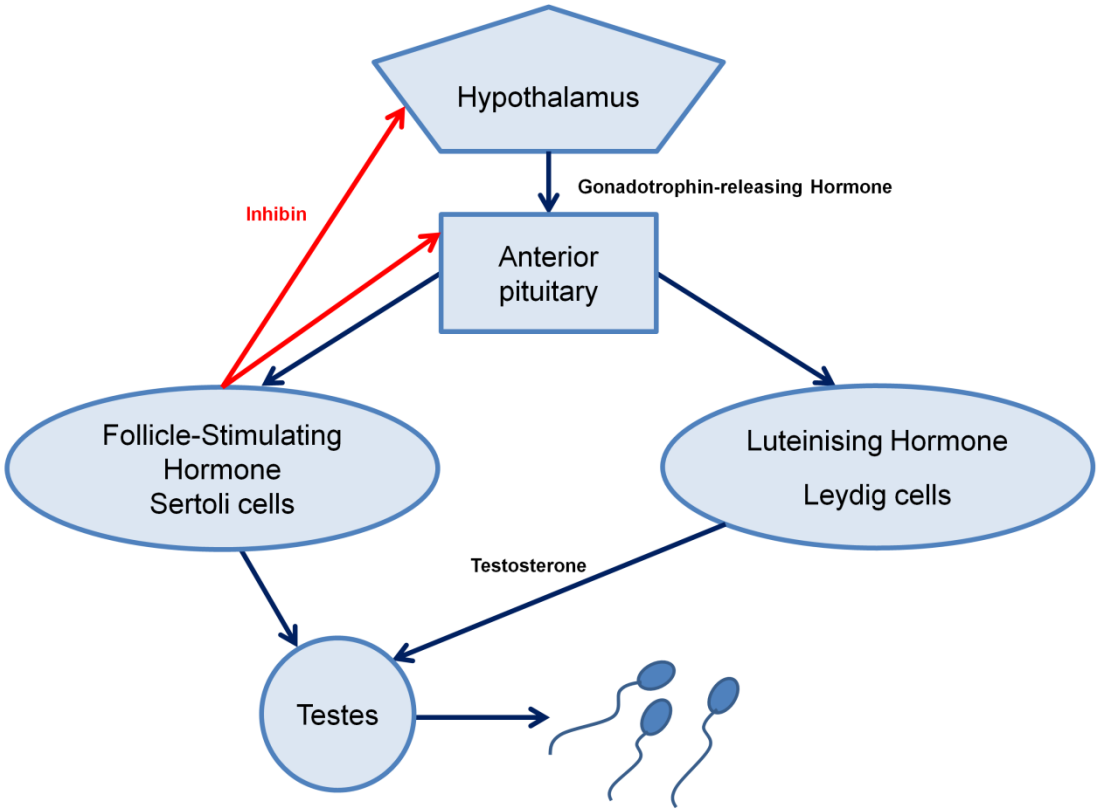

Figure 1. Hormonal regulation of spermatogenesis. Gonadotrophin-releasing hormone (GnRH) stimulates the pituitary to secrete follicle-stimulating hormone (FSH) and luteinising hormone (LH). Leydig cells then secrete testosterone, whilst inhibin is secreted from the sertoli cells in a negative feedback loop. (Adapted from (Raheem and Ralph, 2011, Marieb and Hoehn, 2007))

Sperm quality has been reported to decrease significantly following stressful life events (Gollenberg et al., 2010), such as the Kobe Earthquake and Lebenese civil war (Abu-Masa et al., 2007, Fukuda et al., 1996). Perceived stress, perhaps including 'white coat effects', has also been associated with sperm quality (Janevic et al., 2014). In couples seeking fertility treatment, sperm quality, including motility and concentration, were significantly lower during treatment (Clarke et al., 1999, Harrison et al., 1987, Ragni and Caccamo, 1992). A longitudinal study on the impact of stress during visits to an andrology clinic described a 'vicious cycle' of increasing desire for a child and a subsequent increase in the importance of having a child, which lead to a negative effect on sperm quality (Pook et al., 2004). Whilst the measurement of stress is complex, and ideally involves a combination of psychological and physiological measures, it is important to offer help to ameliorate the effects in cases of male infertility (Nargund, 2015).

1.3.3 *Seasonality*

The circadian clock prepares mammals to anticipate regular events over the course of a 24 hour period (Albrecht and Eichele, 2003). This results in upregulation of physiological pathways required to respond to physiological or behavioural needs at the right time (Gamble et al., 2013). This is aided by a network of organs each with a circadian clock (Schibler and Sassone-Corsi, 2002), in turn coordinated by the suprachiasmatic nucleus (SCN) in the hypothalamus (Buijs and Kalsbeek, 2001).

The daily 24 hour rhythm is synchronised by the light-dark cycle through the retinosuprachiasmatic pathway (Sadun et al., 1984). The neuronal signals from the SCN affect the timing of hormone release and cause body-temperature

fluctuations (Saper et al., 2005). In Antarctica, during winter with no sunlight, circadian rhythms become slightly extended, before synchronising again to the daylight in the spring (Kennaway and Van Dorp, 1991). Melatonin levels are also regulated by photoperiod, in an opposite rhythm to light exposure, peaking at night and providing a signal to reinforce the effect of photoperiod on circadian cycles (Lewy et al., 1992).

Animal studies have demonstrated that changes to photoperiod affect the SCN, which in turn drives the nocturnal melatonin signal, altering reproductive activity such as hormone production and cycle length (Nakao et al., 2008, Scott et al., 1995, Tessonneaud et al., 1995). Melatonin is a hormonal signal that is able to begin the processes leading to sleep (Krauchi et al., 1999), decreasing body temperature, a physiological mechanism to save energy overnight (Cagnacci et al., 1997). When exposed to a bright light, it is possible to shift the circadian cycle, with exposure at dusk delaying the peak in melatonin overnight (Czeisler et al., 1989). Seasonal alterations in temperature, hormone production, and nutrition, have all been linked with changes to human reproductive health, but most effects are attributed to photoperiod (Lawlor et al., 2005, Weber et al., 1998, Huber et al., 2004, Doblhammer and Vaupel, 2001, Ueda et al., 2013). During periods of longer night length, melatonin levels are higher, driving seasonal changes seen in circadian rhythms (Macchi and Bruce, 2004, Gamble et al., 2013).

When natural circadian signals are not adhered, such as in shift work, there is an increased risk of developing many disorders, including cancer, cardiovascular disease, obesity and reproductive issues (Chen et al., 2010, Ha and Park, 2005, Davis et al., 2001, Gamble et al., 2013). Figure 2. highlights the

dysfunction in reproduction associated with female shift workers. Melatonin has been inversely linked with oestrogen levels, ovarian activity and testosterone levels in females during winter (Okatani and Sagara, 1994, Kauppila et al., 1987). Over half of nurses that worked overnight had issues with irregular and painful menstrual cycles (Chung et al., 2005, Wan and Chung, 2012).

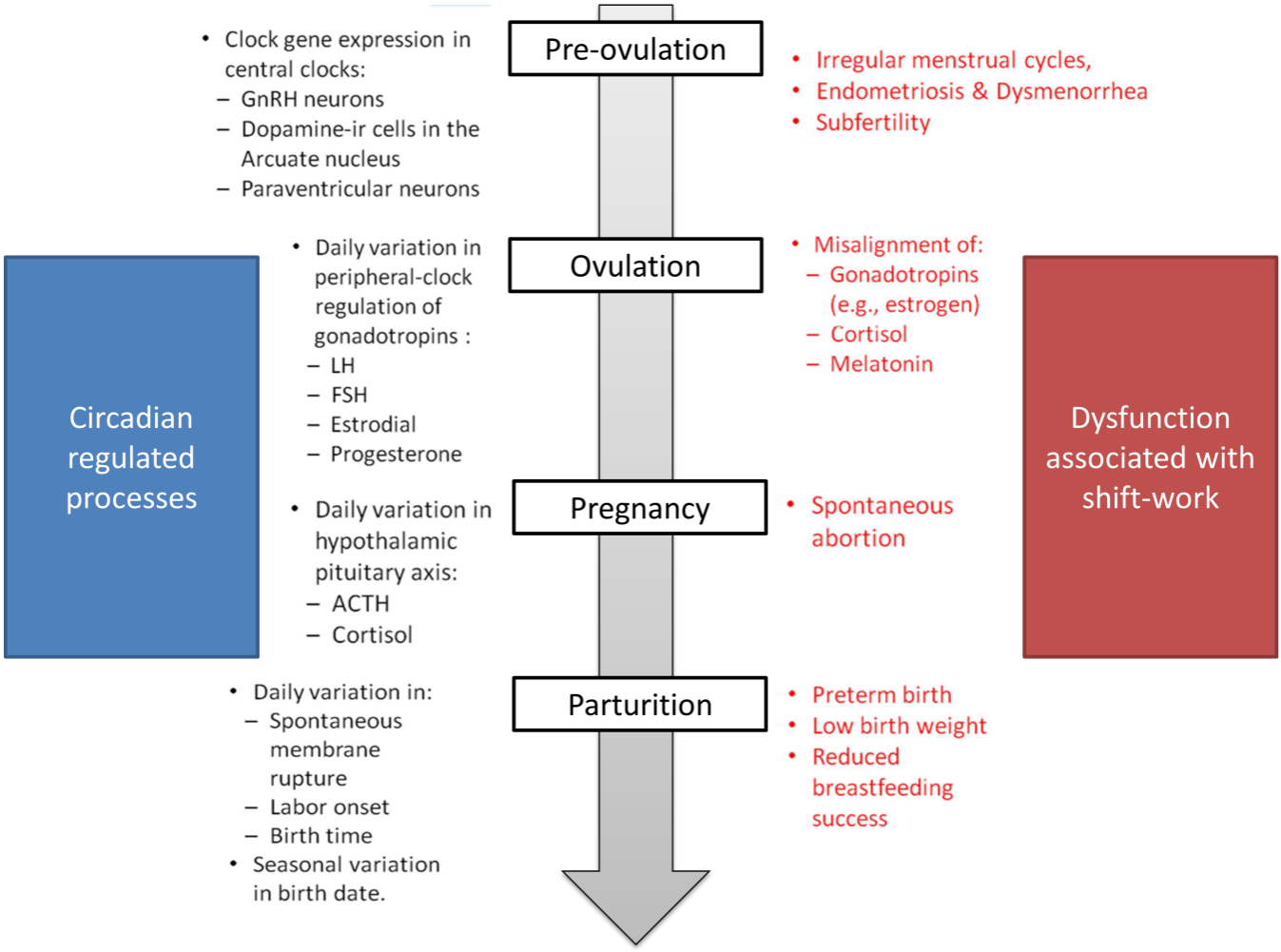

Figure 2. Circadian regulation of reproduction and dysfunction in female shift workers. GnRH - Gonadotropin releasing hormone; LH - luteinizing hormone; FSH - follicle stimulating hormone; ACTH - adrenocorticotropic hormone. Adapted from review (Gamble et al., 2013))

Circadian variation in male or female fertility may contribute to the natural seasonality in birth rates (Lam and Miron, 1994, Smits et al., 1998). Month of birth has been linked with age at marriage, length of reproductive lifespan and numbers of children, with daughters' reproductive success also varying according to their mothers' birth month (Lummaa and Tremblay, 2003). Previously, studies identified that sperm concentration peaked during the autumn and winter, whilst was at its lowest during the summer, with a similar trend found for sperm morphology (Levine, 1999, Sobreiro et al., 2005). However, these studies were conducted in temperate countries. In Singapore, where seasons were less pronounced, no relationship between sperm concentration or volume and seasons was found (Chia et al., 2001). Across studies, it is difficult to make comparisons as there is inconsistency in the method of sperm quality assessment and in which sperm quality parameters are reported.

1.3.4 *Mobile Phones and laptops*

The use of mobile phones and laptops is extensive across many populations, but concerns over adverse effects on human health and sperm quality have been raised (Erogul et al., 2006). Mobile phones and wi-fi from laptops emit electromagnetic radiation (EMR) a low-level radiofrequency (RF), in the microwave range, when being used. EMR exposure has been associated with conditions, such as cancer and Electrohypersensitivity (EHS) (Hardell and Carlberg, 2015, Johansson, 2006). Electromagnetic radiation comes from a number of sources, from low frequency electromagnetic radiation (LF-EMR) emitted from domestic electrical devices and high-voltage power lines, to RF-EMR from mobile phones and wi-fi (Calvente et al., 2010).

At frequencies of 800-2200MHz, RF-EMR is not strong enough to ionise atoms or molecules, but concerns over damage through thermal and non-thermal effects on biological tissue have been highlighted (Challis, 2005, Agarwal et al., 2011). The rate of absorption of RF is described using a Specific Absorption rate (SAR), and is legally limited at 2.0 W/kg in mobile phones (ICNIRP, 1998). As technology improves, the SAR is increasing towards the maximum and potential effects on male fertility have been investigated (Agarwal et al., 2011)

The frequencies of EMR emitted from mobile phones are thought to cause negligible thermal effects (Agarwal et al., 2011). Rats exposed to a mobile phone for an hour a day over a 28 day period, were found to have very little increase (less than 0.1˚C) in facial temperature (Mailankot et al., 2009). However, in humans, cheek temperature rose by up to 2.3˚C following mobile phone use. This was attributed to heat conduction from the handset rather than RF (Anderson and Rowley, 2007). Human testes remain outside of the body to maintain a physiological temperature 2˚C cooler than body temperature to allow optimum spermatogenesis (Agarwal et al., 2011). Therefore, whatever the mechanism, a rise in temperature of 2.3˚C could have a significant negative effect on spermatogenesis (Agarwal et al., 2011).

Non-thermal effects, such as increases in the production of seminal ROS following mobile phone exposure has been demonstrated in rats (Kesari et al., 2011). As discussed earlier, an excess of ROS can lead to oxidative stress, and consequently DNA damage. In humans, EMR emitted with the same frequency range as mobile phones caused sperm to generate an increased number of mitochondrial ROS, which resulted in a decline in motility. DNA base adducts were also found, which ultimately led to DNA fragmentation (De Iuliis et al.,

2009). Agarwal *et al.,* also found ROS levels were increased in the exposed group. However, this did not lead to any significant differences between DNA damage in the two groups (Agarwal et al., 2009).

In addition to radiation from mobile phones, devices that use the internet wirelessly also expose the user to RF-EMR. With laptops typically used in a position close to the male reproductive organs, the effect of using wi-fi with laptop computers on sperm quality requires investigation. Little work has been carried out in this area. One small prospective *in vitro* study using 29 healthy donors, reported a significant decrease in motility and a significant increase in sperm DNA fragmentation following exposure to wi-fi from a laptop, with no change in vitality between the exposed and non-exposed samples (Avendano et al., 2012). Studies on RF-EMR emitted from devices such as mobile phones and laptops have been hindered by low sample sizes and a lack of a suitable control.

1.4 Aims and objectives of thesis

There have been substantial changes in human lifestyles in the past 70 years. However, the impact of these changes on fertility are unclear. My aims in this thesis were to assess how common environmental exposures, including RF-EMR and seasonal changes, altered human sperm quality. In addition, I investigated whether miscarriage rates following ART have changed over time.

To achieve this aim I investigated these topical issues in male fertility using observational, experimental, *in vitro* and *in vivo* study methods. The objectives are:

1. To assess seasonal trends in sperm quality using retrospective analysis of data collated from men referred to a UK fertility clinic
2. To assess the effect of mobile phone exposure on sperm quality using a meta-analysis
3. To assess experimentally the impact of RF-EMR on sperm quality using an *in vitro* factorial experiment and a randomised controlled trial.
4. To assess miscarriage rates over time following ART using data from the HFEA on all ART cycles carried out in the UK.

2. EFFECT OF SEASONAL CHANGES ON SPERM QUALITY IN A UK FERTILITY CLINIC POPULATION

Status: Submitted (PlosOne)

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CONTRIBUTORSHIP STATEMENT

FM and JA conceived and designed the work, JA undertook the data analysis; FM, TG and JA interpreted the data; JA and FM drafted the work and JA, FM and TG revised it critically. All authors gave final approval and all authors agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

2.1 Abstract

Objective

Seasonal alterations in temperature, photoperiod, hormone production, and nutrition, are suggested to affect assisted conception cycles, sperm quality and birth rates. However, patterns are inconsistent. This large scale study, using a contemporary cohort, is a retrospective analysis of circannual rhythms in sperm motility and morphology in the UK.

Method

Data were collected from all men referred to a UK-based fertility clinic for semen analysis between 2008-2012. After excluding vasectomy patients and severely oligospermic samples, our analyses included 1,872 samples. The relationships between season and sperm motility and morphology were assessed using generalised additive models.

Results

There were seasonal trends in both sperm motility and morphology (Generalised additive model (GAM), edf 2.59, $F(1.71)$, $p<0.001$; edf 2.91, $F(1.54)$, $p=0.002$) respectively): the proportions of motile and normal sperm were significantly higher in summer. Mean motility decreased from 54.7% (±SD 13.0) in summer to an average of 51.9% (±SD 14.1) across the other seasons', whilst the mean percentage of morphologically normal sperm decreased from 7.6% (±SD 5.8) to 6.9% (±SD 5.3).

Conclusion

Our study found significant seasonal trends in sperm quality parameters. These seasonal patterns have not previously been reported, but follow patterns of seasonality in birth rates and assisted conception cycles across Europe. The mechanism of seasonal changes in sperm quality, and subsequent implications for fertility interventions and short-and long-term health of conceptuses, needs to be determined.

Keywords: sperm quality; sperm motility; sperm morphology; season;

2.2 Introduction

In temperate countries, there are marked seasonal variations in photoperiod, temperature, diet and activity. These have the capacity to influence human health both directly and indirectly. Alterations in birth weight (Lawlor et al., 2005), growth (Weber et al., 1998), reproductive performance (Huber et al., 2004) and life expectancy (Doblhammer and Vaupel, 2001, Ueda et al., 2013) have been linked with seasonal changes in temperature, photoperiod, hormone production and nutrition during early development. Seasonal variations in birth rates have also been observed (Lam and Miron, 1994), together with increases in female fecundability (conception leading to a live birth) in both June and December (Smits et al., 1998). Overall, data from Europe, including historical population studies, show that most births are recorded in spring, with a secondary peak in September (Lam and Miron, 1994, Rojansky et al., 1992). However, family planning often masks the natural seasonal birth patterns, with shifts towards summer/autumn parturition being reported in the USA (Chandwani et al., 2004), Germany (Lerchl et al., 1993) and Scotland (Russell et al., 1993).

Variations in male or female fertility, or the frequency of coitus, may all contribute to natural seasonality in birth rates. Seasonal changes are largely attributed to increases in photoperiod. Animal studies have demonstrated that changes to photoperiod affect the suprachiasmatic nucleus (SCN), which in turn drives the nocturnal melatonin signal, altering reproductive activity (Nakao et al., 2008, Scott et al., 1995, Tessonneaud et al., 1995). Data from assisted conception cycles have shown peaks in embryo quality, fertilisation, implantation and pregnancy rates between spring and summer (Braga et al., 2012, Wood et al., 2006, Rojansky et al., 2000). However, results between studies are often inconsistent, in part due to chosen exposure times and subsequent season classification, with no evidence of seasonality found in an analysis of over 9,000 IVF cycles in Switzerland and Italy (Revelli et al., 2005, Wunder et al., 2005).

Sperm motility and morphology both significantly affect fertilisation and pregnancy rates (Donnelly et al., 1998). However, it is unclear whether sperm quality varies seasonally. In adult rhesus monkeys, under controlled laboratory conditions, spermatogenesis varied in response to circannual changes in the length of ‘daylight’ (Wickings and Nieschlag, 1980). In humans, higher proportions of sperm motility and morphology have been reported in winter (Sobreiro et al., 2005, Levine et al., 1990) and spring (Andolz et al., 2001), whilst studies at lower latitudes have found no seasonal effect on sperm quality (Chia et al., 2001, Ombelet et al., 1996). If seasonality does affect sperm quality, it may result in fluctuations in assisted conception treatment (ART) outcomes, birth rates and effective fecundability (Smits et al., 1998, Lam and Miron, 1994, Wood et al., 2006). In this study, we present data collected between 2008-2012, from a large cohort of men who attended the Peninsular

Centre for Reproductive Medicine (PCRM), Exeter, UK, for routine semen analysis. This work aims to clarify whether there are seasonal changes to sperm quality in a temperate climate.

2.3 Methods

2.3.1 *Participants*

Data were collected from men referred for semen analysis at PCRM between 2008 and 2012. Azoospermic and severely oligospermic samples (<4 x 10^6/ml) were excluded due to low cell numbers for accurate analyses. Duplicate and repeat samples from the same individual, any incomplete data sets, and samples from vasectomy and vasectomy reversal patients were also excluded. Subsequently, in our initial dataset 1,872 samples were available for analysis. Men included in this study had a mean age of 34.7 (±SD 6.9) years. The majority of men were seeking semen analysis due to a failure to conceive after 12 months, but data on the fertility status of the participants were not available. In some cases, particularly whereby a first sample was suboptimal (according to WHO guidelines (WHO, 2010)), participants were asked to submit a repeat sample. This second dataset was analysed separately to assess whether trends for seasonal variation in sperm quality is replicated in repeat samples from the same individual. Following the same exclusion criteria as our initial data set, there were 878 samples submitted by 388 men.

2.3.2 *Semen analysis*

Semen samples were obtained by masturbation into a wide mouthed plastic container (Sterilin™ 60ml container, Thermo Scientific, UK) and men were instructed to observe sexual abstinence for at least 2 days prior to the

production of a sample. The samples were left to liquefy for at least 30 minutes, and were analysed within 2 hours of collection. All semen analyses were carried out according to WHO guidelines (WHO, 2010, WHO, 1999), with a minimum of 200 sperm cells analysed per sample, for both sperm motility and normal morphology. Minimising potential bias, consistent methods and equipment were used throughout the study period. All technicians were active participants in an external quality assurance programme (UK NEQAS Reproductive Science).

2.3.3 *Statistical Analysis*

Seasons were defined as Winter (Dec, Jan, Feb); Spring (Mar, Apr, May); Summer (Jun, Jul, Aug) and Autumn (Sep, Oct, Nov). Generalised Additive Models (GAMs) were built within *R* v. 3.0.02 (RCoreTeam, 2012) using the package 'mgcv' (Wood, 2014). Month was treated as a smoothed and circular term, and year of collection and patient age were specified as fixed covariates. Given that previous work has indicated differences in seasonal patterns according to concentration (Levitas et al., 2013), a dichotomous classification of whether the sample was oligospermic ($> 4 \times 10^6$/ml $<20 \times 10^6$/ml) was also included as a fixed effect. Potential interactions between the covariates were analysed. Due to the presence of overdispersion, quasibinomial error structures were used for all models. Having established the presence of monthly patterns, changes in sperm quality across the four seasons was assessed using GLMs with quasibinomial error structures, again using the R package 'mgcv', with season in place of month and all other terms and interactions as above. *Post-hoc* pairwise comparisons between levels of season used Boiks method to account for multiple testing (package 'phia'(De Rosario-Martinez, 2013)). Nagelkerke R^2 was calculated for the GLMs using package 'fmsb'((Minato,

2014)). In our second dataset, for the analysis of participants who had submitted repeat samples, Generalised Additive Mixed Models (GAMMs) were built using package 'lme4' (Bates et al., 2015) using a quasibinomial error structure due to overdispersion. Covariates were analysed as in previous models, with the addition of patient ID as a random factor. All models were simplified by manual backwards stepwise deletions until minimum adequate models were obtained. See supplementary information for GAM outputs.

2.4 Results

2.4.1 *Motility*

Sperm motility varied across months and this pattern could be described as a circular sinusoidal wave (Figure 1a)(edf 2.59, F(1.71), p<0.001). In participants who had submitted more than one sample this pattern was replicated (Figure 1b)(edf 2.15, F(3.83), p=0.002). Using the initial dataset, including one sample per individual, GLMs were built to assess contrasts across the four seasons. There were significant interactions between year and season (Year*season interaction change in deviance=200.3; DF=12; p=0.006). The effect size also altered according to oligospermia and participant age (oligospermia*age interaction change in deviance=32.2; DF=1; p=0.03). Accounting for both significant interactions, *post-hoc* contrasts between seasons demonstrated that sperm motility was significantly higher in summer than the other three seasons (Model Nagelkerke R^2=0.49)(Table 1-2).

2.4.2 *Morphology*

Normal sperm morphology also varied across months (edf 2.91, F (1.54), p=0.002) (Figure 2a). In participants who had submitted more than one sample

this change was no longer significant (Figure 2b)(edf 0.64, F(0.11), p=0.31). Nonetheless, using the initial dataset as above, GLMs were built to compare morphology across the seasons. An interaction between year and season was identified (Year*season interaction change in deviance = 220.2; DF=12; p<0.001) .In addition, oligospermia was associated with lower normal sperm morphology (OR 0.60, ChiSq 84.2, p<0.001). However, there was no significant effect of paternal age. Overall, contrasts that allowed for the significant interaction showed that the proportion of sperm with normal morphology was significantly higher in summer than spring, and there was a non-significant trend for higher normal sperm morphology in summer when compared with the other seasons (Model Nagelkerke R^2=0.36)(Table 1, Table 3).

Table 1. Mean seasonal values for sperm quality parameters (±SD).

Season	Total Motility (% ±SD)	Normal Morphology (% ±SD)
Winter	52.03 (± 13.84)	7.09 (± 5.35)
Spring	51.52 (± 14.41)	6.88 (± 5.23)
Summer	54.72 (± 12.95)	7.59 (± 5.75)
Autumn	52.00 (± 14.15)	6.75 (± 5.18)

Table 2. The variation in total sperm motility between seasons using post-hoc comparisons taken from a quasibinomial GLM. OR = Odds ratio. X^2=Chi square

Seasonal contrasts for total sperm motility	OR	X^2	p-value
Winter-Spring	1.04	1.10	0.83
Winter-Summer	0.90	9.31	*0.01*
Winter-Autumn	1.00	0.00	1.00
Spring-Summer	0.86	17.57	*<0.001*
Spring-Autumn	0.96	1.18	0.83
Summer-Autumn	1.12	10.18	*0.01*

Table 3. The variation in normal sperm morphology when comparing across seasons using post-hoc comparisons taken from a quasibinomial GLM. OR = Odds ratio. X^2=Chi square

Seasonal contrasts for normal sperm morphology	OR	X^2	p-value
Winter-Spring	1.05	0.94	1.00
Winter-Summer	0.9	3.12	0.31
Winter-Autumn	1.02	0.17	1.00
Spring-Summer	0.87	7.78	*0.03*
Spring-Autumn	1.03	0.35	1.00
Summer-Autumn	1.12	5.16	0.12

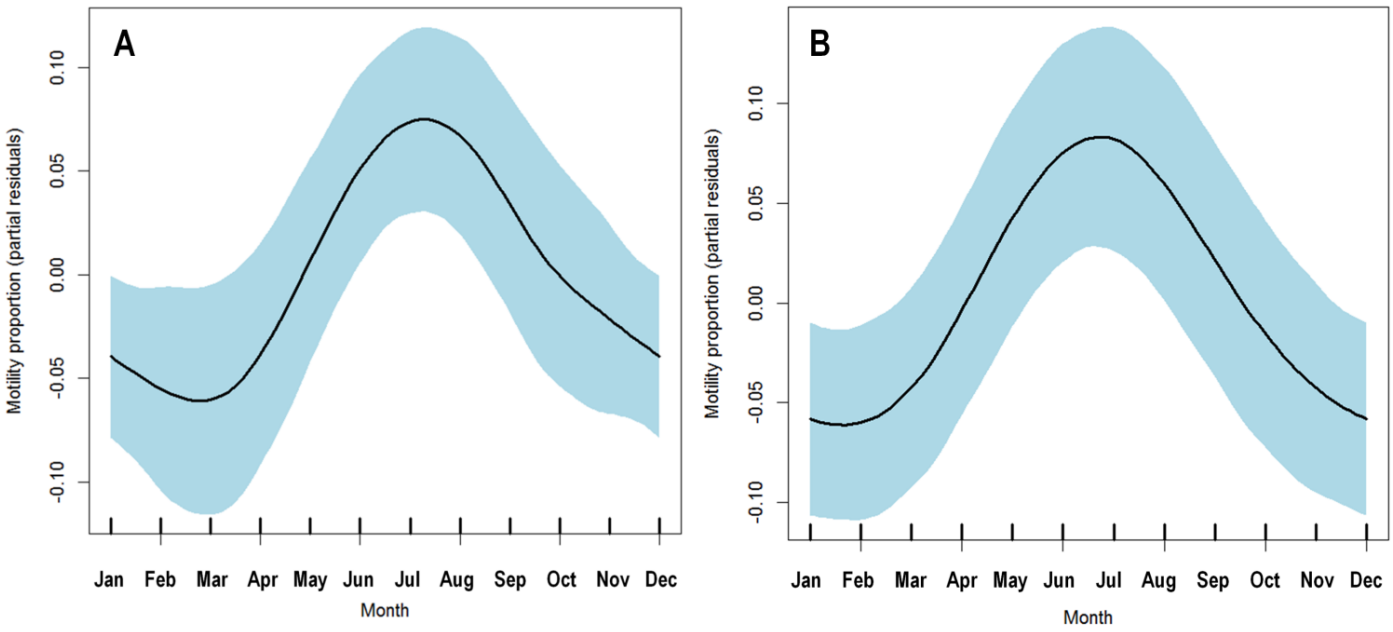

Figure 1. A) Significant monthly pattern of sperm motility from 1872 men, using partial residuals from a quasibinomial Generalised Additive Model (GAM) . Adjusting for significant terms including year (2008-2012), paternal age and oligospermia with month specified as a smoothed circular predictor (edf 2.59, F=1.71, p<0.001) Adj R^2 = 0.07, deviance explained 7.71%. B) Significant monthly pattern of sperm motility, taken from repeat samples from the same individual (878 samples from 388 men), using partial residuals from a quasibinomial Generalised Additive Mixed Model (GAMM). Adjusting for significant terms, including oligospermia, patient ID (included as a random factor) and month (specified as a smoothed circular predictor)(edf 2.15, F(3.83), p=0.002). Adj R^2 = 0.50, deviance explained = 62.6%. Standard errors highlighted in light blue shading.

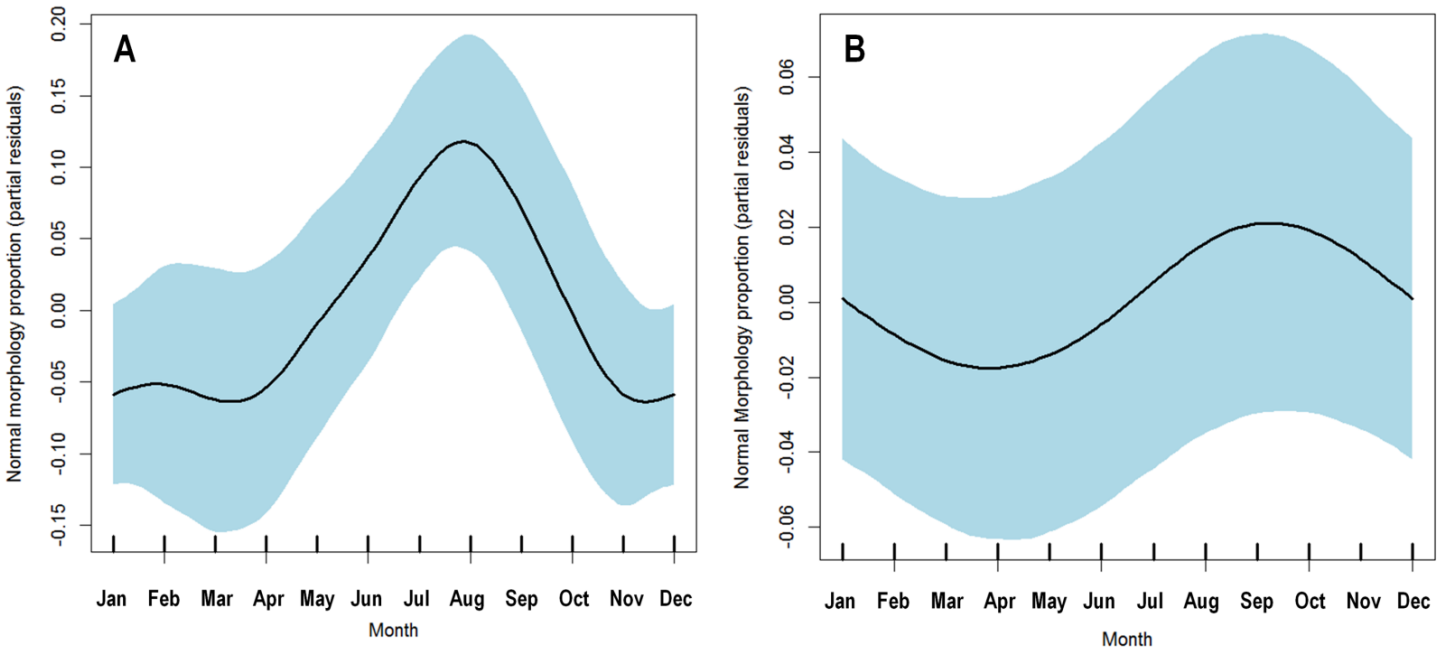

Figure 2. A) Significant monthly pattern of normal sperm morphology from 1872 men, using partial residuals from a quasibinomial Generalised Additive Model (GAM) . Adjusting for significant terms including year (2008-2012), paternal age and oligospermia with month specified as a smoothed circular predictor (edf 2.91, F=1.54, p=0.002). Adj R^2 = 0.08, deviance explained = 8.97%. B) Non-significant monthly pattern of normal sperm morphology taken from repeat samples from the same individual (878 samples from 388 men), using partial residuals from a quasibinomial Generalised Additive Mixed Model (GAMM). Adjusting for significant terms, including oligospermia, year and patient ID (included as a random factor). Month was specified as a smoothed circular predictor)(edf 0.64, F(0.11), p=0.31). Adj R^2 = 0.23, deviance explained = 31.3%. Standard errors highlighted in light blue shading.

2.5 Discussion

In this large dataset, where sperm quality was assessed using consistent methodologies, there is marked seasonal variation in sperm quality. There were trends for greater sperm motility and normal morphology in summer compared with all other seasons. These variations correspond to spring peaks in birth rates across Europe (Lam and Miron, 1994), as well as summer improvements in implantation and clinical pregnancy rates reported in the UK (Wood et al., 2006). Seasonal changes in sperm quality have been studied previously but with inconsistent results (Levine, 1999). In contrast to our findings, previous research in Europe has found no seasonal changes to sperm motility and morphology (Saint Pol et al., 1989, Mortimer et al., 1983, Jorgensen et al., 2001). More recently in the UK, classification of morphology as abnormal was increased in summer (Pacey et al., 2014), with both sperm motility and morphology declining in summer in the US and Brazil (Chen et al., 2003, Sobreiro et al., 2005). Higher ambient temperatures in the latter regions may mean the data are not comparable with our results. However, our findings correspond with an Israeli and Italian study which reported significantly higher total sperm motility in summer than in other seasons (Levitas et al., 2013, De Giorgi et al., 2015), with similar trends for morphology reported in Spain (Andolz et al., 2001).

Seasonal changes are reflected in alterations in photoperiodicity and ambient temperature. In response, a circadian molecular clock may influence reproductive hormones that affect fertility (Gamble et al., 2013). Melatonin levels, which partially control the circadian clock, peak at night and so increase during periods of longer night-length (Macchi and Bruce, 2004). Conversely,

testosterone concentrations peak in June, during periods of shortest night-length (Meriggiola et al., 1996). These changes may explain our findings, as low seminal testosterone has been linked with lower sperm morphology (Huang et al., 1996) and motility (Luboshitzky et al., 2002), whilst increasing melatonin concentrations has been shown to decrease sperm parameters, including motility and morphology in animals (Nunez Favre et al., 2014), and in a small-scale human study (Luboshitzky et al., 2002). This corresponds with the decrease in winter sperm quality found in our data, when melatonin levels are at their highest. However, it is possible that melatonin may affect fertile and infertile men differently, as low serum and seminal plasma levels of melatonin have been reported in infertile men (Awad et al., 2006), with a positive protective effect of the hormone on sperm motility and morphology *in vitro* (Ortiz et al., 2011, du Plessis et al., 2010).

In our study, we also saw a decrease in normal morphology in oligospermic patients and in total motility according to an interaction between paternal age and oligospermia. Many previous studies did not analyse seasonality of motility and morphology according to oligospermia (Andolz et al., 2001, Levine, 1999, Sobreiro et al., 2005, Chen et al., 2003, Saint Pol et al., 1989, Mortimer et al., 1983), which in some cases may have reduced the reported effect of season.

Due to the sensitivity of spermatogenesis to heat, sperm quality seasonality has sometimes been linked to changes in temperature, particularly in the summer heat of equatorial conditions (Gyllenborg et al., 1999, Bronson, 1995, Lam and Miron, 1991). Evidence suggests that sperm quality declines in men with higher scrotal temperature from wearing tight-fitting underwear (Parazzini et al., 1995, Povey et al., 2012) or sedentary lifestyles (Hjollund et al., 2002). However,

many studies report a lack of association with seasonal temperature (Gyllenborg et al., 1999, Zhang et al., 2013, Pacey et al., 2014). In the UK, which has a temperate climate, it would be expected that correlated day length changes may be more important than temperature. Nonetheless, research to understand the relative importance of the mechanisms contributing to seasonal changes, such as photoperiod, temperature and diet, is warranted.

As all samples were taken from men referred to a fertility clinic for semen analysis, the data are likely to represent a higher proportion of infertile men than in the general population. Nonetheless, the mean value for each parameter is above the WHO reference values for sperm quality (Cooper et al., 2010). Information was not available on the many lifestyle features that have been associated with sperm quality, for example, diet (Gaskins et al., 2012) or stress (Fukuda et al., 1996). It is therefore unclear, whether the mechanisms of seasonal variation in sperm quality is partially explained by these factors. Unfortunately, analysing repeat samples from the same individual for sperm morphology did not support the significant seasonal changes that we identified in our initial dataset. However, overall, whilst our study found different seasonal trends when compared with many other studies, our data, taken from a large population, suggests that in particular sperm motility is improved in summer, with consistent results over a 5 year period including when repeated samples were analysed from the same individual and this corresponds with trends in birth rates.

Early maternal environmental conditions can programme birth weight (Lawlor et al., 2005), height (Weber et al., 1998) and future health (Doblhammer, 2004) and survival (Moore et al., 1997), but there has been little investigation of

paternal effects. Given the seasonal changes in sperm quality we have identified, assessment of the clinical implications for fertility and early life health outcomes is needed. Sperm quality, whilst highly heterogeneous, is a widely applied indicator of male fertility (Cooper et al., 2010). Fertilisation and pregnancy rates can be significantly affected by sperm motility and morphology (Donnelly et al., 1998). Across studies, there have been inconsistent results for seasonality in ART outcomes (Rojansky et al., 2000, Stolwijk et al., 1994, Fleming et al., 1994, Revelli et al., 2005). It is often difficult to control for confounding factors, and many studies are based on relatively small samples. However, in a UK based study, including over 1600 treatment cycles, during periods of longer daylight hours, results from assisted conception cycles improved; with better implantation and pregnancy rates (Wood et al., 2006). This may, in part be explained by variations in response to ovarian stimulation. However, this is also consistent with our observation of better sperm quality in summer.

Seasonal effects on sperm quality, may not only affect birth rate but also the outcome of pregnancy. There has been some suggestion that there are seasonal trends in preterm delivery. However, to date, studies are inconsistent (Lee et al., 2006), potentially because they analyse the time of birth rather than time of conception, and so may fail to capture the seasonal impacts on gametes. In addition, there could be effects for long-term health. In Europe, infants born in autumn have greater life expectancy compared with spring births (Doblhammer and Vaupel, 2001, Ueda et al., 2013), this may be due to negative maternal environmental effects from the previous winter, such as nutrition availability and infections (Doblhammer, 2004). In a historical cohort, women born in June were likely to have seven more grandchildren than those

born in October. Month of birth affected age at marriage, length of reproductive lifespan and numbers of children. This effect spanned generations, with the daughters reproductive success also varying according to their mothers' birth month (Lummaa and Tremblay, 2003). Larger scale studies on seasonal impacts on ART, pregnancy outcomes and long-term health, in relation to the month of conception and seasonality in sperm quality, are merited.

2.6 Conclusion

Our study found significant seasonal trends in sperm quality parameters in men from a UK fertility clinic population. These patterns have not previously been reported in the UK, but follow patterns of seasonality in birth rates and in the success of assisted conception cycles. A better understanding of the mechanisms driving these seasonal changes in sperm quality would be helpful for fertility interventions and to determine the potential implications for the short- and long-term health of conceptuses.

3. EFFECT OF MOBILE TELEPHONES ON SPERM QUAITY: A SYSTEMATIC REVIEW AND META-ANALYSIS

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CONTRIBUTORSHIP STATEMENT

JA conceived and designed the work. SE provided raw data. JA undertook the data analysis; FM, TG and JA interpreted the data; JA and FM drafted the work and JA, TG, DP, SE and FM revised it critically. All authors gave final approval and all authors agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

3.1 Abstract

Most of the global adult population own mobile phones. Radio-frequency electromagnetic radiation (RF-EMR) from mobile phones could potentially affect sperm development and function. Around 14% of couples in high- and middle-income countries have difficulty conceiving, and there are unexplained declines in sperm quality reported in several countries. Given the ubiquity of mobile phone use, the potential role of this environmental exposure needs to be clarified. A systematic review was therefore conducted, followed by meta-analysis using random effects models, to determine whether exposure to RF-EMR emitted from mobile phones affects human sperm quality. Participants were from fertility clinic and research centres. The sperm quality outcome measures were motility, viability and concentration, which are the parameters most frequently used in clinical settings to assess fertility.

We used ten studies in the meta-analysis, including 1,492 samples. Exposure to mobile phones was associated with reduced sperm motility (mean difference -8.1% (95% CI -13.1, -3.2) and viability (mean difference -9.1% (95% CI -18.4, 0.2), but no effect on concentration was apparent. The results were consistent across experimental *in vitro* and observational *in vivo* studies. We conclude that pooled results from *in vitro* and *in vivo* studies suggest that mobile phone exposure negatively affects sperm quality. Further study is required to determine the full clinical implications for both sub-fertile men and the general population.

3.2 Introduction

Most men of reproductive age in high- or middle-income countries now own mobile (cell) telephones (phones). Accompanying this increase in mobile phone

ownership, there is concern over the potential effects of mobile phone exposure on human health. Mobile phones emit electromagnetic radiation (EMR), a low-level radiofrequency (RF), at a frequency of between 800-2200 MHz (Agarwal et al., 2011), that can be absorbed by the human body. Mobile phones are legally limited to a specific absorption rate (SAR) of 2.0 W/kg (ICNIRP, 1998), and currently, most have a SAR of ~1.4 W/kg (Agarwal et al., 2011). At this low frequency EMR is unlikely to ionise atoms or molecules (Erogul et al., 2006). However, there is some evidence of potential adverse effects including headaches (Oftedal et al., 2000), increase in resting blood pressure (Braune et al., 1998) and disturbances to electroencephalographic (EEG) activity during sleep (Huber et al., 2000). It has also been suggested that mobile phones, and other electromagnetic devices that emit RF-EMR radiation, are detrimental to human fertility (La Vignera et al., 2012).

Around 14% of couples in industrialized countries experience difficulty with conception at some point in their lives (Wilkes et al., 2009, Oakley et al., 2008, Hull et al., 1985, Templeton et al., 1996). Male factor infertility is involved approximately 40% of the time (Fleming et al., 1995), and a high proportion of cases are unexplained. The oscillating current and rapid transfer of energy generated by the RF electric field can result in rapid heating (Challis, 2005), which could influence sperm quality. There are also non-thermal interactions, including changes to protein conformations and binding properties, and an increase in the production of reactive oxygen species (ROS) that may lead to DNA damage (Challis, 2005, La Vignera et al., 2012). Animal studies have suggested RF-EMR can affect the cell cycle of sperm (Kesari and Behari, 2010), increase sperm cell death (Yan et al., 2007) and produce histological changes in the testes (Dasdag et al., 1999).

Mobile phone exposure has been linked in some animal studies to a reduction in sperm count (Kesari et al., 2010) and motility (Mailankot et al., 2009), suggesting an impairment of male fertility, although these effects are not consistently reported (Dasdag et al., 2003). In humans, the prolonged use of mobile phones decreased motility, sperm concentration, morphology and viability (Agarwal et al., 2008), suggesting a decrease in fertility. However, the evidence is mixed. Some studies have found an effect on sperm motility but not on sperm concentration (Erogul et al., 2006, Fejes et al., 2005), whilst no effect on sperm quality has also been found (Feijo et al., 2011). We therefore conducted a systematic review and aggregated the available published data on the effect of mobile phone exposure on sperm quality using meta-analysis. The aim was to summarise the evidence on RF-EMR exposure from mobile phones and male fertility indices.

3.3 Methods

3.3.1 Search Strategy

We conducted a systematic search using Web of Knowledge and MEDLINE to identify all relevant studies published from 2000-2012. The MESH search terms used were '*phone*' OR 'electromagnetic' AND 'semen' or 'sperm*' OR '*fertil*'. We limited the search to studies using human subjects and those that reported information on basic semen parameters including motility, viability and concentration. Hand searches were carried out of review articles and reference lists. Authors of unpublished or incomplete datasets were contacted to request that they provided information for this meta-analysis. Insufficient information meant that some studies were excluded (Wdowiak et al., 2007, Van-Gheem et al., 2011, Gutschi et al., 2011). Articles were only included if they were written in

English, reported on human participants, did not use workplace RF-EMR exposure and were not review articles. We incorporated both *in vitro* and *in vivo* studies, provided they met with our inclusion criteria (max SAR 2.0 W/kg, frequency 800-2200 MHz, based on previous literature (Agarwal et al., 2011)). We adhered to PRISMA guidelines and provide the PRISMA checklist in the supporting information. Studies were analysed for inclusion independently by two of the authors, any discrepancies were resolved by discussion. Sixty articles were identified from the title. This was reduced to twenty-three potentially suitable articles using the abstract, largely due to the presence of animal and non-mobile phone related EMR exposure studies. From these, ten studies fulfilled all criteria and were included in the meta-analyses (Table 1).

We specified the primary outcome measures *a priori* as sperm motility (mean %); viability (mean %); and concentration ($x10^6$/ml). In clinical settings, these parameters are some of the most frequent measures used for investigations of male fertility. Some of the studies provided data on all three of these outcome measures, and others on just some of them. The following characteristics were assessed for each study: (a) Study design (*in vitro* versus *in vivo*) (b) Data collection methods (e.g. Semen analysis according to WHO guidelines) (c) Sample size.

3.3.2 Statistical analysis

Statistical analysis was undertaken using *R* (i386 2.15.1) (RCoreTeam, 2012) with the package 'Meta' (Schwarzer, 2012). Both fixed effects models (FEM) and random effects models (REM) were fitted, to permit assessment of which model-types were most suited to the data. FEMs were based on the inverse variance method and REMs on the DerSimonian and Laird method. Mean

differences (MD) between exposed and non-exposed groups were calculated to determine the effect size. The heterogeneity of the studies was assessed using I^2 (Higgins and Thompson, 2002) and associated confidence intervals (CI). Where heterogeneity was high, subgroup analyses were carried out to identify potential sources of the heterogeneity. Sensitivity analyses were conducted to assess the leverage of individual studies on the results (see Supplementary Information Figure 1-3). Assessment of potential publication bias is also provided in the Supplementary Information (Figure 4-5).

Table 1. Study characteristics from mobile phone exposure and sperm quality meta-analyses. (- denotes information not provided)

				Sperm parameters						
Reference	**Sample size**	**Study design**	**Participant group**	**Motility**	**Viability**	**Concentration**	**Radio-frequency (MHz)**	**SAR (W/kg)**	**Exposure time**	**Comments**
(Agarwal et al., 2008)	361	*In vivo*	Fertility clinic	✓	✓	✓	-	-	-	Exposed to commercially available mobile phones
(Agarwal et al., 2009)	64	*In vitro*	Fertility clinic	✓	✓	✓	850	1.46	60min	Exposed to Sony Ericsson w300i
(Ahmed and Baig, 2011)	44	*In vitro*	Population	✓			900	1.3	60min	Exposed to Nokia 112 in talk mode
(Dkhil et al., 2011)	40	*In vitro*	Population		✓		850	1.46	60min	Nokia 73 in talk mode
(De Iuliis et al., 2009)	8	*In vitro*	Population	✓	✓		1,800	1	16h	Exposed using a waveguide, connected to a function generator and RF amplifier.
(Erogul et al., 2006)	54	*In vitro*	Population	✓		✓	900	-	5min	Exposed to commercially available mobile phones
(Falzone et al., 2008)	24	*In vitro*	Population	✓			900	2	60min	RF-EMR chamber
(Feijo et al., 2011)	343	*In vivo*	Fertility clinic	✓	✓	✓	-	-	-	Exposed to commercially available mobile phones
(Fejes et al., 2005)	254	*In vivo*	Fertility clinic	✓		✓	-	-	-	Exposed to commercially available mobile phones
(Sajeda and Al-Watter, 2011)	300	*In vivo*	Fertility Clinic	✓		✓	-	-	-	Exposed to commercially available mobile

3.4 Results

All semen analyses were carried out according to WHO guidelines applicable at the time of publication (WHO, 1999, WHO, 2010). Overall, 10 suitable studies were identified, and these included data on 1,492 semen samples. The number of papers included in each meta-analysis varied according to the sperm parameters reported: 9 provided data on motility, 6 provided data on concentration and 5 provided data on viability. All *in vitro* studies were experimental and all *in vivo* studies were observational. Two studies of healthy donors included only normozoospermic individuals, that is, all semen parameters within normal ranges according to the WHO criteria (WHO, 1999). The exposure rates for the *in vitro* studies are reported in Table 1. All used frequencies of 850-900 MHz, with the exception of one study (De Iuliis et al., 2009); SAR, where reported was in the range 1-2; and duration of exposure ranged from 5 minutes to 16 hours, with four of the studies using a duration of 1 hour. Exposure rates were not assessed or reported in the epidemiological studies conducted *in vivo*.

3.4.1 Motility

Nine studies, which included 1,448 samples from 1,353 men, were used in this analysis (Figure 1a). Mean total motility (%) ranged from 36.6-86.8%. Six studies (Erogul et al., 2006, Agarwal et al., 2008, Agarwal et al., 2009, Ahmed and Baig, 2011, De Iuliis et al., 2009, Sajeda and Al-Watter, 2011) reported a significant negative effect of mobile phone exposure on human sperm motility. Overall, both the FEM and REM indicated that mobile phone exposure was linked to reduced sperm motility, FEM -12.2 (95% CI -13.6, -10.7), REM -8.1 (95% CI -13.1, -3.2). Given the high heterogeneity (89.5% (95% CI 82.2%, 93.7%)), the REM is likely to provide the

most appropriate representation of the data. The consistency in the direction of the effect, and overlap of the confidence intervals across studies, increases confidence in the results. Sensitivity analyses (Supplemental Figure 1) indicate that removing the paper by (De Iuliis et al., 2009) reduced the mean difference to -6.65. This was the minimal effect seen, when other studies were removed in turn, the observed pooled effect size was not materially affected (REM -6.65; -9.43).

To assess the causes of the heterogeneity, three subgroup analyses were undertaken (Table 2.). The heterogeneity estimates were not materially affected by performing analyses separately according to study type (*in vivo* versus *in vitro*) or donor type (population versus fertility clinic donors). The effect of how long the samples/participants were exposed to the mobile phone radiation was then assessed (Figure 2), with the studies being split equally into short exposure (≤60 minutes) and long exposure (>60 minutes) groups. All but one of the *in vitro* studies, but none of the *in vivo* studies (De Iuliis et al., 2009), were in the short exposure group. Heterogeneity in the short exposure group was reduced to 35.8%, compared to 90.7% for the long exposure group (Table 2), suggesting that some of the differences between studies are explained by exposure time. The results for the short-exposure treatment were consistent whether a FEM or REM model was used, and suggested mobile phone exposure reduced motility (Table 2). The observed pooled effect size was larger for the long exposure studies, with a greater reduction in motility compared to the short exposed groups.

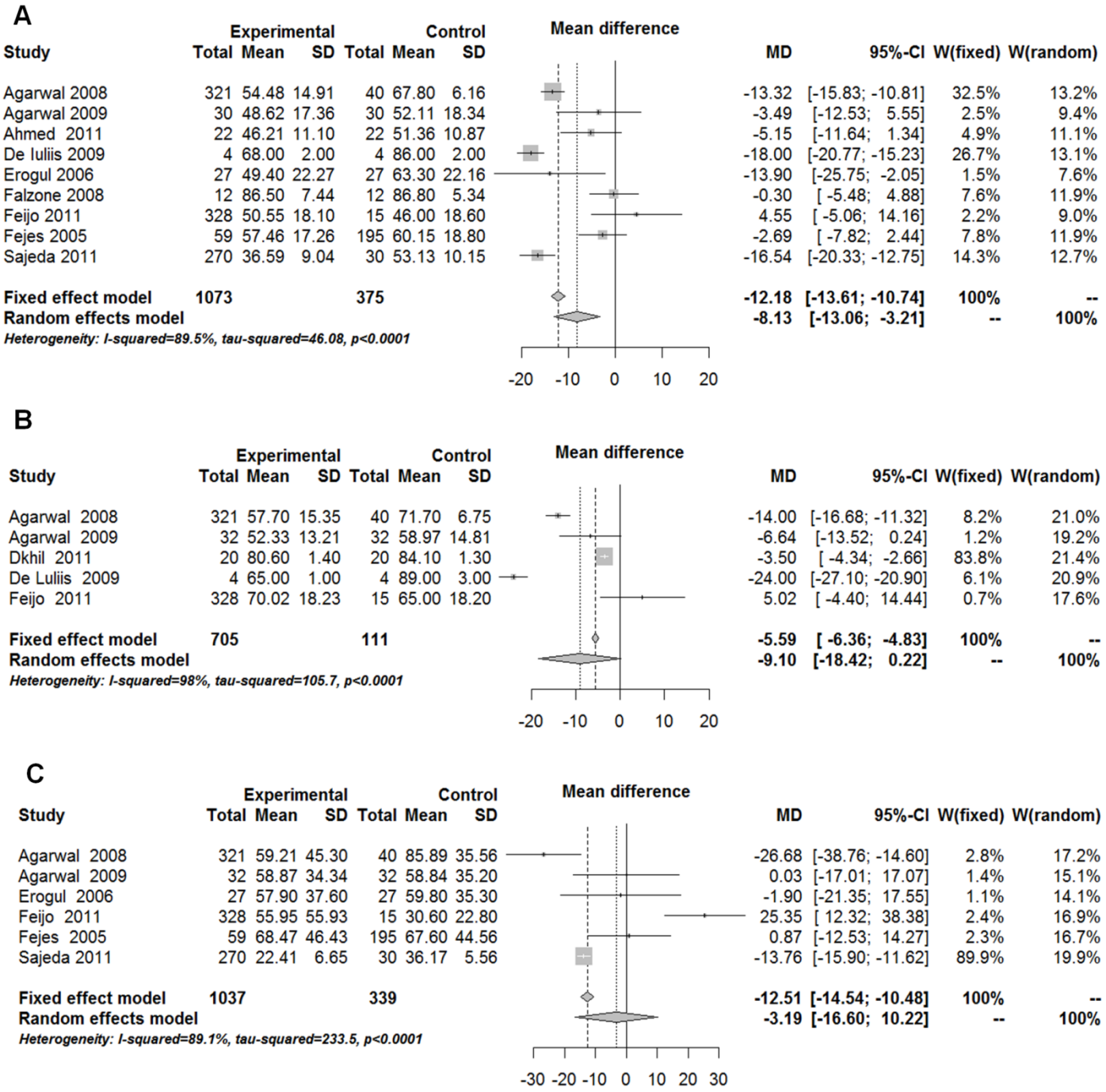
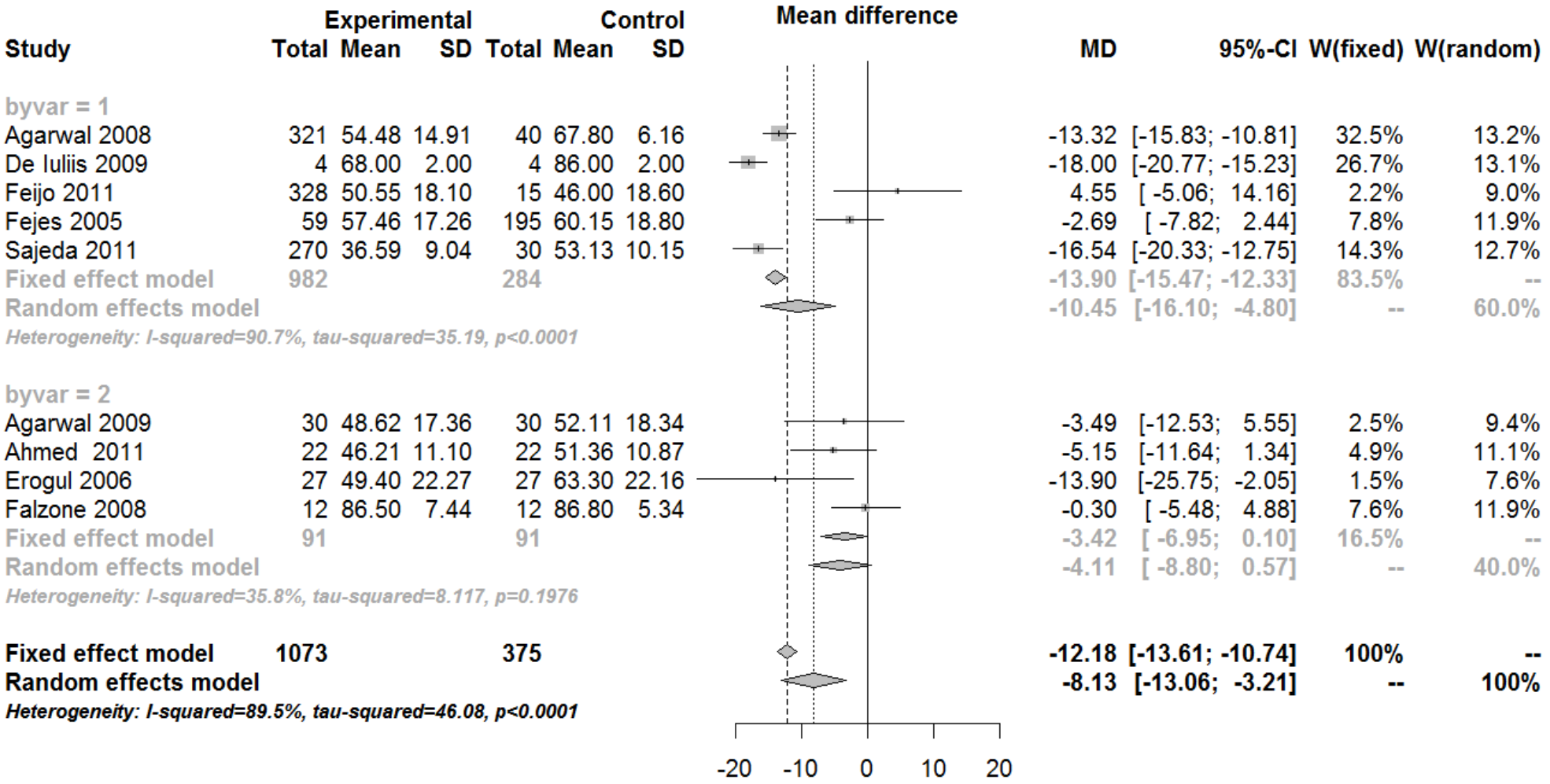


Figure 1. Forest plot showing the effect of mobile phone exposure on human sperm motility (A), viability (B) and concentration (C). A**.** FEM -12.2 (95% CI -13.6, -10.7) REM -8.1 (95% CI -13.1, -3.2**); B.** FEM -5.6 (95% CI -6.4, -4.8) REM -9.1 (95% CI -18.4, 0.2; **C.** FEM -12.5 (95% CI -14.5, -10.5) REM -3.2 (95% CI -16.6, 10.2)



1 **Figure 2.** Exposure time subgroup analyses on the effect of mobile phone exposure on sperm motility

2 (Long exposure (byvar = 1), Short exposure (byvar = 2).

Table 2. Subgroup analyses for motility

Motility Subgroup Analyses	Subgroup	Number of studies (k)	Mean Difference, (95% CI)	I^2 (%)	Statistical model
Study Design	*In vivo* groups	4	-8.1, (-15.14, -1.03)	90.2	REM
	In vitro groups	5	-8.1, (-17.08, 0.78)	91.2	REM
Participant group	Fertility Clinic	5	-7.3, (-13.74, -0.94)	88.2	REM
	Population	4	-9.2, (-19.48, 1.03)	92.7	REM
Time of exposure	Short	4	-3.4, (-6.95, 0.10)	35.8	FEM
		4	-4.1, (-8.80, 0.57)	35.8	REM
	Long	5	-10.5, (-16.10, -4.8)	90.7	REM

3.4.2 Viability

Five studies, which assayed 816 samples, were analysed (Figure 1b). Mean viability ranged from 52.3-89.0%. Four of the five studies reported a significant negative association between mobile phone exposure and sperm viability. The estimated pooled mean reduction in sperm viability was -5.6% (95% CI -6.4, -4.8) by the FEM, and -9.1% (95% CI -18.4, 0.2) by the REM. Heterogeneity (98.0% (95% CI 96.9%, 98.7%)) was high, and the REM is therefore likely to provide a better representation of the data. In subgroup analyses neither the study type, population group or duration of exposure explained the heterogeneity between studies (Table 3). Sensitivity analyses showed that, as with motility, the work of De Iuliis et al. (2009) had a large influence on the results: when this study was removed, the effect size reduced to -5.52 (Supplementary Figure 2). In contrast, the removal of Feijo and others (2011) increased the mean difference to -12.10. These results support the need for further studies to elucidate the relationship between mobile phone exposure and sperm viability.

Table 3. Subgroup analyses for viability

Viability Subgroup Analyses	Subgroup	Number of studies (k)	Mean Difference, (95% CI)	I^2 (%)	Statistical model
Study Design	*In vivo* groups	2	-5.1, (-23.66, 13.56)	93.1	REM
	In vitro groups	3	-11.4, (-26.52, 3.66)	98.7	REM
Participant group	Fertility Clinic	3	-6.0, (-16.26, 4.23)	88.2	REM
	Population	2	-13.7, (-33.78, 6.40)	99.4	REM
Time of exposure	Short	2	-15.6, (-32.61, 1.40)	95.1	REM
	Long	3	-5.1, (-13.82, 3.64)	96.5	REM

3.4.3 Concentration

Six studies, including 1,376 samples, were pooled in this meta-analysis (Figure 1c). Mean sperm concentration (10^6/ml) ranged from 22.4-85.9. There was inconsistent evidence for a reduction in concentration in relation to mobile phone exposure: the FEM, but not the REM, suggested a strong effect on concentration after exposure (FEM MD -12.5 (95% CI -14.5, -10.5); REM MD -3.2 (95% CI -16.6, 10.2)). As heterogeneity was again high (I^2 89.1% (95% CI 79.0%, 94.4%)), the REM is a more suitable analysis, suggesting there is no effect of mobile phone exposure on concentration (Figure 1c). Due to the small number of studies, subgroup analysis was only possible for study type (Table 4). Heterogeneity was reduced to 0% in the *in vitro* groups (n=2) compared to 93% in the *in vivo* groups (n=4), suggesting the majority of the difference between studies is explained by the study type. Sensitivity analyses (Supplementary Figure 3) demonstrated that the removal of Feijo and others (2011) dramatically increased the effect size (to -10.01 from -3.19), as it had for the viability analyses. The removal of any other study from the analyses had no material effect on the results. The overall effect size estimated by the analysis of all the studies may therefore be conservative, due to the influence of Feijo et al.'s study.

Table 4. Subgroup analyses for concentration

Concentration Subgroup Analyses	Subgroup	Number of studies (k)	Mean Difference, (95% CI)	I^2 (%)	Statistical model
Study Design	*In vivo* groups	4	-4.0, (-21.81, 13.77)	93.0	REM
	In vitro groups	2	-0.8, (-13.63, 12.01)	0.0	REM

3.5 Discussion

With evidence of a decline in sperm quality in recent years (Rolland et al., 2013, Swan et al., 2000), there is a need to clarify the relationships between environmental exposures and sperm quality parameters. Studies on the effect of mobile phones on male fertility indices have been contradictory. This meta-analysis summarises the evidence currently available. Mobile phone exposure was associated with reduced sperm motility and viability, whereas there was no apparent effect on concentration. The consistency in the direction of overall effects estimated for both *in vitro* and *in vivo* studies adds confidence to the findings.

The biological plausibility for an effect of mobile phones on sperm quality needs to be considered. RF-EMR may have both thermal and non-thermal effects on biological tissue. Nonthermal interactions are suggested to increase the production of reactive oxygen species (ROS) and this may lead to DNA damage (Challis, 2005). A small amount of ROS has an important functional role in sperm capacitation, the

acrosome reaction, and binding to the oocyte (Garrido et al., 2004). Experimental disruption of the flow of electrons through the mitochondrial electron transport chain has been shown to increase ROS production significantly, with negative consequences for sperm motility (Koppers et al., 2008). *In vitro* evidence found EMR emitted at the same frequency as mobile phones increased mitochondrial ROS production and DNA fragmentation in sperm, and lower motility and viability (De Iuliis et al., 2009) . The trends seen in this meta-analysis are consistent with these effects.

Thermal effects could increase the temperature of the testes − since mobile phones are often carried in trouser pockets near the reproductive organs − hampering spermatogenesis and sperm production (Agarwal et al., 2011). Skin surface temperatures on the face have been reported to rise by up to 2.3˚C after 6 minutes of mobile phone use (Anderson and Rowley, 2007). These thermal effects may be largely due to the heat generated by the handsets rather than the RF-EMR, since the frequencies of EMR released from mobile phones are thought to have negligible heating effects (Agarwal et al., 2011, Challis, 2005, La Vignera et al., 2012). If the impact of mobile phones was mainly due to heating rather than radiation, an effect on sperm concentration rather than parameters such as viability and motility, which are linked with DNA integrity, would be expected.

There are some limitations to this study. Heterogeneity, that is variation between studies that is greater than expected due to sampling error (Higgins and Thompson, 2002), is an issue in most meta-analyses. Heterogeneity was high in all our meta-analyses (I^2>88%). This may partly be due to the inflation of I^2 associated with low study numbers (Huedo-Medina et al., 2006). However, our meta-analysis did include nearly 1,500 samples, which increases confidence in the results. The heterogeneity

in the motility meta-analysis was partially due to the differences in mobile phone exposure times, as the subgroup analysis demonstrated. The high heterogeneity and relatively low number of studies also precluded meaningful assessment of publication bias (Terrin et al., 2003, Ruzni and Idris, 2012, Peters et al., 2007). However, sensitivity analyses demonstrated minimal differences when individual studies were excluded, with a tendency for our results to be conservative.

The possibility of confounding variables influencing the results of the observational studies cannot be ruled out. For example, participant age and smoking status were not consistently reported, so it is possible that these affected the observational studies since they are known to affect some sperm quality parameters, including concentration (smoking only) and motility (Kidd et al., 2001, Ramlau-Hansen et al., 2007a, Sharma et al., 2016). Nevertheless, the inclusion of *in vivo* as well as observational studies, and the consistency of the results between the study types, provides evidence that the observed effects were causal. However, study populations taken from fertility clinics, as used in many studies on male fertility, may not be representative of the general population, as they are likely to contain a higher proportion of men with sperm parameters outside the WHO reference range. This is difficult to assess because even men classified as fertile have high heterogeneity in their semen parameters (Cooper et al., 2010). Nonetheless, in all but two of our studies, the mean values were above the lower reference values given for fertile men (motility (40%, 95% CI (38, 42)); concentration (15, 95% CI (12, 16)); viability (58%, 95% CI (55, 63))) (Cooper et al., 2010), suggesting no marked bias in the study populations. In addition, WHO guidelines for the analysis of the sperm samples were applied consistently across the studies (WHO, 1999) (WHO, 2010), meaning that

standardized methodology and presentation were used, facilitating the pooling of data.

3.5.1 Future research

Mobile phone exposure appears to affect at least two of the most widely-used indices for assessing sperm quality (WHO, 2010). Sperm motility is estimated to be approximately 8% lower in exposed than non-exposed groups. Alone, the clinical importance of an effect of this size may be limited to subfertile men or those at the lower-end of the normal spectrum. However, mobile phone exposure may form part of a cumulative effect of modern day environmental exposures, that collectively reduce sperm quality and explain current trends in infertility. For example, recent evidence found wi-fi from laptops also negatively affected sperm quality (Avendano et al., 2012). A better understanding of the collective influence of environmental factors on sperm quality, and subsequently fertility, will help to improve treatment, advice and support for individuals seeking fertility treatment.

Although the subject of high-profile media attention, the number of available studies on mobile phone exposure and sperm quality is limited. Additional studies, particularly those which assess viability and other sperm parameters, including morphology and subcellular sperm damage such as sperm DNA integrity (not assessed during conventional semen analyses), are required. This would improve the precision of the estimated effect sizes, and allow better judgement of the likely clinical importance of the findings.

The period of exposure is likely to affect sperm quality, as has been demonstrated in other species (Mailankot et al., 2009), and the intensity of exposure is also likely to be important. The exposures observed in the *in vivo* studies are constrained by the

legal limits placed on SARs for mobile phones (ICNIRP, 1998), and data on the maximum SARs for each phone model are available. However, every device has fluctuating SARs, so better methods of monitoring participant exposure levels are urgently required. Long term *in vivo* studies using standardised levels and periods of exposure, ideally a randomized controlled trial in the general population, is needed to assess the importance of mobile phone exposure to public health. The hypotheses of different thermal and non-thermal effects of RF-EMR on sperm quality also need to be tested. It would be advantageous to compare the effects of intermittent exposure (where thermal effects are likely to be small) with continuous exposure to the same total amount of RF-EMR, as has been previously investigated in work on damage to DNA in human fibroblasts from mobile phones (Diem et al., 2005).

3.6 Conclusions

Our analyses indicate negative associations between mobile phone exposure on sperm viability and motility, though not concentration. Further research is required to quantify these effects more precisely and to evaluate the clinical importance of the risk to both sub-fertile men and the general population.

4. RADIO-FREQUENCY ELECTROMAGNETIC RADIATION AND SPERM QUALITY

4.1 PART 1. INVESTIGATION ON THE CUMULATIVE IMPACT OF MODERN TECHNOLOGY ON HUMAN SPERM QUALITY

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CONTRIBUTORSHIP STATEMENT

FM and JA conceived and designed the work, JA undertook laboratory work and the data analysis; FM, TG and JA interpreted the data; JA and FM drafted the work and JA, FM and TG revised it critically. All authors gave final approval and all authors agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

4.1.1 Abstract

Background

RF-EMR emitted from electronic devices has been associated with impaired male fertility. Most of the adult population is exposed to multiple devices, including mobile phones and laptop computers. The relative importance of these devices to sperm quality, and whether their effects are cumulative, is unknown.

Methods

An *in vitro* factorial experiment was carried out. Samples were collected from 10 healthy volunteers. Samples were exposed to RF-EMR from mobile phones, laptops, or the combination of both devices, for 4 hours. There were two replicates of each exposure condition per participant. Sperm motility and morphology were then assessed. Sperm DNA damage was evaluated following exposure to mobile phone RF-EMR only. A computer-assisted sperm analysis (CASA) system was used to assess sperm motility, morphology was manually assessed, whilst DNA integrity was assessed using a Comet Assay. The impact of the exposures on sperm motility, morphology and DNA damage were assessed using a Generalised Linear Mixed Model (GLMM).

Results

Our results demonstrated that exposure to RF-EMR resulted in lower sperm motility and higher levels of sperm DNA damage. *Post-hoc* analysis showed that total sperm motility was decreased by exposure to both mobile phones and laptop wi-fi (Phone Only exposure MD -0.33 95% CI -0.49, -0.16; Laptop exposure MD -0.24 95% CI -0.41, -0.08; Both devices MD -0.34 95% CI -0.50, -0.18). The effects of the

exposures on progressive motility appeared to be additive (Mobile Phone MD -0.14 95% CI -0.29, 0.00; Laptop MD -0.19 95% CI -0.34, -0.04; Both devices MD -0.30 95% CI -0.45, -0.16). No clear effects on morphology were identified. DNA damage was significantly higher following exposure to mobile phone RF-EMR (OR 1.10, 95% CI 1.08, 1.11)).

Conclusions

Sperm quality is negatively affected by exposure to RF-EMR *in vitro*. A cumulative impact on progressive sperm motility has been identified. A randomised controlled *in vivo* study is now required to determine whether sperm quality is affected by exposure to wi-fi and mobile phones in real-world scenarios.

4.1.2 Introduction

Exposure to radiofrequency electromagnetic radiation (RF-EMR) has increased rapidly with the growing use of mobile phones and laptops with wireless (wi-fi) internet access (Mailankot et al., 2009). RF-EMR is a low-level radiofrequency radiation that has been linked with a variety of adverse health effects including headaches (Oftedal et al., 2000), increased blood pressure (Braune et al., 1998) and impaired human fertility (La Vignera et al., 2012). Around 14% of couples report difficulties with conception (Wilkes et al., 2009), and a significant decline in sperm quality has been reported over recent decades (Rolland et al., 2013, Swan et al., 2000). Our previous meta-analysis suggested that exposure to RF-EMR from mobile phones negatively affected sperm quality, such as sperm motility and viability (Adams et al., 2014). It has also been reported that *in vitro* exposure to wi-fi from laptops led to a decrease in sperm quality (Avendano et al., 2012).

The International Commission on Non-Ionising Radiation Protection (ICNIRP) limits mobile phone RF-EMR to a Specific Absorption Rate (SAR) of 2.0W/kg, preventing the negative thermal effects that are documented to occur with exposures >4W/kg (ICNIRP, 1998). Nonetheless, it has been suggested that RF-electric field is still able to cause rapid heating, generating sperm damage through negative thermal effects (Challis, 2005). In addition, non-thermal effects have been reported, including changes to the binding properties of proteins and increases in the production of Reactive Oxygen Species (ROS) (Challis, 2005, La Vignera et al., 2012). RF-EMR may have insufficient energy to cause genotoxic effects such as DNA mutation or strand breaks directly (Baan et al., 2011). No evidence of DNA strand breaks were found in murine fibroblasts (Aitken et al., 2005, Malyapa et al., 1997), human glioblastoma cells (Malyapa et al., 1997) or human white blood cells (Zeni et al., 2008) when exposed to RF-EMR at frequencies similar to mobile phones. However, in human sperm, DNA strand breaks have been reported following exposure to mobile phone RF-EMR (De Iuliis et al., 2009). The conflicting results may reflect differences in redox susceptibility between cell lines (Friedman et al., 2007).

Given the extent of population exposure to RF-EMR from mobile phones and laptops, there is an urgent need to determine whether it is linked with reduced sperm quality. Here we report an *in vitro* experiment that assessed the effect of exposure to RF-EMR from mobile phones and wi-fi from laptops, both separately and in combination, on human sperm quality.

4.1.3 Methods

4.1.3.1 *Ethics*

The University of Exeter, College of Life and Environmental Sciences Research Ethics Committee approved the study (Ref: 2015/864).

4.1.3.2 *Participants and sample collection*

Semen samples were collected from 10 healthy volunteers from the University of Exeter. Participants had no known prior reproductive pathologies, 3 had proven previous fertility, and 2 were current smokers. The participant age ranged from 26-38 years (Mean 31.2, SD 4.4 years). All specimens were obtained by masturbation into a wide-mouthed plastic container (Sterilin™ 60 ml container, Thermo Scientific, UK) following an abstinence period of 2-3 days; all samples were processed within one-hour of production.

Spermatozoa were separated from seminal fluid using a discontinuous density gradient, with an 80% and 40% PureCeption® Phase gradient (Origio LTD, Reigate, UK), following World Health Organisation (WHO) guidelines (WHO, 2010). The isolated spermatozoa were then washed in 6 ml of Quinn's® sperm washing medium (Origio LTD, Reigate, UK) at 1,500 rpm for 10 minutes, before the supernatant was removed. This step was then repeated, before the pellet was re-suspended in 4.2 ml of sperm washing medium and split into 10 aliquots of 400 µl. The aliquots were placed in petri dishes (Sigma Aldrich, UK) and overlaid with ~10 ml of tissue culture oil (Origio LTD, Reigate, UK).

4.1.3.3 *Exposure conditions*

Each aliquot was split between five conditions, with two replicates for each condition (Figure 1). The conditions, adapted from previous studies (Agarwal et al., 2009, Avendano et al., 2012) were:

Control 1: exposure to a mobile phone and laptop which were both disconnected from phone and wi-fi networks;

Control 2: sample not exposed to any devices;

Phone Only: exposure to RF-EMR from a mobile phone in “Talk” mode, with sample placed 10 cm from the mobile phone;

Laptop Only: exposure to a laptop computer actively connected to the internet using a wireless network (wi-fi frequency 2.4 GHz), with sample 3 cm from the wi-fi antenna;

Phone and Laptop: exposure to RF-EMR from both a mobile phone in talk mode and a laptop computer actively connected to the internet as described above.

The following mobile phones and laptops were used in the experiment, Nokia 105 (1.45 W/Kg, GSM 900-1800 MHz) and Dell, Latitude E6520. The duration of exposure was four hours and no other devices were allowed within the study space. The experimental conditions were kept in a separate room to the control conditions. All conditions were carried out at room temperature (21˚C). Power density - the reference measure for exposure of RF-EMR (Agarwal et al., 2009, ICNIRP, 1998) – was measured (Voltcraft, MWT-2G, Conrad, UK) under each control and exposure condition. This experiment was designed to replicate the real world as closely as possible. The exposures were therefore derived from laptops and mobile phones, rather than delivered at a constant frequency. Due to the oscillating nature of the RF-EMR emitted from these devices, we were unable to quantify the amount of radiation each sample received. In the control condition power density varied between 0.01-

0.06 mW/cm^2 whereas in the exposure conditions was 0.1-3.3 mW/cm^2, in line with previous findings (Avendano et al., 2012).

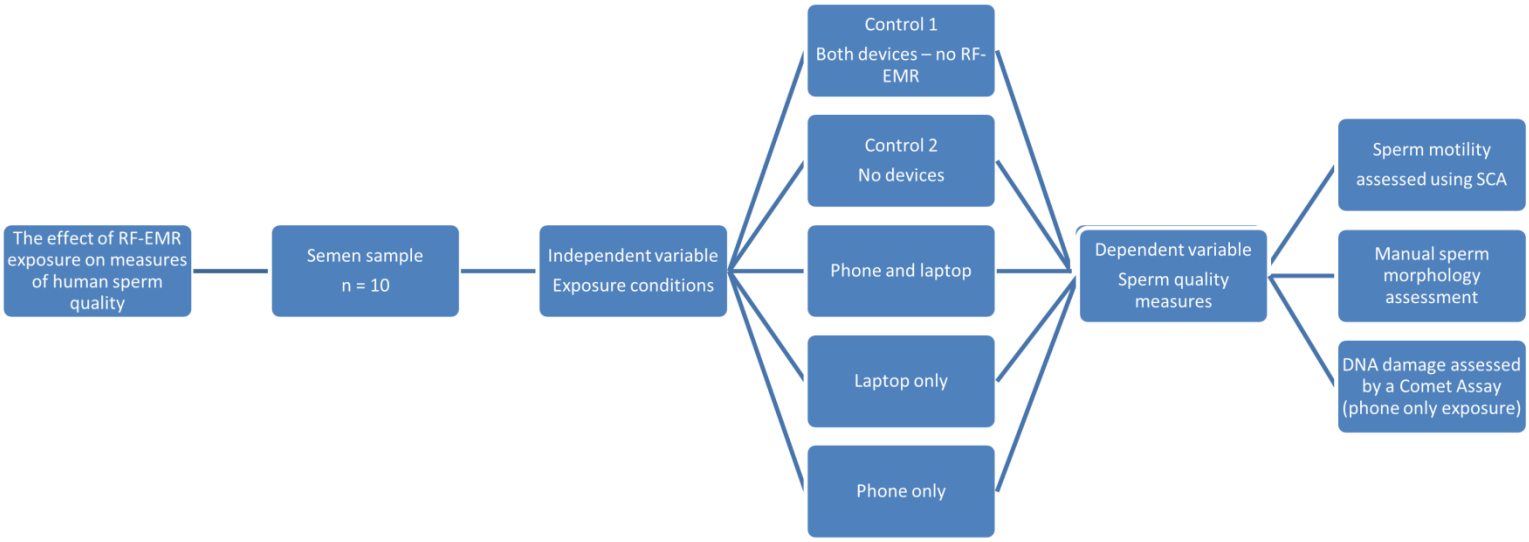

Figure 1. Experimental design diagram for the investigation of the cumulative impact of modern technology on human sperm quality.

4.1.3.4 *Semen analysis*

Assessment of sperm motility and morphology was performed according to WHO guidelines (WHO, 2010) by one practitioner, immediately following the exposure period. The practitioner participated in an external quality assurance programme (UK NEQAS Reproductive Science). Motility was measured using the CASA Sperm Class Analyser (SCA) system (Microptic, S.L, Microm, UK). Two sperm motility parameters were assessed: 1. Total motility 2. Proportion of progressively motile sperm. Sperm morphology was manually assessed using a Phase Contrast microscope (Nikon, UK). At least 200 sperm were assessed from a minimum of four fields of vision for both sperm motility and morphology.

4.1.3.5 *Alkaline Comet Assay*

The protocol for the Comet Assay was based on previously published methods (Donnelly et al., 1999, Lewis and Galloway, 2008). Microscope slides were coated with 100 µl of 1% normal melting point agarose in TAE solution (40 mM TRIS, 1 mM EDTA) at 37˚C and left to solidify. 1 x 10^5 sperm in 10 µl PBS (Ca^{2+} and Mg^{2+} free) were mixed with 90 µl of 1% low melting point agarose in Kenny's salt solution (0.4 M NaCl, 9 mM KCL, 0.7 mM K_2PO_4, 2 mM$NaHCO_3$) at 37˚C and pipetted onto the first agarose layer. A coverslip was then placed over the second agarose layer whilst it solidified at room temperature. The sperm cells were then lysed and the DNA decondensed. Once the coverslip was removed, the slides were placed in a Coplin jar and immersed in cold lysing solution [2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10, with 1% Triton X-100] for 1 hr at 4˚C. 10 mM dithiothreitoll (DTT) was added to the lysing solution and incubated with the slides for 30 min at 4˚C. Finally, 4 mM lithium diiodosalicyclate (LIS) was added to the lysing solution and incubated for 90 min at 20˚C. 10 mM MMS (methyl methanesulfonate) was used as a positive control. The slides were removed from the lysis solution and drained of any remaining liquid. Slides were placed side by side in a horizontal gel electrophoresis tank filled with fresh alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH13) at 12-15˚C, with the agarose end facing the anode. The electrophoresis buffer was filled to a level ~ 0.25cm above the slide surface for 60 minutes. The DNA fragments were separated by electrophoresis at 25 V (0.714 V/cm) for 45 min. The slides were removed from the electrophoresis solution and flooded with three changes of neutralisation buffer (0.4 M Tris, pH 7.5 for 5 min each). The slides were analysed using Comet Assay IV® (Perceptive, UK) on a Nikon Eclipse 50i (Optech Microscopes LTD, UK), with a 420-490 nm excitation filter and a 520 nm emission

filter, using 45 µl Sybr Safe (0.2 µl/ml) to stain the slides. Only nine participants samples were analysed as in one instance the comet assay failed and no results were produced for analysis.

4.1.3.6 *Statistical analysis*

All analyses were conducted using R version 3.0.02 (RCoreTeam, 2012). The effect of RF-EMR on sperm motility, morphology and DNA damage was investigated using generalised linear mixed models (GLMM) with a binomial error structure, in the package ‘lme4’ (Bates et al., 2015). Exposure condition was defined as a fixed factor with five levels: Control 1, Control 2, Mobile Phone, Laptop, Phone and Laptop. Participant identity was included as a random factor. Model fit was assessed by inspection of residuals. Overdispersion was tested using package 'blmeco' (Korner, 2015). The overall importance of including the factor Exposure Condition in the model was judged by a maximum likelihood test for independence and was found to be significant for the models assessed ($p<0.01$). Pairwise comparisons between the exposure conditions were made using Tukey *post-hoc* tests, with the ‘Honest significant differences’ (HSD) method using the package ‘multcomp’(Hothorn et al., 2008). Progressive sperm motility was analysed once an overall effect on total motility was established, as this is a measure of the number of sperm with effective forward motility and therefore may represent a more useful clinical measure.

4.1.4 Results

In all cases, sperm quality parameters were not significantly different between the two control conditions (Total Motility MD -0.01 95% CI -0.05, 0.03; Morphology MD -0.00 95% CI -0.01, 0.01). Therefore, the two control conditions were combined in order to simplify the models.

4.1.4.1 *Motility*

RF-EMR exposure from any source decreased total sperm motility compared with controls (Figure 2). The measure for overdispersion was less than 1.4 for the binomial GLMM models carried out to assess total motility (1.02) and progressive motility (1.21), suggesting they were not overdispersed. The effect of RF-EMR exposure on total motility did not seem to be additive, with the decrease in total motility with both exposures being similar to that observed for mobile phones alone (Mobile Phone Only exposure MD -0.33 95% CI -0.49, -0.16; Laptop exposure MD -0.24 95% CI -0.41, -0.08; both devices MD -0.34 95% CI -0.50, -0.18). Progressive motility declined with exposure to mobile phones and wi-fi, and these effects appeared to be additive (Mobile Phone only exposure MD -0.14 95% CI -0.29, 0.00; Laptop exposure MD -0.19 95% CI -0.34, -0.04; Both devices MD -0.30 95% CI -0.45, -0.16)(Figure 3).

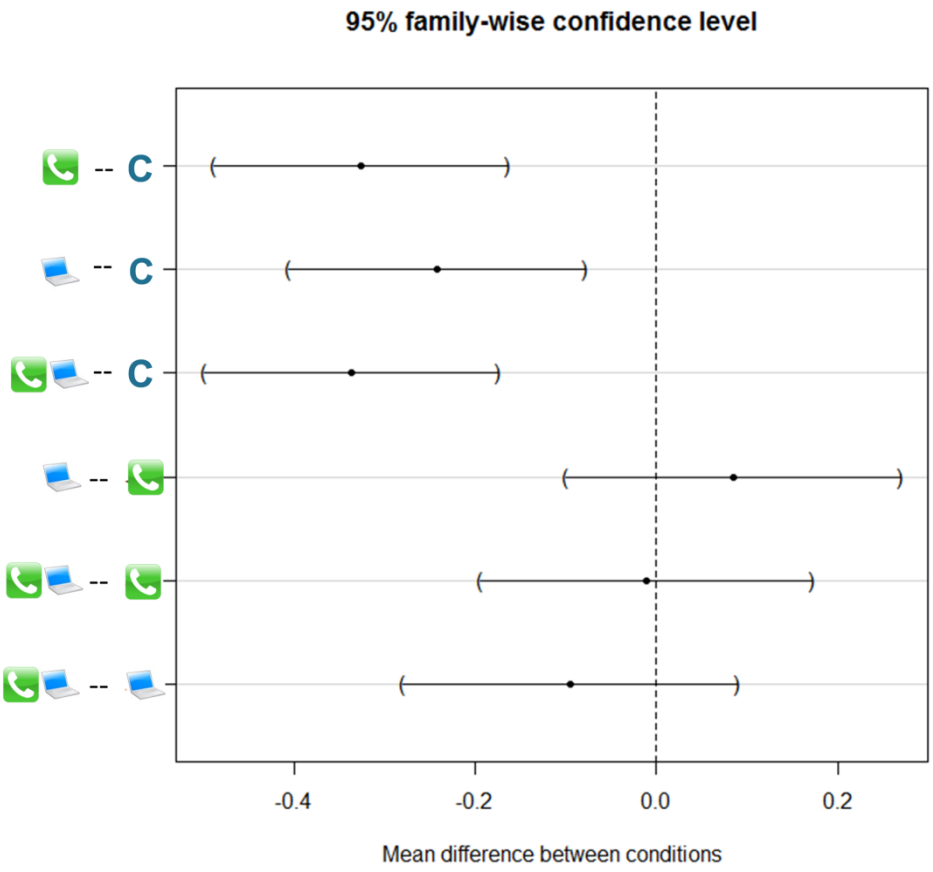

Figure 2. Total sperm motility was significantly lower in all three exposure conditions when compared with the control conditions (Phone Only exposure MD -0.33 95% CI -0.49, -0.16; Laptop exposure MD -0.24 95% CI -0.41, -0.08; Both devices MD -0.34 95% CI -0.50, -0.18)(C=Combined controls, 💻 = Laptop exposure, 📞 = mobile phone exposure).

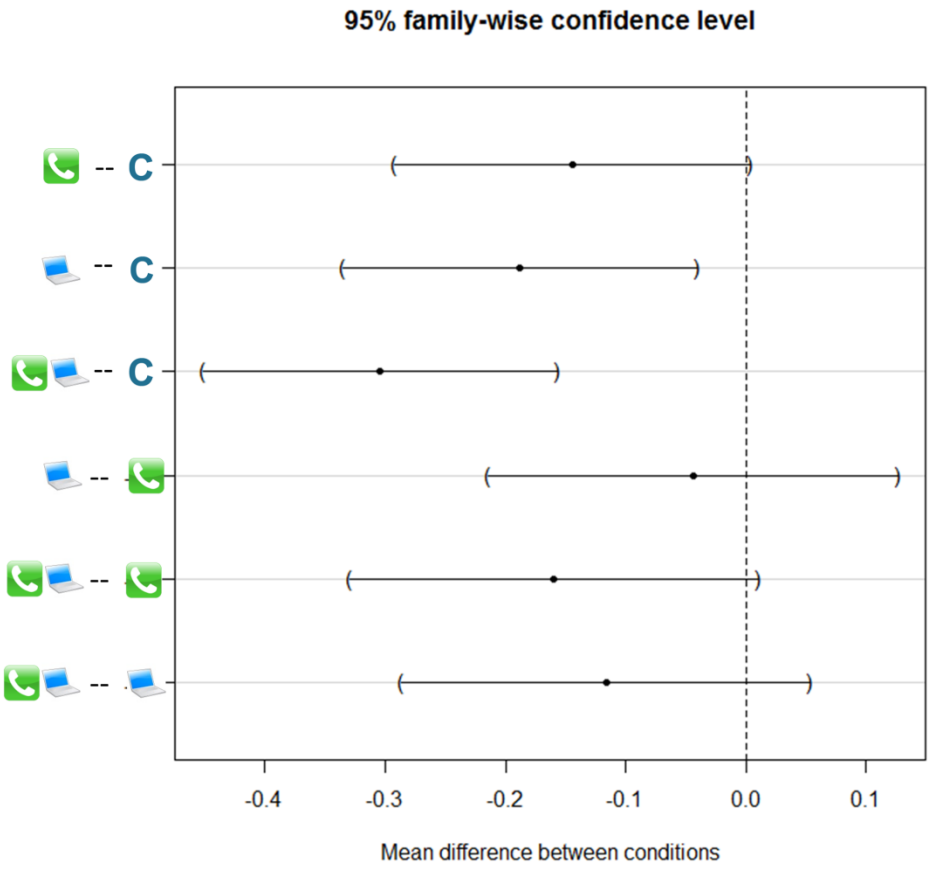

Figure 3. Progressive sperm motility was significantly lower in all three exposure conditions when compared with the control conditions (Mobile Phone only exposure MD -0.14 95% CI -0.29, 0.00; Laptop exposure MD -0.19 95% CI -0.34, -0.04; Both devices MD -0.30 95% CI -0.45, -0.16)(Figure 2) (C=Combined controls, = Laptop exposure, = mobile phone exposure).

4.1.4.2 *Morphology*

RF-EMR emitted from mobile phones and wi-fi did not affect sperm morphology (Figure 3). The measure for overdispersion was less than 1.4 for the binomial GLMM model carried out to assess sperm morphology (0.52), suggesting the model was not overdispersed.

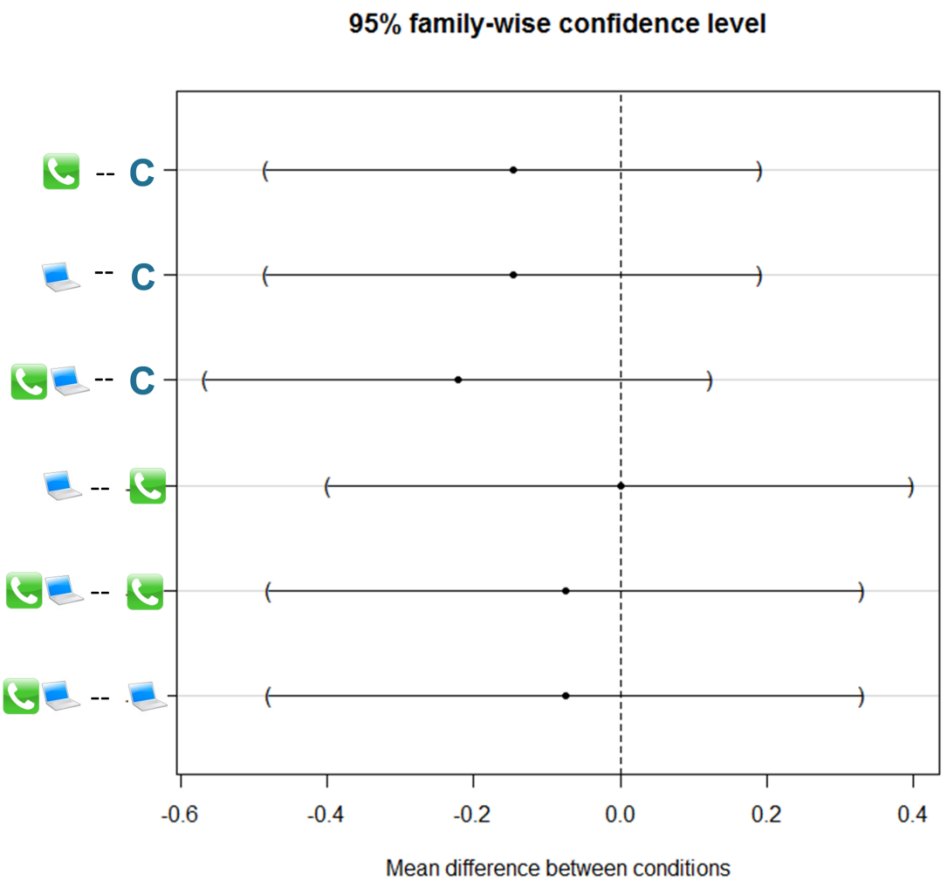

Figure 3. The proportion of sperm with normal morphology did not significantly differ in the three exposure conditions when compared with the control conditions (C=Combined controls, = Laptop exposure, = mobile phone exposure).

4.1.4.3 *DNA damage*

Nine participants could be included for DNA damage assessment following exposure to RF-EMR from a mobile phone. There was overdispersion detected in this model, further data would be of use in drawing strong conclusions from this analysis. Nonetheless, there was a significant increase in DNA damage after exposure to mobile phone RF-EMR (OR 1.10 95% CI 1.08, 1.11).

4.1.5 Discussion

We have shown that RF-EMR from both mobile phones and laptops decrease measures of sperm quality. Further, their effects on progressive sperm motility appear to be additive. This work supports our previous meta-analysis that suggested that exposure to mobile phone RF-EMR had a negative effect on sperm motility (Adams et al., 2014). Further, we provide evidence of a potential mechanism, since DNA damage was increased by mobile phone RF-EMR.

Few studies have investigated the impact of RF-EMR from wi-fi on sperm quality. Our work confirms the impact on sperm motility reported previously (Avendano et al., 2012). The few available animal studies assessing wi-fi exposure, concur with our findings on sperm motility. Negative impacts on measures of rat fertility (Atasoy et al., 2013, Shokri et al., 2015, Dasdag et al., 2015), including sperm concentration and motility have been reported (Shokri et al., 2015), whilst extended exposure led to a decrease in the weight of the epididymis and seminal vesicles (Dasdag et al., 2015). Their additional findings of a negative effect on sperm morphology was not repeated in this analysis (Shokri et al., 2015). However, it is likely that the tolerance to exposure in rats is different to that of humans, and therefore, the results may not be comparable.

Negative thermal and non-thermal effects of RF-EMR have previously been described (Anderson and Rowley, 2007, Challis, 2005, La Vignera et al., 2012). There are two main mechanisms that could explain the observed impacts of RF-EMR from mobile phones and wi-fi on sperm quality. These are (1) DNA fragmentation and (2) thermal effects generated by RF-EMR and/or direct heating from warm devices, especially laptops, being placed near the testes.

Sperm DNA damage was significantly higher following exposure to mobile phone RF-EMR. This effect is likely to be mediated by an increase in ROS (Challis, 2005, La Vignera et al., 2012, Liu et al., 2015). Post meiotic sperm cells are susceptible to oxidative stress due to a lack of cytoplasm and associated antioxidant enzymes, a loss of DNA repair capacity, and an inability to undergo apoptosis (Aitken et al., 2005, De Iuliis et al., 2009). This leaves sperm cells vulnerable to the effects of exposure to RF-EMR when progressing to the cauda epididymis from the seminiferous tubules during spermatogenesis (Turner, 1995). An increase in mitochondrial ROS and subsequent oxidative stress following exposure to RF-EMR, induced DNA fragmentation in sperm (De Iuliis et al., 2009) and lower motility, viability and normal morphology *in vitro* (De Iuliis et al., 2009, Koppers et al., 2008, Aziz et al., 2004). This may explain the increase in DNA damage demonstrated in our results and the observed decrease in sperm motility.

Sperm DNA damage has been inversely associated with live births and an increased risk of pregnancy loss following ART (Simon et al., 2013, Zini et al., 2008). Sperm DNA damage of greater than 25% has been associated with a higher risk of infertility (Simon et al., 2011), with a further study suggesting that there is a threshold of 45% sperm DNA damage leading to male infertility, as measured by the Comet assay (Ribas-Maynou et al., 2013). In one study, a 10% increase in sperm DNA damage

was associated with recurrent miscarriage (23.5% (fertile controls) vs. 33.6% (recurrent miscarriage group) (Ribas-Maynou et al., 2013). Therefore, the increase in DNA damage of ~9% following exposure to RF-EMR from mobile phones in this study may have pathological consequences. Further data is needed to confirm our findings before it is possible to determine the full impact of exposure of RF-EMR on sperm DNA damage.

Warm electronic devices may negatively affect spermatogenesis, for example from laptops used near the testes, whilst RF-EMR emitted from the devices may also cause negative thermal effects (Challis, 2005). If thermal effects were having a significant impact on sperm quality, it would likely be reflected by a decrease in sperm concentration. Although in rats, exposure to RF-EMR increased apoptosis by 91%, reducing sperm count (Liu et al., 2015), our meta-analysis in humans, demonstrated that sperm concentration was not significantly affected (Adams et al., 2014). The decrease seen in other sperm quality parameters, such as motility, suggests RF-EMR damage is largely due to a radiation rather than a heating affect. This is further supported by our findings, which demonstrated no significant difference between our two control conditions on any sperm quality parameter.

4.1.6 Conclusion

Taken together, our meta-analysis and *in vitro* study demonstrate the RF-EMR is negatively affecting various sperm quality parameters. Further, the effects of exposures from different devices can have a cumulative impact on progressive sperm motility. It is now important to replicate this study *in vivo*. Unlike this experiment, sperm cells are usually protected within the body and are not directly exposed to RF-EMR. A randomised controlled study is therefore now required to

conclusively identify if RF-EMR from electronic devices is negatively affecting sperm quality in the general population.

4.2 PART 2. RANDOMISED CONTROLLED STUDY ON THE IMPACT OF MOBILE PHONE EXPOSURE ON HUMAN SPERM QUALITY: A PILOT

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4.2.1 Abstract

Objective

Our previous findings have identified that measures of sperm quality are negatively impacted by exposure to RF-EMR from mobile phones. A causal relationship has yet to be identified. Previous work has shown that both testosterone and smoking can also influence sperm quality. Therefore, in this study, urinary testosterone levels and smoking status will be analysed.

Method

A randomised controlled study was carried out. Fourteen participants were randomly assigned to two groups: 1. Control (phone stored away from testes) 2. Exposure (phone stored in trouser pocket). Participants completed a baseline questionnaire that collected information on age, smoking status and use of electronic devices. Sperm motility was assessed using a computer-assisted sperm analysis (CASA) and morphology, viability and concentration were assessed using light microscopy. Sperm viability was assessed using an eosin-nigrosin stain. Urinary testosterone levels were assessed using Liquid Chromatography - mass spectreometry (LC-MS). The impact of mobile phone exposure on sperm quality was assessed using a Generalised Linear Model (GLM).

Results

Sperm motility was negatively linked to smoking (MD -1.57 95% CI -2.94, -0.33, p=0.04) and hours of usual phone use prior to the study (MD -0.98 95% CI -1.84, -0.21, p=0.04). None of the measured sperm quality parameters were influenced by phone exposure during the experiment group. A positive relationship between

testosterone levels and sperm concentration was identified (MD 4.37 95% CI 1.51, 7.22, p=0.01).

Conclusions

Sperm quality was not negatively affected in the location of mobile phones in this study. However, average phone use prior to the study, and current smoking status, were associated with a decrease in sperm motility. Due to the low number of participants in this study, further large scale randomised controlled studies are required .

4.2.2 Introduction

Androgens such as testosterone are essential for spermatogenesis and therefore male fertility (Sharpe, 1994) (McLachlan et al., 2002). Androgen receptor (AR) knockout (KO) mice have various phenotypes relating to reduced fertility. When AR are removed from leydig cells, enzymes required to synthesise testoserone are reduced (Xu et al., 2007), whilst in sertoli cells, spermatogenesis is halted at meiosis (De Gendt et al., 2004, Chang et al., 2004). Psychological stress and subsequently release of stress hormones including cortisol and adrenocorticotropic hormone (ACTH), are also able to disrupt testosterone production, resulting in poorer sperm quality (Klimek et al., 2005, Bhongade et al., 2015). Therefore, assessment of testosterone may be useful as an indicator of male reproductive health.

Smoking may also have a negative effect on male reproductive health (Vine et al., 1994, Yang et al., 2016, Sharma et al., 2016). The effect of smoking on sperm quality is conflicting (Vine et al., 1994, Harlev et al., 2015, Kunzle et al., 2003, Joo et al., 2012, Ramlau-Hansen et al., 2007a, Martini et al., 2004, de Jong et al., 2014,

Jensen et al., 2004, De Bantel et al., 2015, Sharma et al., 2016). However, smoking has been identified as harmful to germ cells (Yauk et al., 2007), and may negatively affect the secretary function of leydig cells resulting in a sperm maturation and spermatogenesis deficiency (Parazzini et al., 1993, Yamamoto et al., 1998). In addition, heavy paternal smoking has been associated with early pregnancy loss (Venners et al., 2004) and lower pregnancy rates following ART (Zitzmann et al., 2003).

Our previous work, including a meta-analysis and *in vitro* study, has identified that RF-EMR from electronic devices, influences sperm quality. Here we have assessed the relationship between RF-EMR and sperm quality using a randomised controlled study. As men often keep their mobile phone in their trouser pockets, close to the testes, we have investigated whether this 'real world' exposure to RF-EMR is limiting sperm quality. Due to the relatively low sample size of the project, adjustments were made for differences between the groups in the potential confounding variables of urinary testosterone concentration and smoking status.

4.2.3 Methods

4.2.3.1 *Ethics*

Ethical approval was granted by the University of Exeter, Biosciences Research Ethics Committee.

4.2.3.2 *Participants and sample collection*

Fourteen anonymous volunteers were randomly assigned into one of two groups (Figure 1). The participant age ranged from 22-43 years old (Mean 29.6 ±SD 6.8 years). Two participants were current smokers. The exposure group were asked to

store their mobile phones in their trouser pockets during normal waking hours, for 5 days prior to producing a semen sample for analysis; whereas the control group were asked to ensure their phone was kept out of their trouser pocket. Participants were asked to abstain from sexual activity for 2 days prior to participation. Urine samples were produced prior to production of a semen sample; all samples were collected within one-hour post production. All participants were asked to complete an anonymous questionnaire (see supplementary information), that collected information including such as their age (years), smoking status (Yes/No) and normal mobile phone use per day (hours/day). After the exposure period, participants were asked to produce a urine and semen sample. All specimens were collated into a wide-mouthed plastic container (Sterilin™ 60ml container, Thermo Scientific, UK).

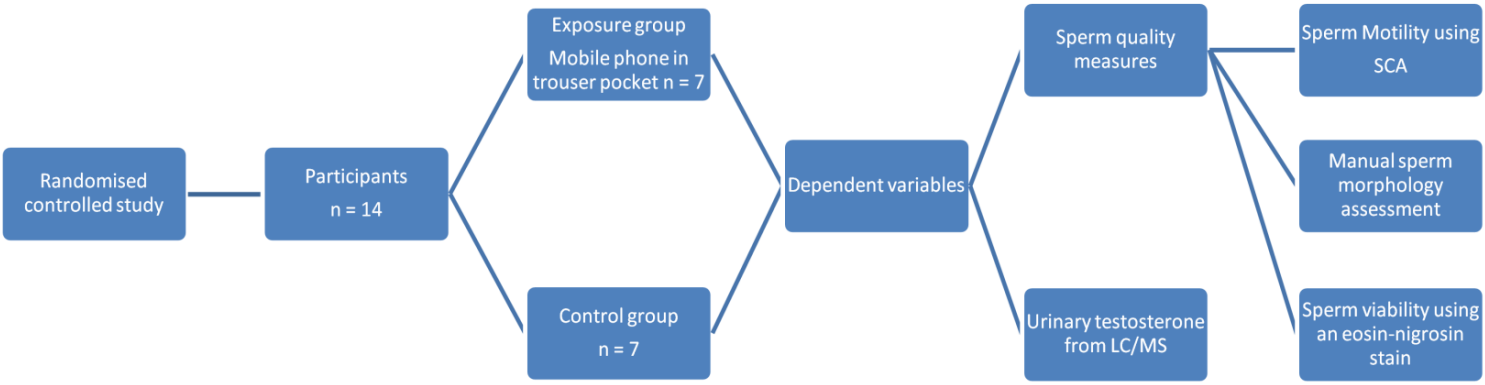

Figure 1. Experimental design diagram for a randomised controlled study on the impact of RF-EMR from mobile phones on sperm quality.

4.2.3.3 *Semen analysis*

Assessment of sperm motility, morphology, vitality and concentration were performed according to WHO guidelines (WHO, 2010) within one hour of production. The practitioner participated in an external quality assurance programme (UK

NEQAS Reproductive Science). Motility was assessed using the Sperm Class Analyser (SCA) system (Microptic, S.L, Microm, UK). A minimum of 200 sperm cells, across at least 4 fields of vision were assessed for sperm motility, morphology and viability. Viability was assessed using an eosin-nigrosin stain (VitalScreen®, Microm, UK). 50 µl of semen were mixed with two drops of 1% Eosin Y. After 30 seconds, three drops of 5% nigrosin were added and vortexed briefly to mix thoroughly. Within 30 seconds a thin smear of the mixture was placed on a microscope slide and assessed immediately.

4.2.3.4 *Testosterone analysis using LC/MS*

The protocol was adapted and carried out according to manufacturer's instructions (Biotage, UK). 2.325 ml of formic acid were added to 2.5 ml of urine, to enhance the extraction efficiency of the columns, and spiked with 1mg/ml of testosterone. The samples were then purified on Isolute® SPE C8 cartridges (Biotage, UK). The urine solution was added to the cartridge, a vacuum was applied for 2-10 sec to initiate loading and the sample was left for 5 min to absorb into the cartridge. The columns were then washed with 8 ml of ethyl acetate, flowing for 5 min under gravity, completing the elution with 2 minutes under vacuum. This was repeated a further two times. The solution was then concentrated using a SpeedVac® system (ThermoScientific, UK) and the residue reconstituted in 500 µl methanol (100%). This was centrifuged and the supernatant was analysed.

Analyses were performed on a 6520 Accurate Mass Q-TOF LC/MS (Agilent technologies). Aceto Nitrile and 1mM ammonium fluoride were used as mobile phases, on a gradient of 0-100% over 20 min. The mass spectrometer was used in the negative ion mode with a spray voltage of -3,500 V. The vaporiser temperature

was adjusted to 325˚C. The Q2 nitrogen collision gas was set at a pressure of 1 x 10^5Torr and the dwell time was set at 50 ms.Threshold levels were set at 1,000 counts with a max coverage of 0.1 m, to ensure maximal coverage. To optimise the mass spectrometer parameters the internal standards were analysed using testosterone (Sigma-Aldrich, UK) and a standard curve produced (0.24-31.25 pg/ml). Preferential mass detection was set at mass levels associated with the target compound.

Following this, the method was optimised further using the 6410 Triple Quad LC/MS (Agilent technologies). The standards were run without separation to allow for Selective ion monitoring (SIM). This method improves ion transmission to increase sensitivity and the detected abundance of the ion. For each product mass the fragmentor voltage and collision energy were optimised to find the voltages that recorded the largest abundance of the standard under investigation. As SIM analysis detected fragments, the samples were suitable for Multiple reaction monitoring (MRM). This is designed to obtain the greatest sensitivity to detect the required compounds (Cox et al., 2005).

4.2.3.5 *Statistical analysis*

All analyses were conducted using R version 3.0.02 (RCoreTeam, 2012). The data collated were assessed using Generalised Linear Models (GLM) using the package ‘stats’ (RCoreTeam, 2012) . Overdispersion was detected in models assessing the effect of phone exposure sperm motility, viability and morphology, therefore standard errors were corrected using a quasibinomial error structure. Gaussian error structures were appropriate for the model of sperm concentration. Normal mobile phone use per day, urinary testosterone concentration and age were included as

covariates. Exposure to phone in pockets and smoking status were included as fixed factors with two levels. All models were simplified by manual backwards stepwise deletions until minimum adequate models were obtained. Residuals were checked to ensure model fit was appropriate.

4.2.4 Results

Sperm motility was negatively linked to being a smoker and increasing hours of usual phone use prior to the study : Motility was lower in smokers (MD -1.57 95% CI -2.94, -0.33, p=0.04) and decreased by ~1.0% per hour of increased phone use (MD -0.98 95% CI -1.84, -0.21, p=0.04). None of our sperm quality parameters were associated with exposure group (motility: MD 1.25 95% CI 0.14, 2.54, p=0.06; morphology: MD -0.50 95% CI -1.11, 0.08, p=0.12; viability: MD 0.12 95% CI -0.46, 0.70, p=0.69; concentration: MD -13.33 95% CI -77.33, 50.67, p=0.68). Increased testosterone levels were associated with an increase in sperm concentration (Figure 2)(MD 4.37 95% CI 1.51, 7.22, p=0.01) but was not associated with the other sperm quality parameters assessed (Figure 3). However, the effect between concentration and urinary testosterone was not significant when a potential outlier was removed (see Figure 2)(MD 5.75 95% CI -0.91, 12.40, p=0.08).

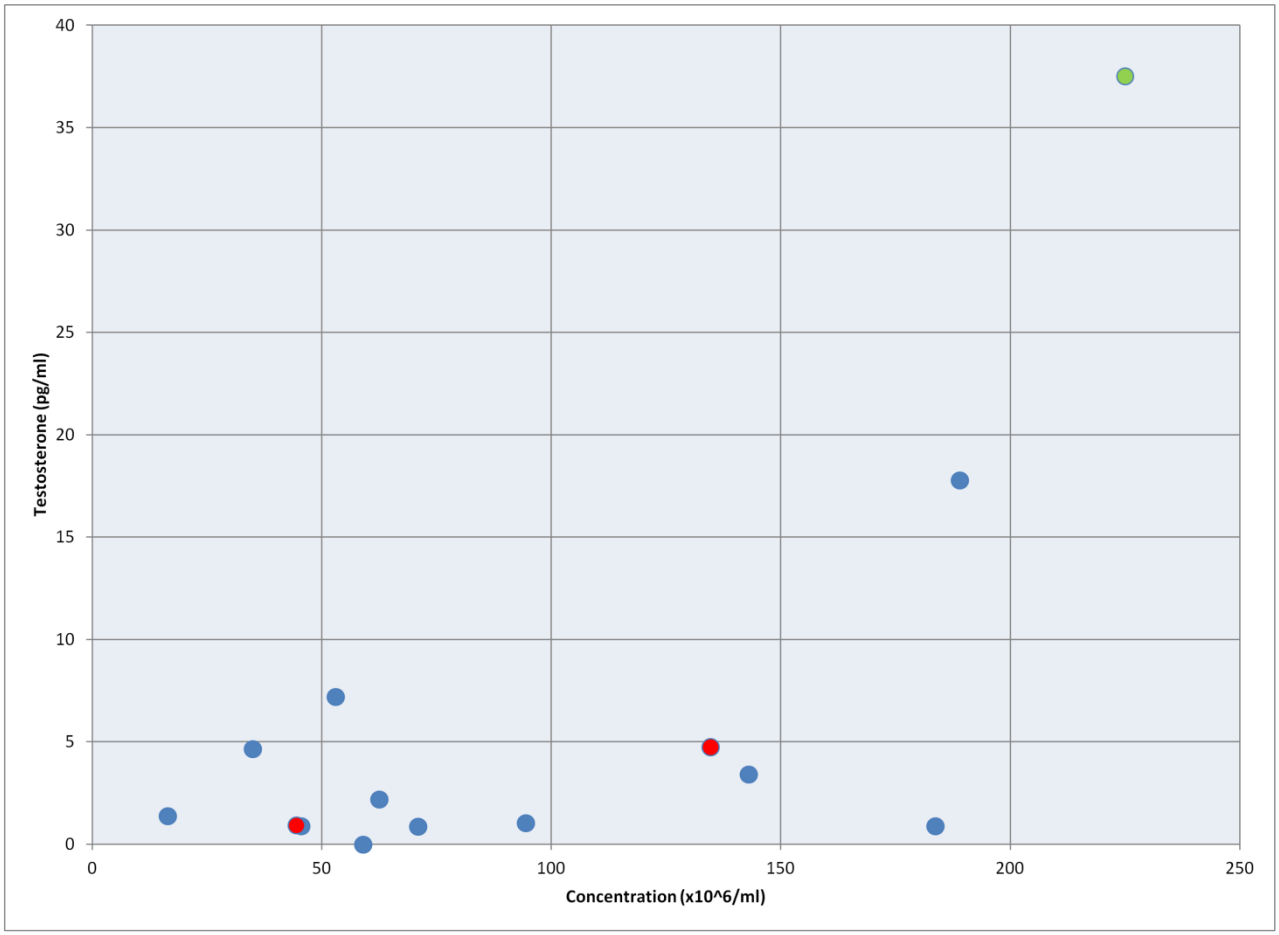

Figure 2. The relationship between urinary testosterone levels and sperm concentration. Smokers are marked in red. Potential outlier marked in green.

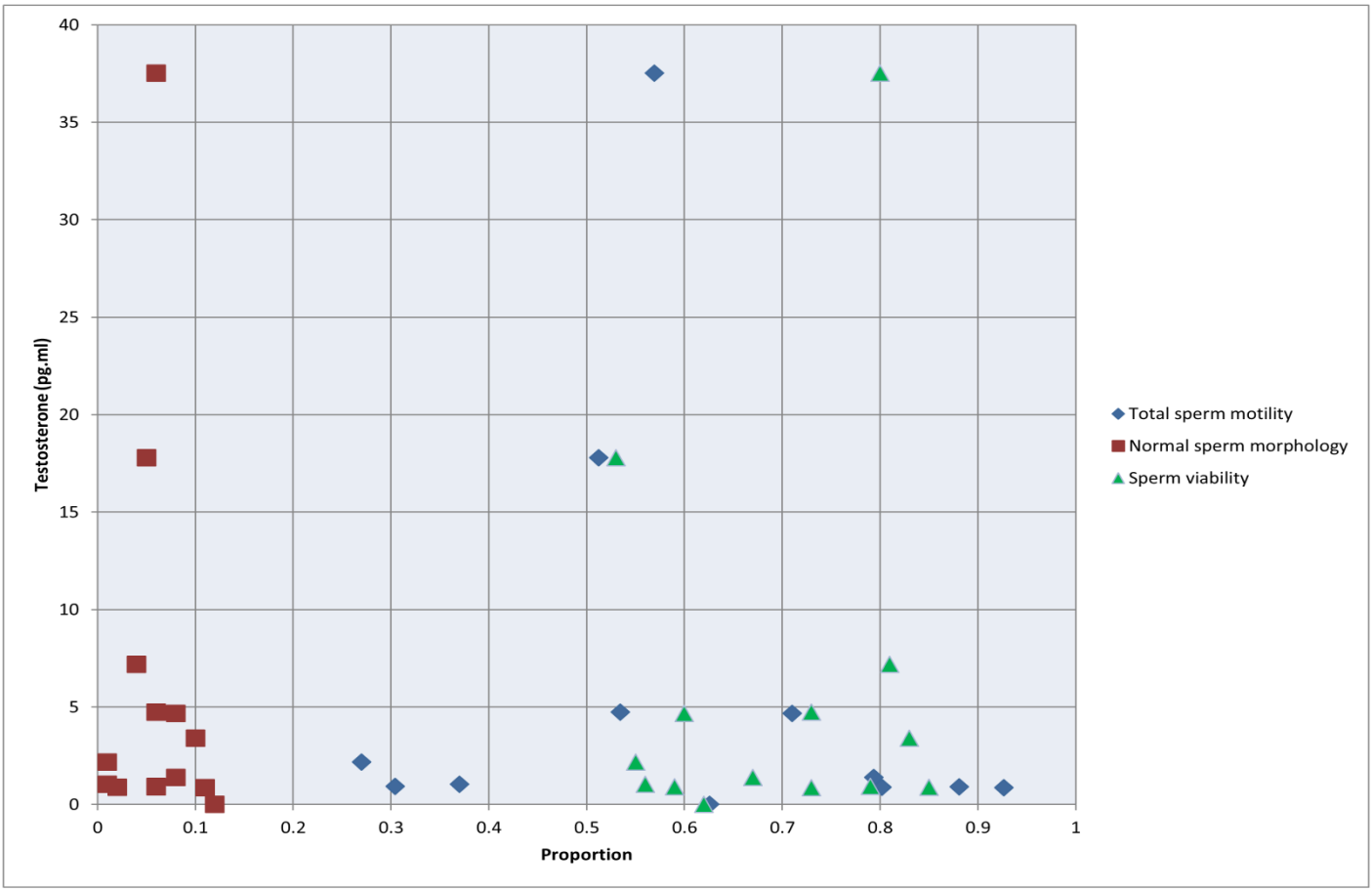

Figure 3. Urinary testosterone levels were not associated with sperm quality parameters such as total sperm motility, normal sperm morphology and sperm viability.

4.2.5 Discussion

To the best of our knowledge, this is the first randomised controlled study to assess whether RF-EMR from mobile phones kept in trouser pockets are negatively affecting sperm quality. Our findings in this pilot study suggest that there is no alteration to sperm motility, morphology, viability or concentration according to where mobile phones are stored in relation to the testes. However, we have identified that motility decreases in smokers and by increased normal phone use. In addition, sperm concentration is likely to be greater in men with higher urinary testosterone levels.

The finding of no effect on sperm quality, in our exposure group, is at odds with our previous findings. Nonetheless, we identified that average mobile phone use was negatively associated with sperm motility. The variation in our results may reflect the small study numbers in our analyses and we are unable to explain the increase in sperm motility seen with the device kept close to the testes. Nonetheless, taken together with the evidence described previously in this chapter, the effect seen on motility, rather than concentration, adds further evidence that RF-EMR from mobile phone use, may be acting through a non-thermal pathway by increasing DNA fragmentation and OS (Koppers et al., 2008, De Iuliis et al., 2009, Aziz et al., 2004).

This work has suggested two other influential factors on sperm quality: testosterone and smoking status. Testosterone levels can be altered by exogenous factors such as psychological stress, for example, following ART treatment (Klimek et al., 2005, Bhongade et al., 2015). Our findings suggested that there was a positive relationship between sperm concentration and testosterone levels, whilst an increase in the participant number may strengthen our findings, this is supported by previously

described biological mechanisms. Without testosterone or androgen receptors (AR), spermatogenesis is unable to progress past meiosis, rendering the male infertile (Haywood et al., 2003, Chang et al., 2004, De Gendt et al., 2004). Testosterone is involved in maintenance of a number of reproductive functions. This includes maintaining the blood testis barrier (BTB), to inhibit meiotic germ cells coming under autoimmune attack and sertoli-spermatid adhesion, to ensure round spermatids are not release prior to conversion to elongated spermatids. Testosterone also aids the process of mature spermatozoa release from the sertoli cells: if this does not happen phagocytosis of the germ cells occurs (Smith and Walker, 2014, Walker, 2011). Serum testosterone levels have previously been positively associated with sperm concentration (Meeker et al., 2007).

Our findings of an effect of smoking status on sperm motility follows a history of conflicting evidence (Kunzle et al., 2003, Vine et al., 1994, Harlev et al., 2015, Sharma et al., 2016). Recently, evidence of a genetic susceptibility to the negative effect of smoking on fertility has been identified, which may help to explain the inconsistencies in other research. Certain polymorphisms in a transcription factor involved with expression of antioxidant genes (NFR2) has been found more frequently in heavy smokers with poorer sperm quality, than those with normal sperm quality (Yu et al., 2013). Previously, it had been identified that these polymorphisms decreased transcriptional activity of NFR2 increasing the risk of oligoastenozoospermia (Yu et al., 2012). If a genetic susceptibility increases the risk to fertility posed by environmental exposures, there are a number of avenues for future research into this relationship.

Previous studies, such as those included in our meta-analysis, largely included men from fertility clinics who may not be representative of the general population, as they

are likely to contain a higher proportion of men with sperm parameters outside the WHO reference range. Alongside this studies were observational, meaning cause and effect could not be established. We attempted to address this by carrying out a randomised controlled study on mobile phones and sperm quality. However, the time frame we studied, of 5 days prior to participation, may not have been long enough to demonstrate a significant effect of storing phones in trouser pockets, but was constrained by the length of time that was deemed acceptable to participants. Further, whilst participants were randomly assigned to a exposure group, it was not possible to blind the study to the single researcher carrying out the lab analyses In addition, there was a limited number of volunteers to our study reducing the statistical power of our analyses.

4.2.6 Conclusion

This pilot study shows that testosterone concentration may influence sperm concentration, and that increased use of mobile phones and smoking may negatively impact on sperm quality. However, it is possible that the variations in the size of effect demonstrated across studies is in part, mediated by a personal genetic predisposition that requires further investigation. In addition, larger scale randomised controlled studies would be advantageous before strong conclusions can be formed on whether exposure to RF-EMR *in vivo* can can negatively impact sperm quality.

5. MISCARRIAGE RATES FOLLOWING FERTILITY TREATMENT IN THE UK: A LONGITUDINAL STUDY

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CONTRIBUTORSHIP STATEMENT

FM and JA conceived and designed the work, JA undertook the data analysis; FM, TG and JA interpreted the data; JA and FM drafted the work and JA, FM and TG revised it critically. All authors gave final approval and all authors agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

5.1 Abstract

Objective

Miscarriage is a common pregnancy outcome, and the risk is elevated among couples receiving Assisted Reproductive Technologies (ART). In this study, we examine whether the risk of miscarriage is increasing over time for women undergoing their first IVF/ICSI cycle.

Methods

The Human Fertilisation and Embryology Authority (HFEA) collates information on all ART treatments in the UK. Using data from 126,498, singleton pregnancies in women undergoing their first cycle using autologous gametes, excluding frozen cycles, we assessed the change in miscarriage rates between 2003 and 2011 using a Generalised linear model (GLM).

Results

The success of ART as measured by both clinical pregnancies and live births has increased over time (OR 1.045 95% CI 1.040-1.050; OR 1.040 95% CI 1.035-1.046, respectively). However, the risk of miscarriage was also higher (OR 1.046 95% CI 1.032-1.059). The results are not explained by an increase in the proportion of older mothers (≥38 years) undergoing ART – a known risk factor for miscarriage – as this has remained stable. In addition, patients treated for male factor infertility with ICSI had a lower risk of pregnancy loss compared with IVF.

Conclusions

Previous reports of the increasing success of ART are corroborated by our findings, but we have also identified an increased risk of miscarriage over time among women undergoing ART. Hence the number of livebirths has not kept pace with the numbers of successful conceptions and clinical pregnancies. It is also the first large scale study to identify that treating male factor infertility with ICSI, in place of IVF, reduces the risk of pregnancy loss. Further work is required to identify the causal factors involved.

Keywords**:** Miscarriage, ART, fertility, pregnancy loss, IVF, ICSI, HFEA.

5.2 Introduction

Between 10 and 15% of all conceptions end in miscarriage (Nybo Andersen et al., 2000, de la Rochebrochard and Thonneau, 2002). In the US general population, self-reported miscarriage rates steadily increased between 1970-2000. Explanatory factors include improved (earlier) pregnancy diagnosis and negative environmental exposures, such as occupational exposure to solvents or pesticides (Lang and Nuevo-Chiquero, 2012), with risk factors for pregnancy loss including maternal age, BMI, stress and alcohol consumption previously identified (Nybo Andersen et al., 2000, Maconochie et al., 2007, de la Rochebrochard and Thonneau, 2002, Feodor Nilsson et al., 2014, Veleva et al., 2008). Paternal factors including age (de la Rochebrochard and Thonneau, 2002) and sperm DNA damage (Zini et al., 2008) have also been implicated in miscarriage.

The number of people accessing Assisted Reproductive Technologies (ART) increased by 40% from 2006 to 2013 (HFEA, 2014, Kurinczuk and Hockley, 2010). A steady rise in clinical pregnancy and live birth rates resulting from these interventions has also been observed (HFEA, 2014). Pregnancies achieved via ART treatment

have a higher risk of miscarriage when compared with natural pregnancies (OR 1.2) (Wang et al., 2004). This is a burden on success rates and has psychological implications for patients (Thapar and Thapar, 1992). In this study, we use data from all women in the UK undergoing their first IVF/ICSI cycle, to assess changes in miscarriage rates over time, in a well-defined cohort of women undergoing their first autologous (own gametes) IVF/ICSI cycle. The research focused on whether the increased use of ART treatment is altering miscarriage rates.

5.3 Methods

5.3.1 *Data collection*

The HFEA collates baseline information and birth outcomes from all treatment cycles carried out in the UK. Anonymised data were extracted from this register for the years 2003-2011 (later data were excluded since they have not yet been verified). Only women undergoing their first IVF or ICSI treatment cycle were assessed (n=195,374 women). Cycles that did not achieve an embryo transfer, and those involving gamete donation, surrogacy, frozen embryos or resulting in multiple pregnancies were excluded, leaving 126,498 women for analysis. Information on each cycle including maternal age, reason for infertility and cycle outcome, were extracted from the HFEA register. A clinical pregnancy was diagnosed following detection of a fetal heartbeat using ultrasound at 7-8 weeks gestation (n=36,562 cases). Subsequently, miscarriage was defined as a loss following a detection of a fetal heartbeat up to and including 24 weeks (n=3,949 cases) (HFEA, 2007).

5.3.2 *Statistical Analysis*

Generalised linear models (GLMs) with binomial error structures were built within *R* v.3.0.02 using the ‘stats’ package (RCoreTeam, 2012). Overdispersion was not detected. The aetiology of infertility was split into male or female, each defined as a fixed factor with two levels (yes/no). Treatment type was also specified as a fixed factor with two levels, *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). Maternal age and year of treatment were specified as continuous covariates. All potential interactions were assessed. The model was simplified by manual backwards stepwise deletion until minimum adequate models were obtained. The 'effects' (Fox, 2015, Fox and Hong, 2009) package was used to assess and plot higher-order effects within the model.

5.4 Results

The success of ART, as measured by the proportion of cycles achieving clinical pregnancies and live births, significantly increased over the study period (OR 1.0448 95% CI 1.0397-1.0499; OR 1.040 95% CI 1.0350-1.0455, respectively). The overall increase in the proportion of clinical pregnancies was larger than the increase in live births seen (Figure 1). In total, 630 clinical pregnancies were not classified as a miscarriage or livebirth, from these 31 were classified as ectopic, 158 stillbirths and 441 were lost to follow up. Over the same period miscarriage rates were significantly increased (OR 1.0455 95% CI 1.0321-1.0591) (Figure 2). The percentage of older mothers (≥ 38 years) - a known risk factor for miscarriage - remained stable throughout the study (Figure 3).

A significant interaction between male factor infertility and treatment type was identified. In cases of male factor infertility, miscarriage rates were higher following treatment with IVF (MD 0.034 95% CI 0.030, 0.038), but were lower following ICSI (MD 0.029 95% CI 0.028, 0.031)(Figure 4). Together, maternal age and female factor infertility also altered miscarriage risk. Maternal age was positively associated with miscarriage (18-34 years MD 0.029 95% CI 0.028, 0.031; 45-50 years MD 0.045 95% CI 0.040, 0.052), but not where infertility was classified as female factor (18-34 years MD 0.031 95% CI 0.029, 0.033; 45-50 years MD 0.031 95% CI 0.025, 0.038), (Figure 5).

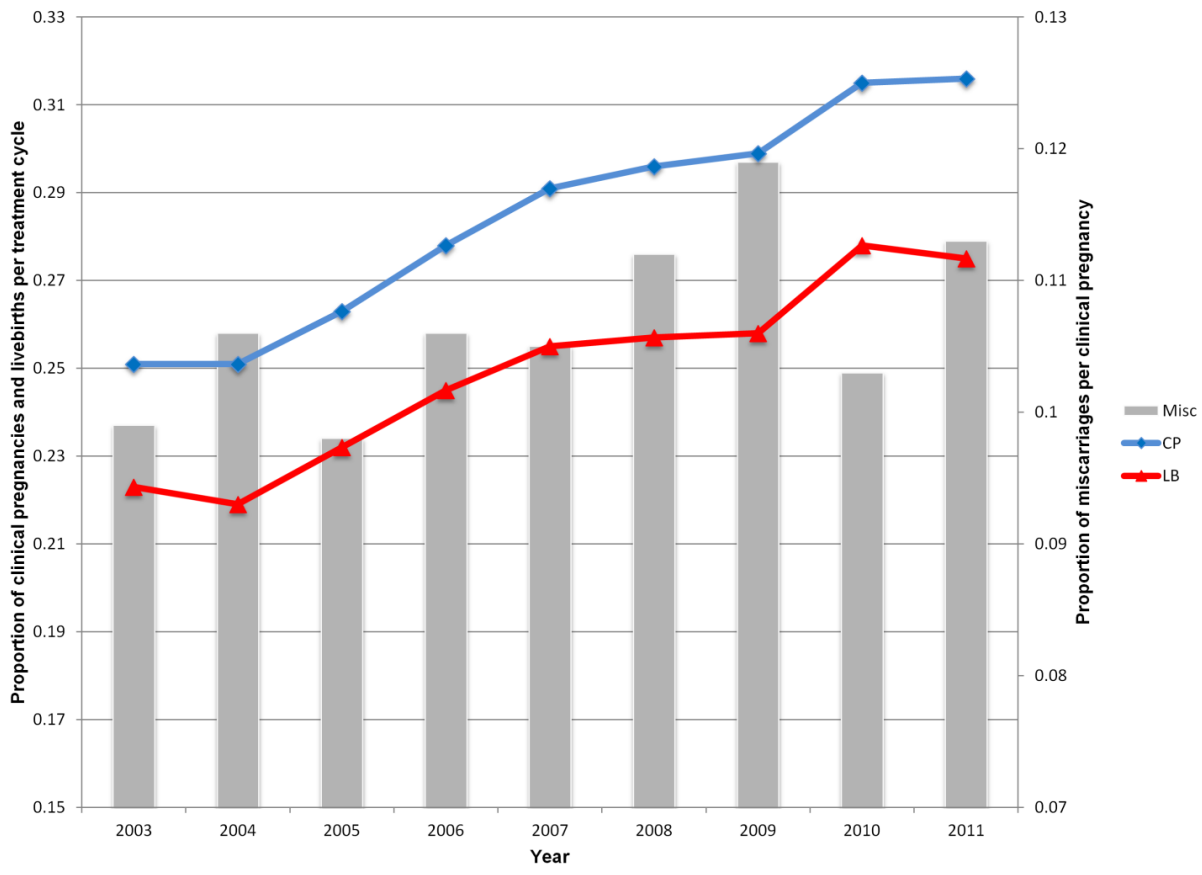


Figure 1. Change in the yearly proportion of clinical pregnancies (CP), livebirths (LB) and miscarriages (Misc) from 2003-2011, taken from data collated on women undergoing their first ART cycle in the UK.

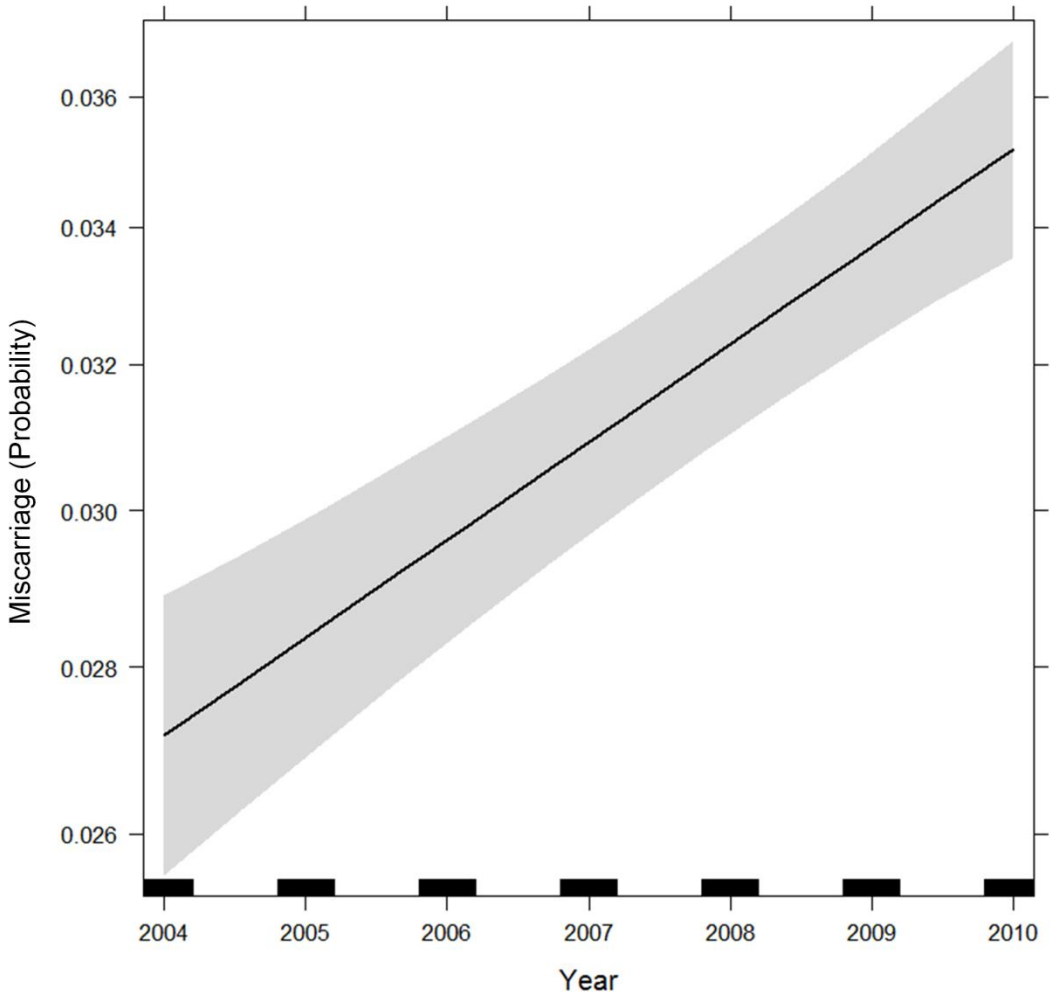


Figure 2. Miscarriage rates consistently increased over time in women completing their first ART cycle (OR 1.05 95% CI 1.03, 1.06).(Plot produced using 'effects' package (Fox, 2015) accounting for multiple interactions)(Confidence intervals = grey shading).

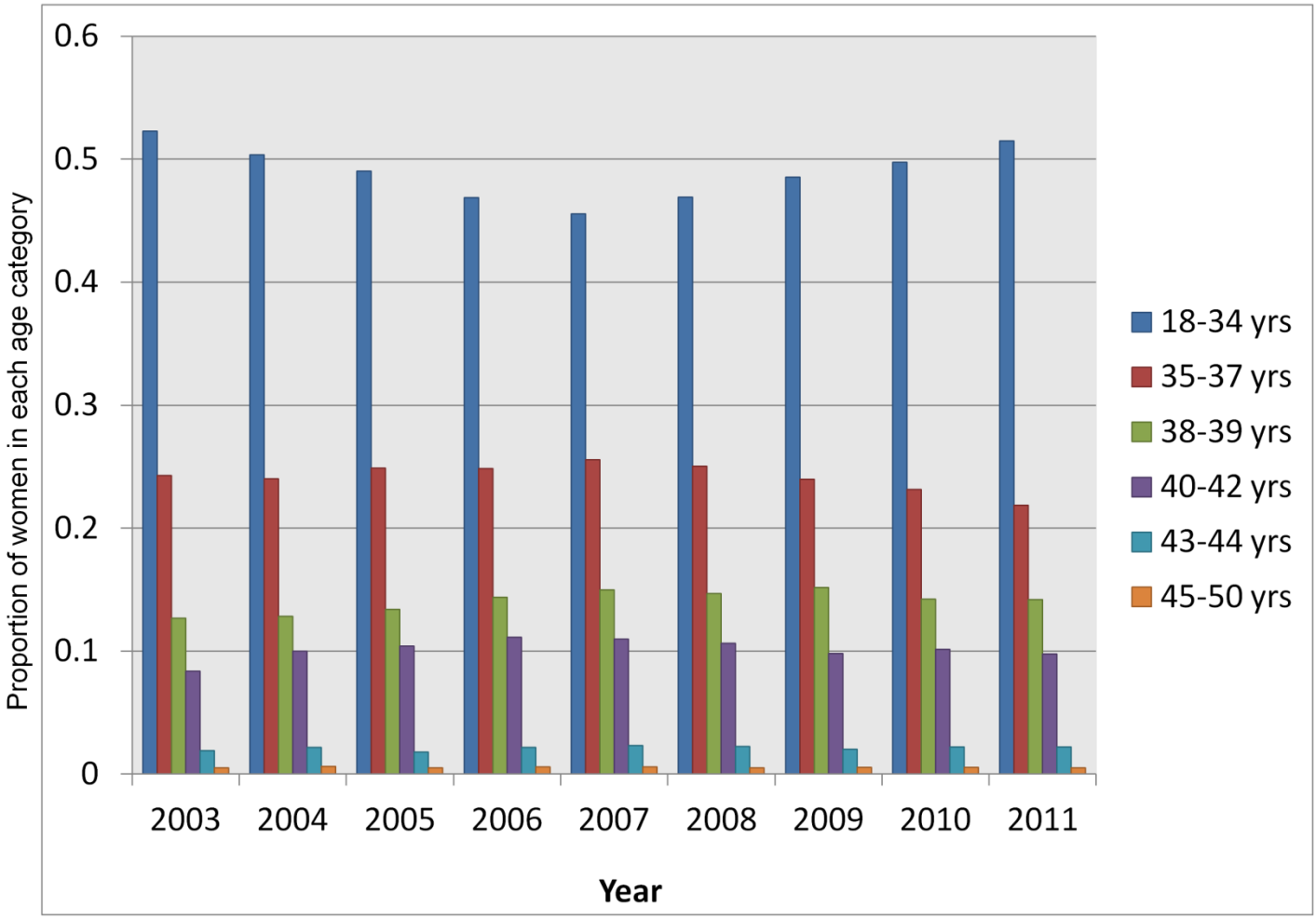


Figure 3. The age of women who sought their first ART cycle between 2003-2011 in the UK. The proportion of older mothers (>38yrs) remained largely unchanged through the study period.

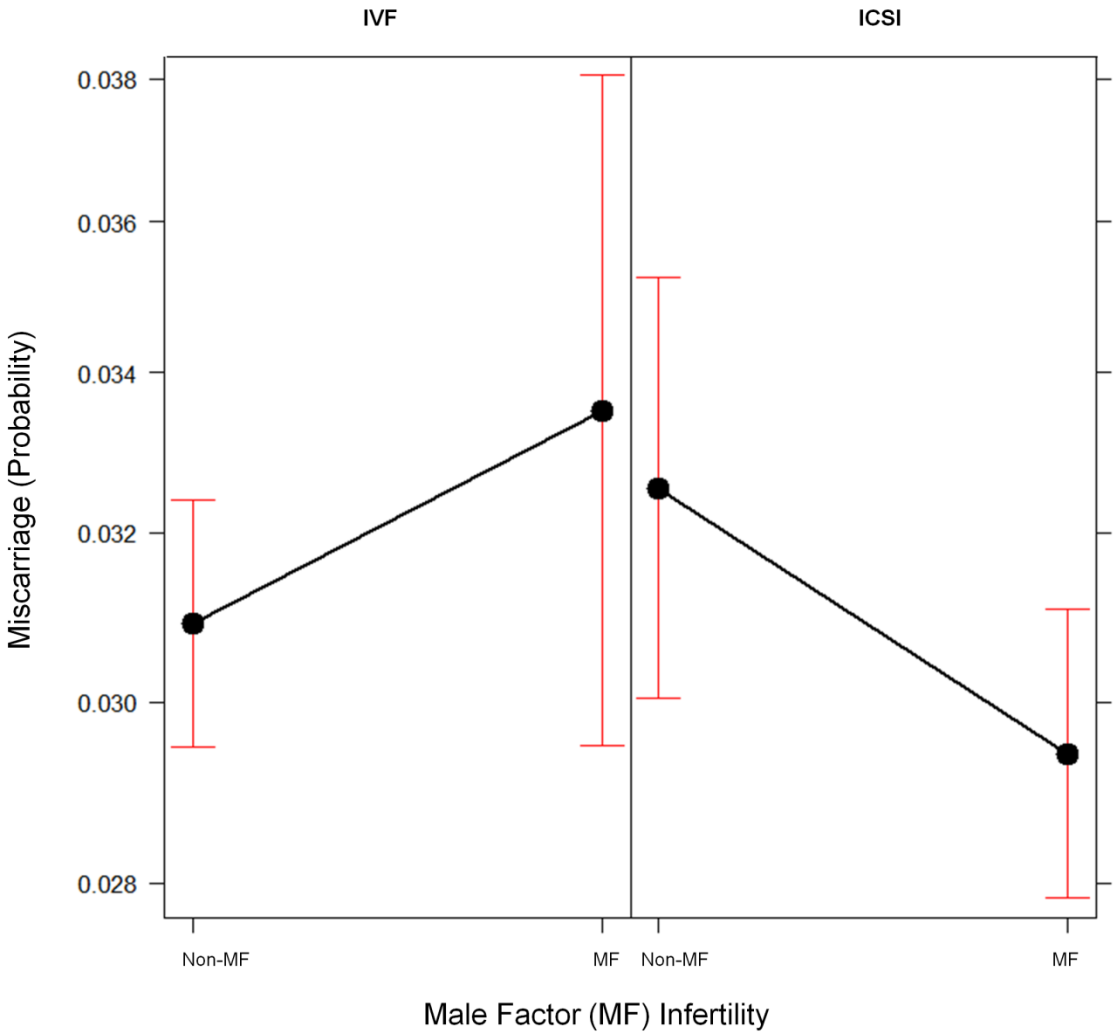


Figure 4. The effect display for the interaction between male factor infertility and treatment type and subsequent impact on the probability of miscarriage in women seeking their first ART cycle. In cases of male factor infertility miscarriage rates using IVF were higher (MD 0.034 95% CI 0.030, 0.038) than when treated using ICSI (MD 0.029 95% CI 0.028, 0.031)(p=0.03)(MF = Male factor infertility, Non-MF = Cause of infertility not categorised as male factor)(Confidence intervals demonstrated in red)

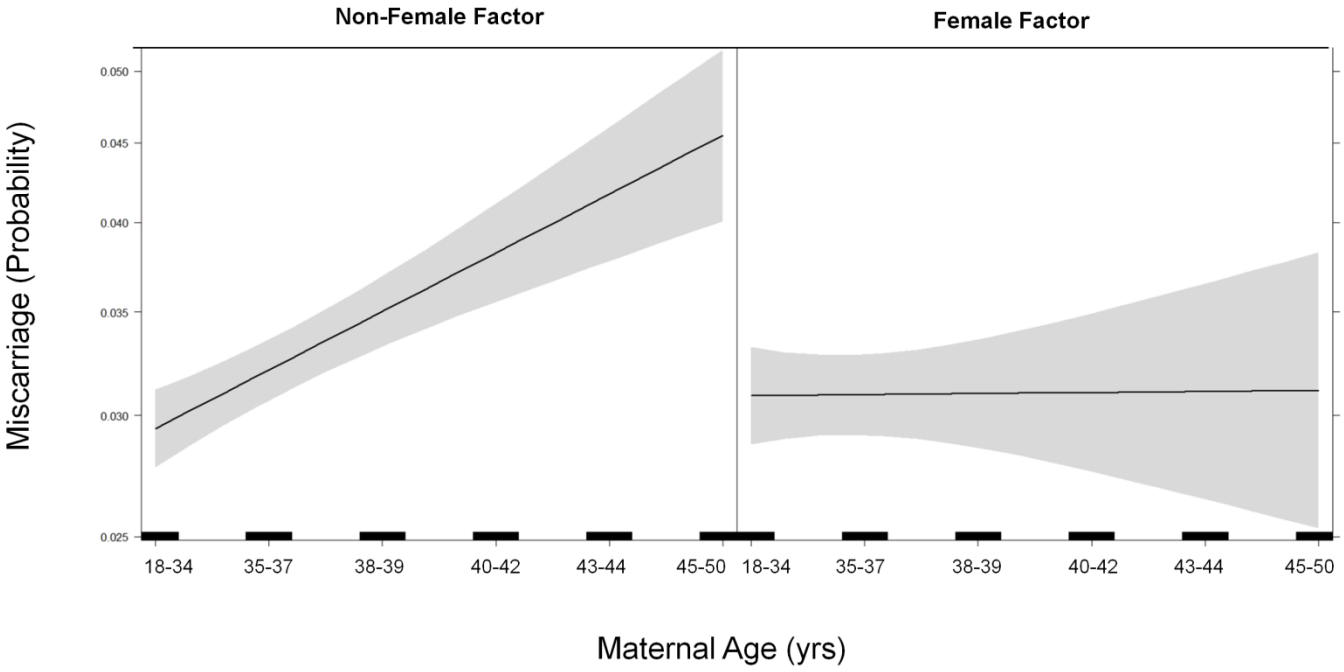


Figure 5. The effect display for the interaction between maternal age and treatment type and subsequent impact on the probability of miscarriage. Maternal age was positively associated with miscarriage (18-34 years MD 0.029 95% CI 0.028, 0.031; 45-50 years MD 0.045 95% CI 0.040, 0.052), but not where infertility was classified as female factor (18-34 years MD 0.031 95% CI 0.029, 0.033; 45-50 years MD 0.031 95% CI 0.025, 0.038)(p=0.003). (Non-female factor = Cause of infertility not categorised as female factor)(Confidence intervals = grey shading)

5.5 Discussion

The chance of ART treatment resulting in a successful outcome (live birth) is undoubtedly improving (HFEA, 2014), and this pattern is clearly shown by our data, which includes all first cycles conducted within the UK over a 9-year period. In addition, we have identified a small but steady increase in miscarriage rates over time, from a proportion of 0.099 in 2003, to 0.114 in 2011. To our knowledge, this is the first report that has identified that miscarriage rates are increasing among women undergoing ART. Comparable data are not available from the general population and therefore it is impossible to determine whether this risk reflects changes in all women, or just those receiving fertility treatment.

In this extensive cohort, the proportion of clinical pregnancies increased slightly more than the live birth rate over the study period, and there was a corresponding increase in miscarriage rates. It is likely that our analyses of miscarriage rates are conservative: we note that in total the number of recorded clinical pregnancies is 630 fewer than the sum of miscarriages and livebirths. From these, a total of 189 ectopic pregnancies and stillbirths were recorded and the remaining lost to follow up. We suggest that it is more likely that unsuccessful, rather than successful pregnancies, are lost to follow-up, due to the emotional demand of discussing a pregnancy loss with the clinic.

Previous studies have attempted to use the data collated on all ART treatment in the UK by the Human Fertilisation and Embryology Authority (HFEA), to assess factors influencing live births (Templeton et al., 1996, Nelson and Lawlor, 2011, Bhattacharya et al., 2013) but has not been utilised to assess miscarriage. In the US, self-reported miscarriage rates increased by 1% per annum from 1970-2000. This

trend was greatest in pregnancies <7 weeks gestation and was largely attributed to increased awareness of early pregnancy (Lang and Nuevo-Chiquero, 2012). Despite this, in our population, a significant increase of 4.6% per year in miscarriages between 7-24 weeks gestation was demonstrated, suggesting factors other than improved pregnancy detection are involved. Our project uses the most current data available on fertility treatments, offering an insight into the efficacy of modern fertility treatment. Our results may also contrast with the US data due to the different populations studied, and therefore, our data from couples seeking fertility treatment may reflect improved success of ART in terms of clinical pregnancies, but that these pregnancies are not viable and therefore do not result in a live birth.

It is unclear whether the increase in miscarriage rates reported in this study are specific to women undergoing ART or are applicable more widely. The careful monitoring of fertility patients from conception onwards means that the results cannot be attributed to improved diagnosis of early pregnancy, unlike the case in the general population (Lang and Nuevo-Chiquero, 2012). Environmental factors have been implicated in miscarriage. These include *Chlamydia trachomatis* (Chlamydia) (Baud et al., 2011, England, 2015), tobacco smoke (Pineles et al., 2014), and electromagnetic radiation (EMR) (Ouellet-Hellstrom and Stewart, 1993, Li et al., 2002). In addition, environmental pollutants, including pesticides such as the organochlorine dichlorodiphenyltrichloroethane (DDT), and contaminants from the electronics industry, such as polychlorinated biphenyls (PCBs) (Buck Louis et al., 2013, Venners et al., 2005) have been associated with pregnancy loss. Chlamydia is screened for in the fertility referral process (NICE, 2013) and our recent meta-analysis, found a decrease in sperm quality from mobile phone use (Adams et al., 2014), suggesting they are unlikely to be a primary explanation of our findings. In

addition, smoking in pregnant mothers is declining (HSCIC, 2014) and smoking in enclosed public spaces in the UK has been banned since 2007 (Government, 2006). The contaminants mentioned above are also banned, suggesting these factors do not explain the trends we have found. It is not possible to rule out all environmental factors, but at present there is insufficient evidence on which to base inferences about causal pathways.

Our analysis is based on a very large and well defined cohort of over 120,000 women. Investigating only fresh first cycles, including only autologous gametes, and singleton pregnancies removes repeated analyses of individuals, which has previously been an issue when analysing fertility data (Templeton et al., 1996, Nelson and Lawlor, 2011). In addition, the study utilises national data from a 9-year period. However, we were unable to obtain information on duration of infertility and centre data due to confidentiality. Further information on the couples, including paternal age, BMI or smoking status, were also unavailable. These factors have previously been linked with miscarriages (Rittenberg et al., 2011, de la Rochebrochard and Thonneau, 2002, Chatenoud et al., 1998). Further improvement in information gathered from patients having ART by the HFEA would be advantageous in identifying key factors in infertility. This will become increasingly important if current trends of decreasing male fertility continue (Swan et al., 2000, Rolland et al., 2013).

Our finding of an elevated risk of miscarriage in older mothers is supported by a number of previous studies (Maconochie et al., 2007, Feodor Nilsson et al., 2014, Templeton et al., 1996, Bhattacharya et al., 2013, Nybo Andersen et al., 2000, de la Rochebrochard and Thonneau, 2002). Maternal factors including BMI, stress and alcohol consumption have also previously been linked with a higher risk of

pregnancy loss (Maconochie et al., 2007, Feodor Nilsson et al., 2014, Veleva et al., 2008, Arck et al., 2008). However, our findings of an interaction between female factor infertility and maternal age on miscarriage rates (Figure 5), to our knowledge, have not been previously described. In cases of female factor infertility, miscarriage rates remained steady across all age groups (Figure 5). However, the overall increase in the risk of miscarriage in the cohort cannot be attributed to an increasing proportion of older women seeking treatment, as this remained constant over time (Figure 2).

We also show, for the first time using a large-scale cohort that the use of ICSI rather than IVF as treatment for male factor infertility is linked with a lower risk of pregnancy loss. This supports previous findings, that treating male factor infertility with IVF is unlikely to increase the chance of a successful outcome (Nelson and Lawlor, 2011). The introduction of ICSI in the treatment of severe male factor infertility has significantly improved outcomes, in addition to helping in cases of low or no oocyte fertilisation (Orvieto et al., 2000). Previous concerns about the efficacy and safety of ICSI, as there is no natural selection of sperm, have been well-documented, but epidemiological evidence suggests few negative effects on subsequent children to date (Sutcliffe et al., 2001, Belva et al., 2007, Katalinic et al., 2004, Retzloff and Hornstein, 2003).

5.6 Conclusion

We have demonstrated that using ICSI to treat male factor infertility decreases the risk of miscarriage. There is growing demand for ART. The likelihood of a successful outcome following ART is increasing, but there is also a significant increase in miscarriage rates over time. Hence, the live birth rate is slightly lower than would be

anticipated from the clinical pregnancy rate. The reason for this increase is currently unknown and requires further investigation.

6. DISCUSSION

Male factor infertility is a global health issue, affecting at least 30 million men around the world (Agarwal et al., 2015), with around 1 in 7 couples experiencing difficulty with conception (Wilkes et al., 2009). There is much evidence to suggest that sperm quality is declining, and testicular disorders are increasing (Rolland et al., 2013, Swan et al., 2000, Skakkebaek et al., 2016). In addition, more people than ever before are turning to fertility treatment (HFEA, 2014, HFEA, 2008). In part, this has been linked to changes in lifestyle and the modern day environment (Homan et al., 2007, Sharpe and Franks, 2002). However, there have been conflicting reports on the impact of environmental factors on male reproductive health. Overall, male fertility has been under-researched when compared with maternal factors. In this thesis, using an integrated approach that combined epidemiological research with laboratory investigations, I have analysed environmental impacts on male reproductive health, from assessing seasonal changes on sperm quality to the impact of radiofrequency electromagnetic radiation (RF-EMR). In addition, I assessed temporal trends in miscarriage rates following fertility treatment, and investigated the links between miscarriage risk and the type of treatment received. The following table outlines the thesis chapters and key findings (Table 1):

Table 1. Thesis chapters: key findings and context

Chapter (Status)	Key findings	Previous information	What has been added by this thesis?
2. Seasonality and sperm quality (Submitted to PLOS one)	Increased motility and morphology in summer. Relationship can be altered by oligospermia.	*Animal studies*: spermatogenesis varied in response to circannual changes in the length of daylight. *Human studies*: Unclear: some found no seasonality, in others sperm motility and morphology highest in spring and winter.	First report of these seasonal patterns. Findings correspond with trends in birth rates and ART success.
3. Mobile phones and sperm quality: Meta-analysis (Published in Environment International)	Exposure to mobile phones reduced sperm motility and viability consistently across *in vitro* and in vivo studies.	Effect was unclear, partly because of small sample sizes within each study.	First meta-analysis on this topic. Showed that mobile phone exposure was negatively associated with sperm quality
4. *In vitro* and *in vivo* randomised controlled studies of mobile phone exposure on sperm quality	Sperm motility and morphology were lower, and DNA damage increased by RF-EMR from electronic devices *in vitro*. The effect can be cumulative.	Our meta-analysis had demonstrated a negative effect from mobile phones. Previously, only one paper had investigated laptop RF-EMR exposure on sperm quality.	First report to analyse the cumulative impact of mobile phones and laptops RF-EMR on sperm quality. First to assess mobile phone exposure in a randomised controlled trial.
5. Changes in miscarriage rates over time	The proportion of miscarriages following ART has increased over time. The risk of pregnancy loss is lower following ICSI compared with IVF.	In the US, miscarriage increased by ~1% per year, but this was attributed to earlier pregnancy diagnosis. Risk factors for miscarriage had been identified – including maternal age, BMI, stress, alcohol, paternal age, sperm DNA damage.	First paper to identify that miscarriage rates following ART are increasing. First to link treatment of male infertility with ICSI as reducing the risk of miscarriage.

6.1 ART and Society

Since the birth of the first ‘test tube’ baby in 1978, approximately 3.4 million children have been born via ART (Chambers et al., 2009). In the UK, the number of people accessing fertility treatment annually increased by 40% from 2006-2013 (HFEA, 2014, Kurinczuk and Hockley, 2010). Couples are now more likely to have a successful outcome following ART. The research in this thesis indicates however, that the risk of miscarriage following ART has also increased over time. However, whilst it is now more likely a couple will achieve a clinical pregnancy, the increase in the miscarriage rate has reduced the overall increase in livebirths. The cause of this increase is currently unclear. It deserves further research due to the negative and often severe psychosocial consequences for childless couples (Dyer et al., 2009, Wischmann et al., 2009).

Despite the higher miscarriage rates we identified following ART, it has offered many couples an opportunity to have a child whereby no other means were possible. However, access to ART varies considerably and there is sometimes controversy over the ‘right’ to seek fertility treatment. There are a number of considerations for ART, including the significant psychosocial impacts, whether infertility should be considered a disease and equitable access (Ombelet et al., 2008). Nonetheless, in the UK, NICE guidelines set out the criteria for NHS access to ART as follows:

- ‘In women aged under 40 years who have not conceived after 2 years of regular unprotected intercourse or 12 cycles of artificial insemination (where 6 or more are by intrauterine insemination), offer 3 full cycles of IVF, with or without ICSI.’

- ‘In women aged 40–42 years who have not conceived after 2 years of regular unprotected intercourse or 12 cycles of artificial insemination (where 6 or more are by intrauterine insemination), offer 1 full cycle of IVF, with or without ICSI, provided the following 3 criteria are fulfilled:
    - They have never previously had IVF treatment
    - There is no evidence of low ovarian reserve
    - There has been a discussion of the additional implications of IVF and pregnancy at this age.’ (NICE, 2013)

However, in reality there is a ‘postcode lottery’ regarding access to fertility treatment (Figure 1).

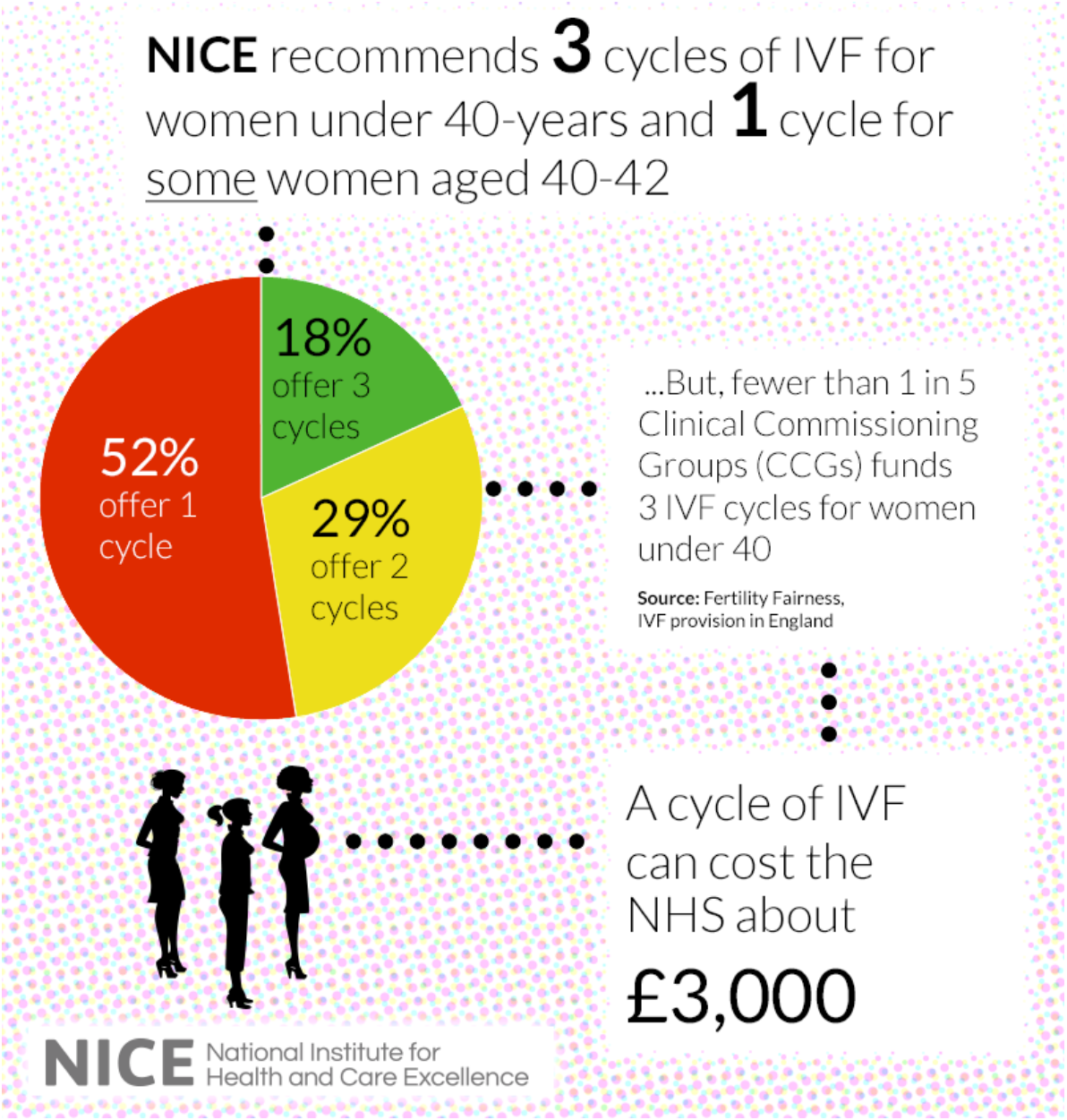


Figure 1. Variation in number of cycles funded by the NHS across CCGs. (Adapted from (NICE, 2014)).

Alongside the social aspect of treatment funding, ART has raised a number of ethical and legal questions. With the emergence of new technological advances, this will continue into the future (Brezina and Zhao, 2012). There are substantial differences between countries in the approach taken to regulation. For example, the USA has no central regulatory authority (Chang and DeCherney, 2003). Whilst guidelines are offered, clinicians are able to individualise treatment according to the patients 'circumstances' (ASRM, 2008). This has important consequences. For example, the risk multiple births is elevated following fertility treatment (Sunderam et al., 2014, Parazzini et al., 2016, Braude, 2006), and are at greater risk of complication and greater socioeconomic costs (Lawlor and Nelson, 2012). In the UK, the HFEA state that each centre cannot exceed a 10% multiple birth rate (Fasht, 2012), with no more than 2 embryos to be transferred per cycle, in women under 40 (HFEA, 2009); whereas this may not be the case in the USA. Such differences alter the inherent risks associated with treatment and can therefore complicate comparisons of studies conducted in different countries.

The HFEA collates information on each treatment cycle carried out in the UK. From these data, I have assessed trends in miscarriage rates over time. As may be expected, there is female bias for data collated for each treatment cycle and the potential effect of male-factor infertility on miscarriage has not been investigated thoroughly. Our finding that male factor infertility was associated with increased miscarriage rates when treated with IVF, rather than ICSI, provides information that may guide clinical and patient decisions on their treatment route. However, the data were limited to classification of male factor infertility as either Yes or No, thereby limiting the scope of possible analysis. I would therefore advocate the collection of detailed information on sperm quality parameters by the HFEA. Such detail would

allow improved assessment of how male infertility, and subsequent treatment, impacts on miscarriage rate.

6.2 Wider health implications of electronic devices and RF-EMR

The intensity of RF-EMR emitted from an electronic device such as mobile phones, is well below the safety limits set out by the International Commission on Non-Ionising Radiation Protection (ICNIRP, 1998). Nonetheless, this thesis has added further evidence that there is a negative impact on male fertility. In addition, we identified that exposure to mobile phone RF-EMR was able to cause DNA damage in human spermatozoa. The mechanism for this effect is likely to be mediated by oxidative stress. Mobile phone RF-EMR is able to increase the production of reactive oxygen species (ROS) by spermatozoa (De Iuliis et al., 2009). In high concentrations ROS, highly oxidative radicals, and/or a depleted antioxidant capacity, can cause oxidative stress, resulting in DNA damage (Song et al., 2006). One review found that 93 of 100 articles reported significant oxidative stress following RF-EMR exposure, across animal and human models (Yakymenko et al., 2015). However, there has been no meta-analysis of these data and therefore interpretation of the magnitude of effect of RF-EMR on oxidative stress is not possible.

Wider implications of increased ROS levels, and subsequently oxidative stress, from RF-EMR emitted from electronic devices, can include oncogenic transformations (Valko et al., 2006, Ralph et al., 2010). In individuals who use mobile phones 'heavily', epidemiological studies have indicated an increased risk of cancer (Hardell et al., 2007, Hardell and Carlberg, 2015, Sadetzki et al., 2008, Sato et al., 2011). These included an increased risk of melanomas (Hardell et al., 2011), brain tumours (Cardis et al., 2010) (Hardell et al., 2007) and parotid cell tumours (Sadetzki et al.,

2008). However, these findings are not universally supported (Repacholi et al., 2012). Consideration needs to be given to the latency period for any potential carcinogenic impact, as some studies have been limited to <10 years latent exposure whereby an effect may not have yet developed (Hardell et al., 2007).

Assessing the effects of EMR on human health outcomes is difficult due to confounding variables, exposure classification errors and difficulty in identifying a true negative control (Calvente et al., 2010). In addition, the oscillating nature of EMR emitted from electronic devices, makes quantitative measures difficult. For example, there has previously been a high-profile debate about the potential link between EMR emitted from power lines and childhood leukaemia (Milham and Ossiander, 2001). Children living within 4-6 km of radio and TV transmitters were reported by two independent studies to have an elevated risk of childhood leukaemia (Michelozzi et al., 2002, Hocking and Gordon, 2003). However, other studies failed to find an association (Merzenich et al., 2008). This highlights the difficulty of demonstrating a causal link in observational studies, particularly when there are a number of confounding variables.

Following exposure to EMR, some individuals report Electrohypersensitvity (EHS). In people living <300 m from mobile phone base stations, symptoms have included headaches, sleep disturbance, depression, nausea and decreased appetite (Santini et al., 2002). Sweden officially recognises EHS as an ‘impairment’ generated by exposure to EMR (Johansson, 2006). In some individuals, a chronic stress state has been described, with increased adrenaline and noradrenaline and a decrease in dopamine (Buchner and Eger, 2011). It has been estimated that this disorder may affect up to 11% of the total population in Europe, increasing from 0.06% in 1985 (Hallberg and Oberfeld, 2006). However, there is a difficulty in differentiating

between the greater exposure to EMR over this time period and an increased awareness of EHS as a condition: the report did not fully explain the methodology leading to their conclusion so the prevalence reported is likely to have been over-estimated. Despite considerable scepticism from many about the existence of EHS (Rubin et al., 2005, Rubin et al., 2010, Seitz et al., 2005, Roosli, 2008), an allergic-type response has been proposed as a mechanism (Yakymenko et al., 2015). In skin samples taken from individuals suffering from EHS, an increase in mast cells following exposure to EMR devices has been demonstrated (Johansson et al., 2001). However, with just 13 participants it is difficult to extrapolate these results to the general population. If a larger scale study could replicate these initial findings, it has been proposed that as mast cells are able to generate ROS (Nagata, 2005), this may lead to EHS-like symptoms (Yakymenko et al., 2015).

This thesis demonstrated a negative link between RF-EMR exposure from electronic devices and sperm quality in both experimental research and meta-analysis. It also showed that sperm DNA damage was induced by mobile phone exposure. Research elsewhere has implicated EMR exposure in cancer and EHS. Exposure to wi-fi and other sources of EMR are increasing and children are at the greatest risk. Modern lifestyles mean children are exposed from their homes, schools and general daily environment, this is the first generation to be exposed from childhood. It is possible that the latency period of any resulting impact on health means we have not yet seen the true implications of exposure to EMR.

6.3 Interaction between genes and the environment

There is clear evidence that a variety of environmental factors can affect male fertility. This thesis shows that sperm quality is decreased in smokers and DNA

damage increased following exposure to mobile phones (Agarwal et al., 2006, Agarwal et al., 2009, Aitken et al., 2014). Often evidence on specific environmental influences is inconclusive, for example, evidence on the link between smoking and male fertility is inconsistent (Vine et al., 1994, Joo et al., 2012, Kunzle et al., 2003, Ramlau-Hansen et al., 2007a, De Bantel et al., 2015, de Jong et al., 2014, Jensen et al., 2004, Martini et al., 2004, Sharma et al., 2016). This may reflect the interplay of the environment and genetic susceptibility (Yu and Huang, 2015, Yarosh et al., 2015). Given that polymorphisms in transcription factors associated with antioxidant genes may reduce protection against oxidative stress (Yu et al., 2013), work is needed to assess whether genetic polymorphisms can moderate the impact of phones and other oxidative agents such as smoking, on sperm quality.

Polymorphisms in genes that code for key antioxidant defence enzymes have been associated with variation in male fertility (Yarosh et al., 2015, Chengyong et al., 2012). Meta-analyses have identified that the *GSTM1/GSTT1* null genotypes are associated with an increased risk of male infertility (Kan et al., 2013, Li et al., 2013, Song et al., 2013, Chengyong et al., 2012, Safarinejad et al., 2012). Environmental toxins and carcinogens that act to form ROS are also metabolised by the cytochrome P450 (CYP) family to reduce oxidative stress (Yu and Huang, 2015). In Russian men, certain polymorphism combinations in GST genes have been shown to be associated with infertility in smokers (Yarosh et al., 2015). Polymorphisms in CYP genes have been associated with lower sperm concentration, motility and morphology (Zalata et al., 2015). It has been demonstrated that specific variants of the CYP and GST genes in combination, gives a 6.9 fold increase in risk for infertility in those individuals (*GSTM*-null & *CYP1A1* Val/Val or *CYP1A1* Ile/Val compared with *GSTM*-null & *CYP1A1* Ile/Ile) (Aydos et al., 2009). Taken together, this research

highlights how an individual's genetic profile can affect their susceptibility to negative influences on male fertility.

6.4 Future research and clinical implications

It is thought that around 80% of the variability in sperm quality is due to environmental factors (Storgaard et al., 2006). However, compared with female fertility, relatively little research has been conducted to identify them. There is substantial intra-individual variation and this means that large sample sizes, or very careful longitudinal studies, are required in order to overcome the inherent errors in estimating fertility parameters from a single sample per individual. In addition, following the introduction of ICSI, only one sperm is required per mature oocyte collected. Therefore, even in cases of severe male factor infertility, there is a treatment of proven success. This means that finding the causes of male fertility is not always a clinical priority, as ultimately it does not change the treatment pathway.

The lack of research on male reproductive health is also hindered by an apparent reluctance of men to participate. This posed a considerable barrier to the research undertaken in this thesis (particularly the randomised controlled trial of *in vivo* mobile phone exposure). It has also been reported elsewhere that of 1,409 men approached to participate, just 15.8% men agreed to be involved (Eustache et al., 2004). The nature of providing a sample by masturbation under 'standardised conditions' is a potentially embarrassing procedure for many men, which may be reflected in the low participation to our recruitment process (Handelsman, 1997). Differences have also been identified in individuals who are willing to volunteer. In fertility clinic populations, or men who have previously experience a negative pregnancy outcome, curiosity or concern regarding their fertility, means they are more likely to participate than the

general population (Handelsman, 1997, Muller et al., 2004). In men who were interested in becoming sperm donors, their psychological and attitudinal profiling was distinctive from the general male population (Handelsman et al., 1985), whilst men from lower educational backgrounds or those who smoked were less likely to volunteer. Outside of fertility research, males were more likely to participate for financial gain (Gerstein et al., 2004). However, there are questions over the ethics of using incentives and local rules regarding recruitment meant we were unable to use financial incentives to recruit participants (Grant and Sugarman, 2004).

Despite the difficulties in assessing male fertility, the evidence suggesting sperm quality is declining highlights the importance of the topics included in this thesis. Taken on their own, the declines in sperm associated with the environmental exposures studied in this thesis are unlikely to have a significant impact on an individual's fertility. However, I found three distinct environmental factors that may be able to alter sperm quality: seasons, RF-EMR and smoking. I also identified a trend for a cumulative impact of RF-EMR from multiple devices. It is possible, that the additive effect of a number of negative environmental factors, will lead to the decline in male fertility that has been described. In particular, this puts men who have borderline fertility at greater risk of infertility. Currently, an understanding of how the decrease in male fertility from certain environmental factors, relates to chance of conception, subsequent pregnancy outcome and future offspring health, is not clear. This gap in knowledge makes it difficult to ascertain how the modern day environment will affect fertility in the long-term. This kind of information would be of use, particularly in view of the evidence from this thesis, that miscarriage rates are increasing following ART, due to as yet unknown causes.

Our understanding of infertility needs improvement: 25**%** of people in western societies are still classified as having unexplained infertility (NICE, 2013). There are also cases of normospermic men who are unable to conceive (Pasqualotto et al., 2001). Research has suggested that men have a susceptibility to environmental exposures due to the structure of spermatozoon membranes, due to polymorphisms in their genetic profile that can lead to poorer sperm quality and a greater risk of infertility. This opens up avenues for further research and potential treatments. At the very least, an understanding of whether a patient is susceptible to oxidative stress due to a GST null genotype, for example, may mean patients can be offered ways of mitigating that risk.

I have identified a number of priority research areas that should be addressed in the near future:

- Investigation into the mechanism of seasonal alterations to sperm quality. Assessment of whether the effects could be due to light exposure; if so what are the impacts of altered working patterns and nocturnal use of electronic devices such as TVs on male fertility.
- Long term prospective analysis on the effect of exposure to RF-EMR on sperm quality, time to pregnancy, pregnancy outcome and future fertility of the child.
- Large scale randomised controlled study on RF-EMR exposure from mobile phones and laptop wi-fi on sperm quality.
- Miscarriage rates over time in the general population. Prospective data collection from early pregnancy assessment centres and GP surgeries could be utilised to monitor pregnancy loss rates.

- The impact of RF-EMR from electronic devices, in tandem with assessment of the genetic profile of the participants.
    - In particular, GST and CYP genes, to assess whether the individuals ability to metabolise ROS produced from exposure to RF-EMR, affects the risk to sperm quality.

6.5 Conclusion

Infertility affects up to 45 million couples worldwide. This research has addressed a number of key environmental impacts on sperm quality, in addition to addressing whether there have been changes in temporal trends in miscarriage within a fertility clinic population. I have identified that male fertility can be affected by both the natural environment, in seasonal changes, and from man-made sources, including RF-EMR. Modern day environmental exposures, such as mobile phones, are now ubiquitous across the population. However, previously research was unclear as to the impact on sperm quality. I have consistently identified, using a meta-analysis, *in vitro* and *in vivo* studies, that exposure to RF-EMR from mobile phones and laptop wi-fi, is able to negatively affect sperm quality. In couples seeking ART, I have also identified that miscarriage, the most common negative pregnancy outcome, has increased over time, for reasons that are not clear. Future research on the relationship between environmental exposures, male fertility and subsequent reproductive outcomes are required. In addition, understanding the role of genetic susceptibility in male fertility, may offer further insight into some of the inconclusive research on the effect of environmental exposures.

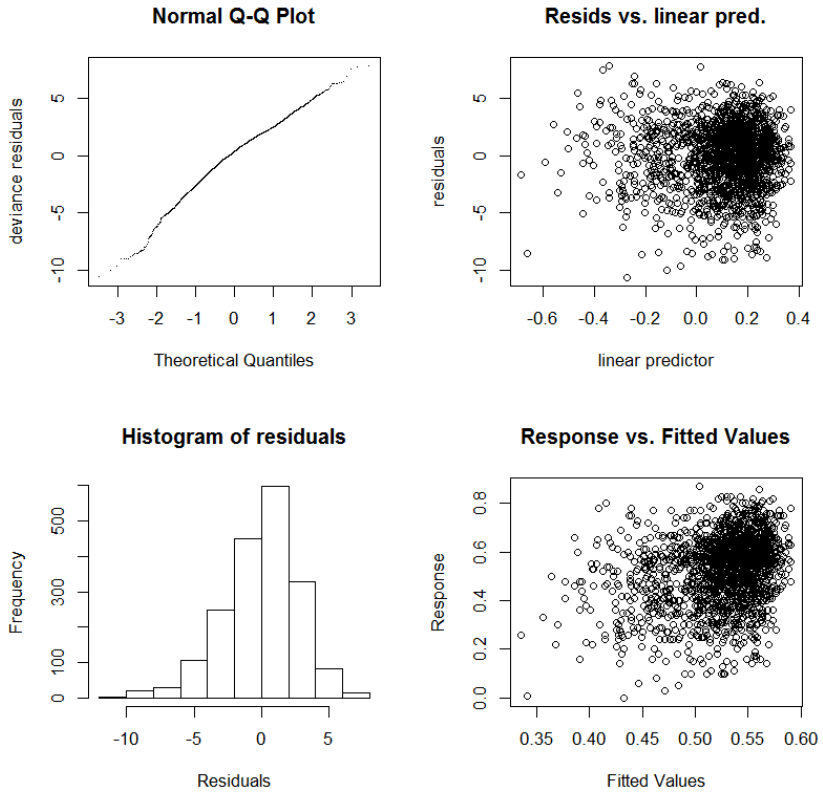
Supplementary Information

a. Information and plots for GAMs in seasonal data analysis.

Model 1. The effect of season on human sperm motility

Model1<-gam(y ~ s(month, bs="cc") + year + Age + Oligospermic + month:year + Oligospermic:Age,

+ data = dframe1, family = quasibinomial())



gam.check(Model1)

Method: GCV Optimizer: outer newton

full convergence after 3 iterations.

Gradient range [2.017891e-08,2.017891e-08]

(score 7.600286 & scale 7.536993).

Hessian positive definite, eigenvalue range [0.003192288,0.003192288].

Basis dimension (k) checking results. Low p-value (k-index<1) may

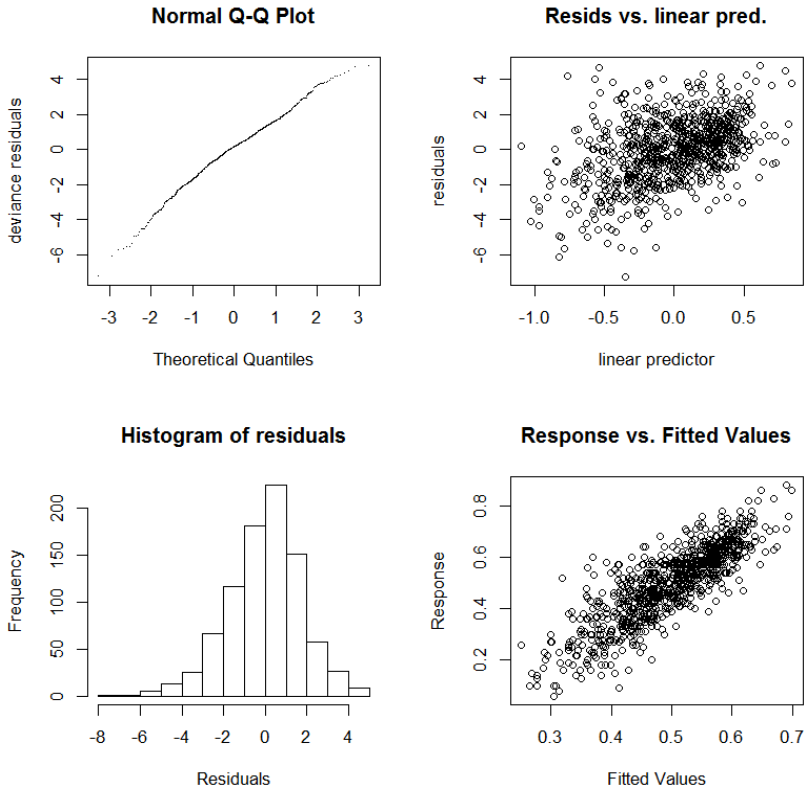
indicate that k is too low, especially if edf is close to k'.

	k'	edf	k-index	p-value
s(month)	8.00	2.59	1.04	0.98

Model 2. The effect of seasonal changes on human sperm motility when assessing repeat samples from the same individual.

Model2<-gam(y ~ s(Month, bs="cc") + s(PatientID, bs="re") + Oligospermic,

+ data = dframe1, family = (quasibinomial))



gam.check(Model2)

Method: GCV Optimizer: outer newton

full convergence after 5 iterations.

Gradient range [-8.009458e-10,1.478548e-09]

(score 6.312683 & scale 4.467027).

Hessian positive definite, eigenvalue range [0.01091296,0.7880667].

Basis dimension (k) checking results. Low p-value (k-index<1) may

indicate that k is too low, especially if edf is close to k'.

k' edf k-index p-value

s(Month) 8.000 2.151 0.998 0.5

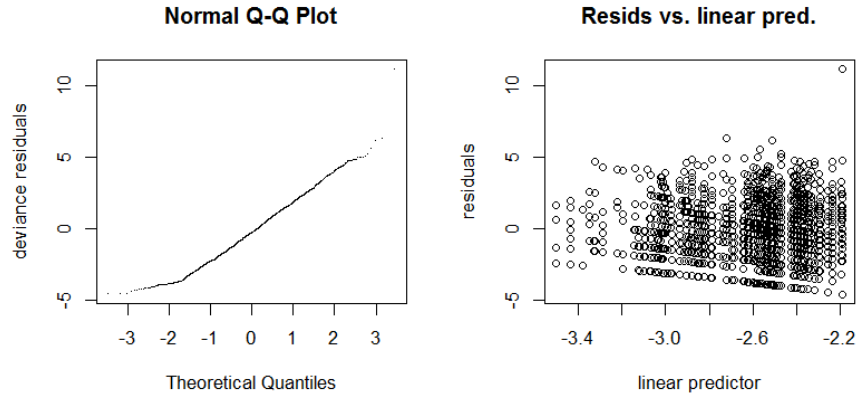
s(PatientID) 388.000 252.552 NA NA

Model 3. The effect of seasonal changes on human sperm morphology

Model3<-gam(y ~ s(month, bs="cc") + year + Oligospermic + month:year,

data = dframe1, family = quasibinomial())

>



gam.check(Model3)

Method: GCV Optimizer: outer newton

full convergence after 3 iterations.

Gradient range [1.638596e-10,1.638596e-10]

(score 4.220946 & scale 4.189591).

Hessian positive definite, eigenvalue range [0.0006624293,0.0006624293].

Basis dimension (k) checking results. Low p-value (k-index<1) may

indicate that k is too low, especially if edf is close to k'.

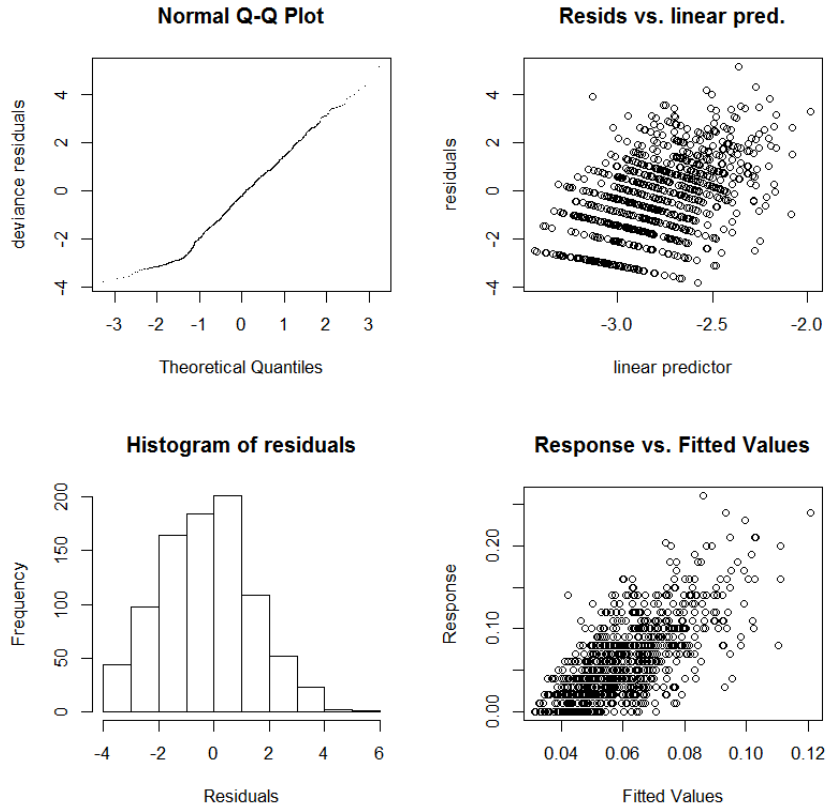
k' edf k-index p-value

s(month) 8.000 2.906 0.919 0

Model 4. The effect of seasonal changes on normalsperm morphology when assessing repeat samples from the same individual.

ModelGAM1<-gam(y ~ s(Month, bs="cc") + s(PatientID, bs="re") + Year + Oligospermic,

+ data = dframe1, family = (quasibinomial))



gam.check(Model4)

Method: GCV Optimizer: outer newton

full convergence after 5 iterations.

Gradient range [-2.473536e-10,-2.350868e-11]

(score 3.829243 & scale 3.278465).

Hessian positive definite, eigenvalue range [0.0008193563,0.1770089].

Basis dimension (k) checking results. Low p-value (k-index<1) may

indicate that k is too low, especially if edf is close to k'.

k' edf k-index p-value

s(Month) 8.00 0.64 0.98 0.62

s(PatientID) 388.00 119.65 NA NA

b. Meta-analysis supplementary information

Figure 1. Sensitivity analysis for effect of mobile phone exposure on sperm motility.

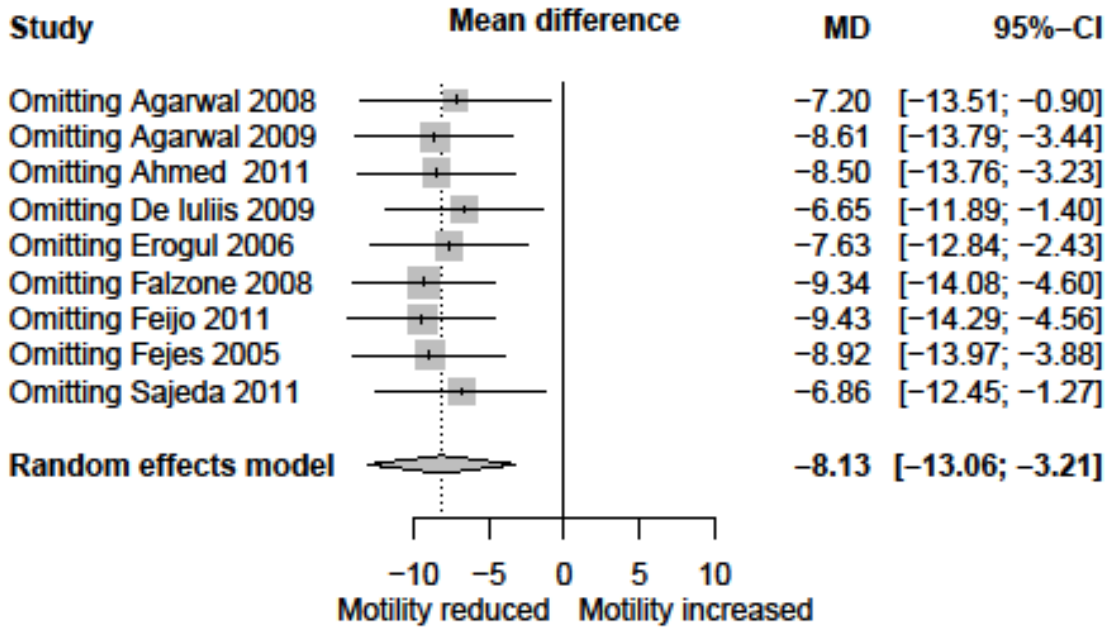


Figure 2. Sensitivity analysis for effect of mobile phone exposure on sperm viability.

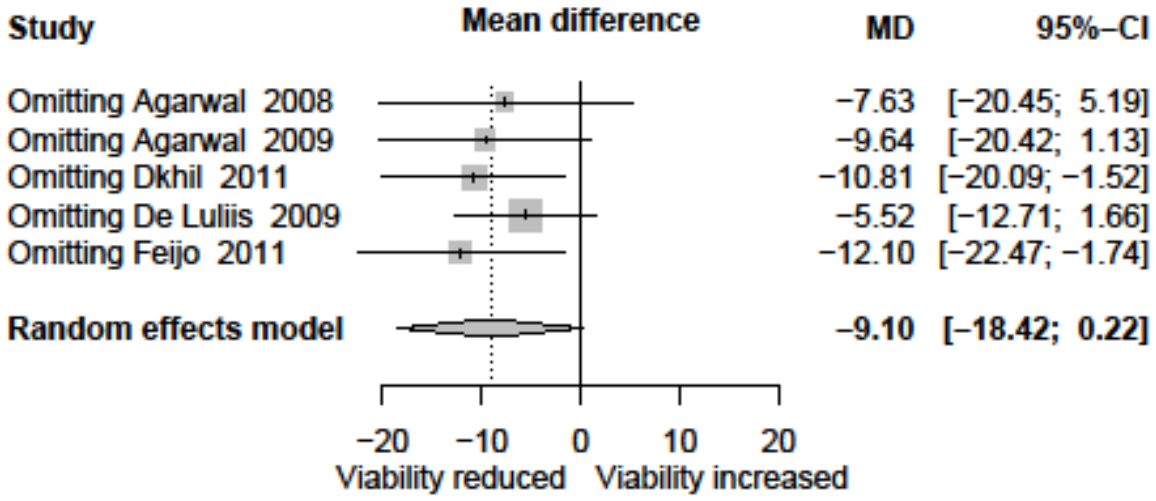
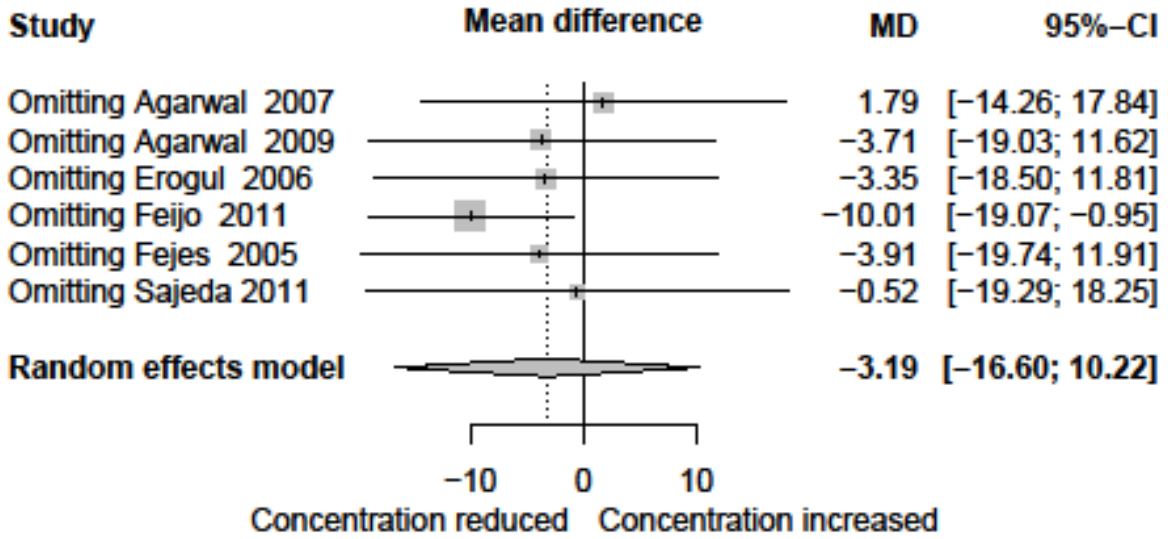


Figure 3. Sensitivity analysis for effect of mobile phone exposure on sperm concentration.



Publication Bias

Publication bias is an issue in any meta-analysis as significant studies are more likely to be published (Ruzni and Idris, 2012). Funnel plots are helpful in detecting publication bias (Supp. Fig. 4). However, high heterogeneity and low study numbers in meta-analyses often preclude meaningful assessment of publication bias, as there is potential for adjusted estimates of effect sizes to be biased (Terrin et al., 2003, Peters et al., 2007, Ruzni and Idris, 2012). We therefore only assessed publication bias for motility, as this was the outcome where most data was available. We used the trim-and-fill method, as is recommended for continuous data sets (Ruzni and Idris, 2012)(Supp. Fig. 5). The estimated effect size was increased by the adjustment (Table 1).

Table I. Meta-analysis results for the effect of mobile phone exposure on sperm motility before and after adjustment for potential publication bias (Trim and Fill)

		Motility Meta-analysis	**After Trim and fill analysis**
FEM	MD (%)	-12.18	-14.97
	95% CI	(-13.61, -10.74)	(-16.28, -13.67)
REM	MD (%)	-8.13	-14.39
	95% CI	(-13.06, -3.21)	(-19.64, -9.15)
Heterogeneity (I^2 %)		89.5	92.7

Figure 4. Funnel plot for the meta-analysis of mobile phone exposure on sperm motility.

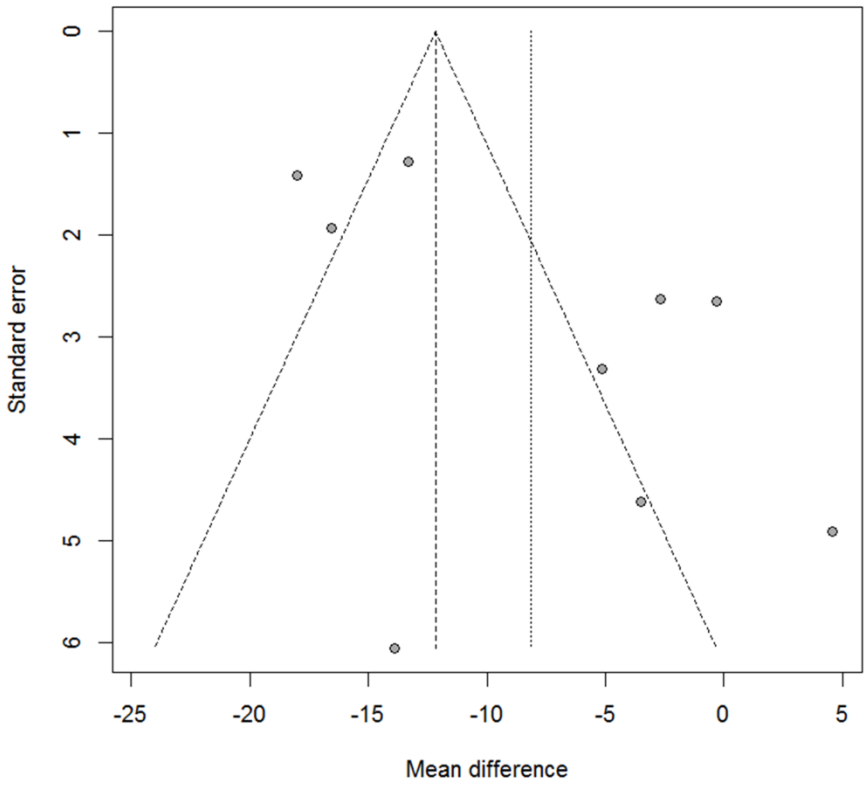
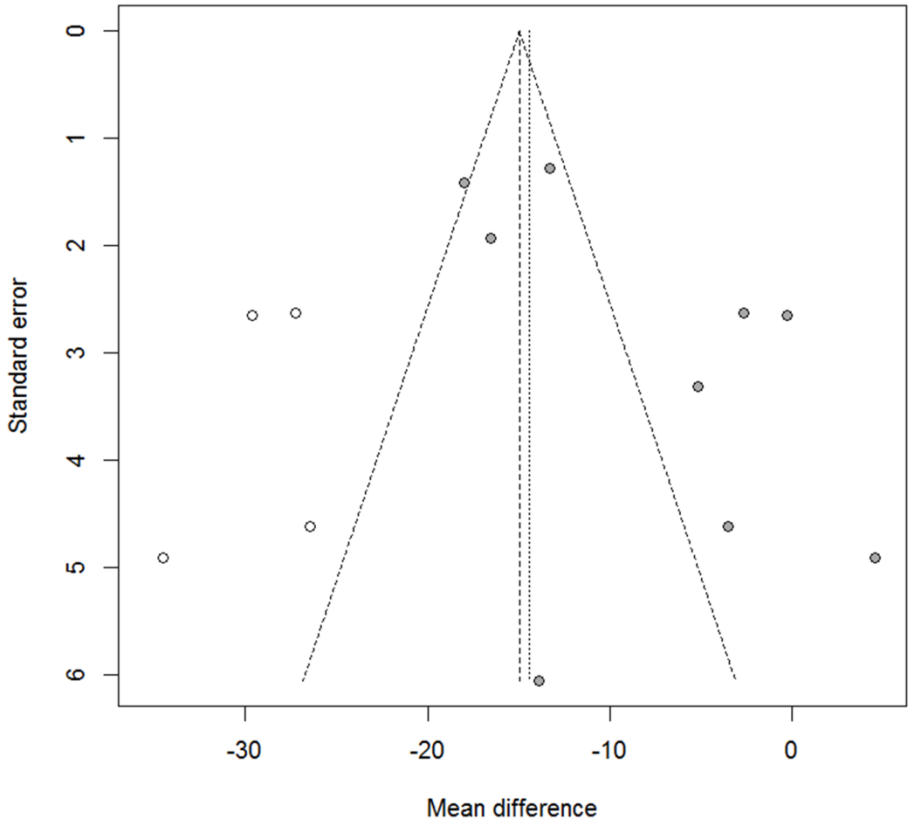


Figure 5. Trim and fill funnel plot for the meta-analysis of mobile phone exposure on sperm motility.



PROTOCOL FOR SYSTEMATIC REVIEW AND META ANALYSIS

Background

Aim and rational for project

Most men of reproductive age in high- or middle-income countries now own mobile (cell) telephones (phones). With this has come increasing concern over the effect of mobile phone exposure on human health. By undertaking a cohesive examination of existing literature and databases, the work will identify whether mobile phone exposure is negatively affecting human sperm quality.

Objective of review

The overall objective is to perform a systematic review of available studies to investigate the impact of mobile phone exposure on human sperm quality, and to estimate the effect size using meta-analysis.

Method

Identification of potential studies: Search strategy

1. The search will be run in Medline on the Ovid platform and Web of Science on the Thomson Gale platform
2. Only published literature in English from 2000 will be considered
3. The search terms will be

Phone/Electromagnetic/Semen/Sperm*/ *fertil*/

4. Citations identified from electronic searches will be downloaded to a Reference Manager database. Abstracts will be screened for relevance and full copies of studies that may meet the inclusion criteria will be obtained. Reference lists of relevant systematic reviews and of included studies will also be screened for potentially relevant primary studies. Authors will be contacted for further information or relevant unpublished data where necessary.

Inclusion and exclusion criteria

1. Type of measure

Inclusion:

1. Studies including exposure to a maximum SAR value of 2.0W/kg will be included.
2. Studies including exposure to mobile phone frequency of 800-2200Mhz will be included.

Exclusion:

1. Studies which were not written in English will be excluded.
2. Studies based on non-human participants will be excluded.

2. Method

Inclusion:

1. Original observational *in vivo* and *in vitro* studies with described study design will be included.

Exclusion:

1. Those with incomplete definitions of, or missing data on the population or study design will be excluded.

Screening studies

Inclusion and exclusion criteria will be applied first to abstracts and then to full manuscripts. Full manuscripts will be obtained for those studies where the abstract appears to meet the criteria or where there is insufficient information to be certain about excluding them. The inclusion and exclusion criteria will be re-applied to the full manuscripts and those that do not meet the criteria will be excluded.

Characterising the studies

The included studies will be characterised based on population type, study design and exposure time.

Quality assurance process

Data will be extracted by JA and checked by FM. Any disagreements will be resolved by discussion, and if necessary by discussing with others in the team. Where data are not available in the published report, the authors will be contacted for the missing information. Where relevant data is not available or the author not contactable, then the data will be excluded.

Methodological quality of included studies will be assessed considering study design, selection bias, confounders, data collection methods and integrity of the

study method. Two reviewers (JE and FM) will assess quality of studies independently. Any disagreements in quality ratings will be resolved by discussion.

Process of synthesis

All studies will be included in the synthesis, according to the inclusion and exclusion criteria identified in previous sections. The outcome data will be separated into individual sperm quality measurements, motility, viability and concentration.

The primary analyses will examine the output using meta-analysis methods (fixed and random effect models; the choice of appropriate model being influenced by the heterogeneity of the studies) and appropriate graphical methods (forest plots).We will explore heterogeneity using quantitative measure (I-square statistics) and graphical measures (funnel plot) and confounding effects will be looked by performing secondary analyses using subgroup analysis

Deriving conclusions

We will use a participatory means of drawing inferences and conclusions from our results. In order to do so, preliminary findings will be synthesised and integrated as a report by JA and FM and circulated to TG, SE and DM. The final conclusions will result from debate and discussion within the team. We will then share the findings as a manuscript published in a reputed journal.

d. Patient information documents

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E: spermhealthproject@exeter.ac.uk

Patient Information Document

Investigating the cumulative impact of modern technology on human sperm quality.

INFORMATION FOR POTENTIAL PARTICIPANTS

You have been giving this information sheet as you have expressed an interest in participating in our research. Before you decide whether or not to participate, we would like you to understand why the research is being carried out and what it would involve for you. If you have any questions please contact us using the details above.

WHAT IS THE PURPOSE OF THE STUDY?

Recent evidence, including our own meta-analysis, found electromagnetic radiation (EMR) from mobile phones and laptop Wi-Fi, are negatively affecting human sperm quality. Alone, these exposures may not have significant impacts on male fertility. However, we want to investigate whether together, there is a cumulative impact on sperm quality.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

If you wish to take part, please take a sample pot and submission form and agree the day you wish to drop in the sample with the study researchers. We ask you to submit the semen sample by 9.00am, to the postal table in Hatherly. We ask that you ensure the sample is dropped off within 1-hour post production and you abstain for 2 days prior to participation. Please complete and sign the submission form before handing in alongside your sample. Your sample will be exposed to EMR radiation and analysed for sperm quality according to WHO guidelines. Researchers at the University of Exeter will carry out all analyses. We ask that you submit your sample anonymously. No identifying information will be required. Sperm samples begin to degrade quickly following ejaculation and no viable samples will be stored for this project. If you decide to take part you are free to withdraw at any time and without giving a reason.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

We cannot promise the study will help you but the information we gain may help improve our understanding of the effect of environmental exposures on sperm quality.

CONFIDENTIALITY

We are not collecting any identifiable information about you during the course of the research.

WHAT SHOULD I DO IF I WANT TO TAKE PART?

If you do decide to take part, completing and signing the submission form (before returning it with your sample on the agreed date) demonstrates your agreement to participate in this study.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

The research has been organised by staff and postgraduate researchers at the University of Exeter. Funding has been provided by the University of Exeter and Natural Environment Research Council.

WHO HAS REVIEWED THE STUDY?

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This study has been reviewed and given a favourable opinion by the University of Exeter ethics committee.

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Investigating the cumulative impact of modern technology on human sperm quality.
Submission form.

If you would like to participate, please complete this form and hand in alongside your sample. We ask that you ensure the sample is dropped off within 1-hour post production and you abstain for 2 days prior to participation. Please hand in this sample before 9.00am on your chosen day.

1. Please state your age_____
2. Are you a current smoker? (Please circle appropriate answer)

   Yes No
3. Do you have children of your own? (Please circle appropriate answer)

   Yes No
4. Please state the period since your last ejaculation_____
5. Please state the time of sample production _____

If you have read the information provided and are happy for your sample to be used in the above study, please sign below.

....
Signature Date

UNIVERSITY OF EXETER

Group A

BIOSCIENCES

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Hatherly Laboratories
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Exeter UK EX4 4PS
t +44 (0)1392 72517
f +44 (0)1392 72343

E: spermhealthproject@exeter.ac.uk

Randomised control trial investigating the link between sperm quality and mobile telephones.

Thank you for agreeing to participate in this research. Please read this **BEFORE** signing up for a donation time-slot.

You have been assigned to Group A. This means you **MUST NOT** carry your mobile telephone in your trouser pocket during waking hours for a period of **FIVE DAYS** prior to donation. Please book a time-slot for donation immediately after this five day period using your participant number, not your name. Your participant number is
It is also shown on your sample pots and questionnaire. The time-slot sheet is on the clipboard next to the participation packs.

Your participation pack contains two sample containers, one for semen and one for urine. All donations may be carried out at home. The semen sample needs to be produced by masturbation (all other methods are likely to interfere with the test results), and should be collected after two days of sexual abstinence. The urine sample should be produced first thing in the morning. The samples need to be returned at your allotted time. This must be within one hour of producing the sample because all the laboratory analyses need to be completed within two hours of donation. There is no need to refrigerate the samples.

You are also asked to complete a questionnaire. This will enable us to look at your normal patterns of mobile telephone use. The questionnaire also asks questions regarding diet, smoking status, psychological stress and use of electronic devices.

Please return the urine and semen samples, and also the questionnaire in the brown envelope. This needs to be put in the box labelled 'Sperm Health Project Envelopes' which can be found on the table under the pigeon holes in Hatherly.

If you have any queries please do not hesitate to contact us on; spermhealthproject@exeter.ac.uk

Thank you for your participation.

Group B

BIOSCIENCES

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f +44 (0)1392 7234

E: spermhealthproject@exeter.ac.u

Randomised controlled trial investigating the link between sperm quality and mobile telephones.

Thank you for agreeing to participate in this research. Please read this **BEFORE** signing up for a donation time-slot.

You have been assigned to Group B. This means you **DO** need to carry your mobile telephone in your trouser pocket during waking hours for a period of **FIVE DAYS** prior to donation. Please book a time-slot for donation immediately after this five day period using your participant number, not your name. Your participant number is
It is also shown on your sample pots and questionnaire. The time-slot sheet is on the clipboard next to the participation packs.

Your participation pack contains two sample containers, one for semen and one for urine. All donations may be carried out at home. The semen sample needs to be produced by masturbation (all other methods are likely to interfere with the test results), and should be collected after two days of sexual abstinence. The urine sample should be produced first thing in the morning. The samples need to be returned at your allotted time. This must be within one hour of producing the sample because all the laboratory analyses need to be completed within two hours of donation. There is no need to refrigerate the samples.

You are also asked to complete a questionnaire. This will enable us to look at your normal patterns of mobile telephone use. The questionnaire also asks questions regarding diet, smoking status, psychological stress and use of electronic devices.

Please return the urine and semen samples, and also the questionnaire in the brown envelope. This needs to be put in the box labelled 'Sperm Health Project Envelopes' which can be found on the table under the pigeon holes in Hatherly.

If you have any queries please do not hesitate to contact us on; spermhealthproject@exeter.ac.uk

Thank you for your participation.

BIOSCIENCES

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w biosciences.exeter.ac.uk

Randomised control trial investigating the link between sperm quality and mobile telephones

Information for potential participants

Thank you for your interest in helping with this project. This document contains important information about the purpose of the work, the collection protocol, and the way in which data will be managed. The project has been approved by the Ethical Committee of the College of Life and Environmental Sciences. If, having read the document, you would like to assist with the project, please sign two copies of the informed consent statement. You should retain one for your records and return the other to Fiona Mathews, Hatherly Laboratories.

Background and Aims of Project

Infertility effects up to 1 in 7 couples. 40% of these cases are attributed to the male partner. However, for a significant number of infertile couples a cause cannot be found. A significant body of work has focused on the female's impact on fertility. However, with evidence of a decline in sperm quality in the last 50 years there is a need for a better understanding of male factor infertility.

Developing research and innovative technologies are aiding in identifying new causes of infertility. There is currently considerable interest in the hypothesis that mobile telephones, and other portable electronic devices, have a negative impact on fertility. However, all of the available evidence is derived from *in vitro* experimental exposure of semen to heat and/or radio-waves. Therefore, there is a need to assess the real-life effect of mobile phones on sperm quality.

What is required from participants?

We understand confidentiality is a key issue. Therefore, if you want to participate please send your consent form in a sealed envelope FAO Fiona Mathews or Jessica Elliott-Friend, Hatherly laboratories. This will not be opened. Once you have done this you can come to the postal table in Hatherly to collect a participation pack and sign up for a delivery time-slot. The pack contains an instruction sheet, questionnaire and two sample containers, one for semen and one for urine. The pack and its contents will be labelled with a random participant number. This ensures your anonymity. Please read

the instruction sheet before using the participant number to sign up for your chosen time-slot.

The instruction sheet will tell you which group you are being assigned to. One group will be asked to store their mobile phones in their trouser pockets during the day for five days prior to donation. The second group will be asked to ensure the phone is not stored in their trouser pockets for the same period of time prior to donation. Once you have been assigned to a group please sign up for a delivery time-slot (in at least 5 days time).

At the time allotted, you will need to bring the completed questionnaire and your samples. The short questionnaire asks for the following information: age; numbers of cigarettes smoked per day; alcohol consumption per day; diet; usual location for storing your mobile phone; usual weekly usage of your mobile telephone; use of other portable electronic devices (eg laptops). The samples can be produced at home. The semen sample needs to be produced by masturbation (all other methods are likely to interfere with the test results), and should be collected after two days of sexual abstinence. The urine sample should be produced first thing in the morning. The samples need to be returned at your allotted time within one hour after donation.

You will be asked to take your samples and questionnaire to a box on the Hatherly postal table at your allotted donation time. It is vitally important that the laboratory analyses are completed within two hours of producing the semen sample and therefore, that you return the samples within one hour after donation.

Initially we are requesting a single donation. However, you will be asked to indicate on the consent form whether you agree to being contacted again by letter to ask for a further sample if required. You may decline this request if you wish.

Who is doing the work?

Dr Fiona Mathews (Mammalian Biologist) and Prof. Tamara Galloway (Ecotoxicologist) are leading the project, and supervise PhD student Jessica Elliott-Friend. Mr Jonathan West, Fertility Consultant at the Royal Devon and Exeter Hospital is providing expert input.

What happens to my semen/ sperm at the end of the study?

All sperm will be made non-viable within 2 hours of your having produced the sample. No viable sperm will be stored as part of this project.

Will I be told my results?

The results of the will not be reported to donors. This is because the clinical value of any particular outcome is not clear at the present time. However, if you would like to know the overall findings of the research please give your email address on the consent form.

Confidentiality

We recognise that potential donors may have concerns about confidentiality. It is not possible for the process of donation to be made completely anonymous because we are bound by ethical guidelines which require donors to sign an informed consent form. However, a strict protocol is in operation which means that your consent form data will be kept securely and unopened. The use of a random participant number given when you collect a participant pack from Hatherly ensures anonymity of your samples.

Should you prefer to have a face-to-face meeting with a researcher, either to go through the questionnaire or to answer queries, this can be arranged. Contact details are on the consent form.

Will I be paid?

Payment will not be given because this is expressly forbidden by the UK's Human

Fertilisation and Embryology Authority.

Who is funding the project?

University of Exeter and NERC are funding this project

STATEMENT OF INFORMED CONSENT

Randomised control trial investigating the link between sperm quality and mobile telephones

I confirm that I have read and understood the document providing information about this project, and I would like to participate in the research.

I understand that I may withdraw from the project at any time.

I will return this form in a sealed envelope FAO Fiona Mathews or Jessica Elliott-Friend, Hatherly laboratories. Using the random participation number on this pack I will sign up for an appointment to give my donation.

NAME:……

SIGNED: ……

DATE:……

If you have any queries please contact spermhealthproject@exeter.ac.uk

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Questionnaire 1. Randomised controlled trial investigating the link between sperm quality and mobile telephones.

Please complete the following questionnaire for use in the above study. This will be used to link with the results gained from your sperm and urine sample. If you do not wish to answer a question please feel free to leave it blank. You are reminded that you are free to withdraw from the study at any time.

1. Please give your age.....

Section A. Smoking.

2. Are you a current smoker?

☐ Yes — If Yes please go to question 3

☐ No — If No please go to question 4

3. How many cigarettes do you smoke a day?

☐ 0-15

☐ 16-25

☐ 26-39

☐ 40+

If you smoke a pipe or cigars, please describe quantity

4. Have you ever been a smoker?

☐ Yes

- ☐ Less than 1 year ago
- ☐ 1-5 years ago
- ☐ >5 years ago

☐ No

Section B. Diet

5. Are you a vegetarian or vegan?

☐ No

☐ Yes

IF YES, please circle: Lacto-ovo vegetarian; vegan; other (describe)

6. This question aims to find out some information about your usual diet. Please put a cross (x) in the box to show us how often, on average, you consume the food listed in the last year. *eg. if you eat 2 slices of bacon once a week then you need to put a cross in the 2-4per week column.*

	Never or less than once/mo	1-3 per month	Once a week	2-4 per week	5-6 per week	Once per day	2-3 per day	4-5 per day	6+ per day
FRUIT AND VEGETABLES									
5 fresh fruit and vegetables a day							N/A	N/A	N/A
MEAT AND FISH									
Beef: roast, steak, mince, stew									
Beefburger (one)									
Port: roast, chops, stew, slices									
Lamb: roast, chops, stew									
Chicken or other poultry									
Bacon (1 rasher)									
Ham (1 slice)									
Sausages (one)									
Fried fish in batter (as in fish and chips)									
White fish eg cod, plaice, haddock									
Oily fish, fresh or canned, eg. Tuna,kippers, sardines, salmon									
SWEETS AND SNACKS									
Home made desserts eg Cakes, pies, buns etc									
Ready made desserts eg. Cakes, pies, buns etc									
Ice cream									
Chocolate bars eg Mars									
Crisps or other pack snack eg Wotsits									
DRINKS									
Tea (cup)									
Coffee, instant or ground (cup)									
Cofee, decaffeinated (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits eg gin brandy (single)									
Fizzy drinks eg Coca cola (glass)									
Pure fruit juice eg orange (glass)									

Section C. Psychological Stress

Perceived Stress Scale

The questions in this scale ask you about your feelings and thoughts **during the last month**. In each case, you will be asked to indicate by circling *how often* you felt or thought a certain way.

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

7. In the last month, how often have you been upset because of something that happened unexpectedly?..... **0 1 2 3 4**

8. In the last month, how often have you felt that you were unable to control the important things in your life?..... **0 1 2 3 4**

9. In the last month, how often have you felt nervous and "stressed"? **0 1 2 3 4**

10. In the last month, how often have you felt confident about your ability to handle your personal problems?..... **0 1 2 3 4**

11. In the last month, how often have you felt that things were going your way?..... **0 1 2 3 4**

12. In the last month, how often have you found that you could not cope with all the things that you had to do? **0 1 2 3 4**

13. In the last month, how often have you been able to control irritations in your life?..... **0 1 2 3 4**

14. In the last month, how often have you felt that you were on top of things?..... **0 1 2 3 4**

15. In the last month, how often have you been angered because of things that were outside of your control? **0 1 2 3 4**

16. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?..... **0 1 2 3 4**

Section D. Electronic devices

17. Do you currently own a mobile phone?

☐ Yes — If Yes, please go to Q18

☐ No — If No please go to Q23

18. How long have you owned a mobile phone?

☐ Less than 1 year

☐ 1-4 years

☐ 5-10 years

☐ Over 10 years

19. On average, how many hours a day are you actively using your mobile phone? (e.g on phone calls or texts)

.....hours

20. Where do you usually carry your mobile phone?

.....
.....

21. If you carry your phone in your trouser pocket, how many hours a day do you store your mobile phone here?

.....hours

22. What is the make and model of your mobile phone?

.....

23. Do you regularly use a laptop?

☐ Yes

☐ No If No, go to the end of the questionnaire.

24. On average, how many hours a day do you use your laptop on your lap?

.....hours

25. Do you connect to the internet wirelessly?

☐ Yes

☐ No If No, please go to the end of the questionnaire.

26. When you are connected to the internet wirelessly, for how many hours is your laptop on your lap?

.....hours

You have now completed this questionnaire. Thank you for your time.

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