Variations in semen quality and sperm DNA damage in a longitudinal study

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine, and Health

2020

Muaamar Noori Al-Khaiqani

School of Health Sciences

Division of Population Health, Health Services Research and Primary Care

Centre for Epidemiology

Centre for Occupational and Environmental Health

List of contents

Word count: 54,686 words

List of Abbreviations

8-oxoG	7, 8-Dihydro-8 oxoguanine
8-OHdG	8-Hydroxy deoxyguanosine
AOT	Acridine orange test
AP	Apurinic/apyrimidinic site
ART	Assisted reproductive technology
ASB	Atl1 slot blot
ATP	Adenosine triphosphate
Atl1	Alkyltransferase like protein-1
AZF	Azoospermia factor
BER:	Base excision repair
BMI:	Body mass index
BPDE	Benzo(a)pyrene-diol-Epoxide
BSA	Bovine serum albumin
cAMP	cyclic Adenosine monophosphate
CASA	Computer-aided sperm analysis
CHAPS-UK	Chemicals and Pregnancy Study -UK
CFTR	Cystic fibrosis transmembrane conductance regulator
CO	Carbon monoxide
CO_2	Carbon dioxide
CT-DNA	Calf thymus DNA
CV	Coefficient of variation
DE-I	diol epoxide-1
DEFRA	Department of Environment, Food and Rural Affairs
dH_2O	Distilled water
DF	DNA fragmentation
DFI	DNA fragmentation index
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DPBS	Dulbecco's phosphate buffered saline
DSB	Double strand break
DTT	Dithiothreitol
dUTP	Deoxyuridine triphospate

ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
Fapy G	2, 6-diamino-4-hydroxy-5-formamidopyrimidine (Fapyguanine)
fmole	Femtomoles
Fpg	Formamidopyrimidine-DNA glycosylase
FSH	Follicle-stimulating hormone
GC	Gas Chromatography
GDI	Genetic damage indicator
GGR	Global genome repair
GnRH	Gonadotropin-releasing hormone
H_2O_2	Hydrogen peroxide
H2AX	H2A histone family, member X
HDS	High DNA stainability
HDG	Highly DNA damaged
HF	High Fat
hOGG1	Human 8-hydroxyguanine DNA-glycosylase 1
HR	Homologous recombination
HRP	Horseradish peroxidase
HNO	Nitroxyl anion
HNO_3	peroxynitrous acid
HPTaxis	Hypothalamic-pituitary -testicular axis
ICSH	Interstitial cell-stimulating hormone
ICSI	Intracytoplasmic sperm injection
ISB	Immunoslot blot
ISNT	In situ nick translation assay
IVF	*In vitro* fertilization
KS	Klinefelter's syndrome
Ku70	Ku protein 70
Ku80	Ku protein 80
LC-MS_2	Liquid chromatography-mass spectrometry
LDG	Low DNA damage
LH	Luteinizing hormone
LMP	Low melting point
LSI	Leisure score index

MDG	Medium DNA damage
MEM	Minimum essential medium
MGMT	O^6-Methylguanine-DNA- methyltransferase
MMR	Mismatch repair
MMS	Methyl methanesulphonate
MN	Micronuclei
MNU	N-nitroso-N-methyl urea
MS	Mass spectrometry
mZp3	Mouse zona pellucida glycoprotein 3
N_2O	Nitrous oxide
N7-MeG	N7-methyldeoxyguanosine
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
NMP	Normal melting point
NNOC	N-nitroso compounds
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_{3-}	Peroxynitrite
NOA	Non-obstructive azoospermia
O_2-	Superoxide anion
O^6-MeG	O^6-methylguanine
OAT	Oligoasthenoteratozoospermia
OH^-	Hydroxyl radical
OPs	Organophosphorus pesticides
OTM	Olive tail moment
PAH-DNA	Polycyclic aromatic hydrocarbon DNA adduct
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PIS	Participant information sheet
PK	Proteinase K
PM_{10}	Particulate matter with an aerodynamic diameter between 2.5 µm to 10 µm
$PM_{2.5}$	Particulate matter with an aerodynamic diameter of less than or

	equal to 2.5 µm
PSG	Penicillin Streptomycin-Glutamine
RNA	Ribonucleic acid
RIA	Radioimmunoassay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
SAM	S-adenosylmethionine
SCD	Sperm chromatin dispersion
SCOS	Sertoli cell-only syndrome
SCSA	Sperm chromatin structure assay
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SSBs	Single strand breaks
TBE	Tris-borate-EDTA
TBST	Tris buffer saline – Tween 20
TCR	Transcription-coupled repair
TDS	Testicular dysgenesis syndrome
TdT	Terminal deoxynucleotidyl transferase
TE	Tris-EDTA
TLS	Translesion synthesis
TM	Tail moment
TMZ	Temozolomide
TUNEL	TdT-mediated dUTP nick-end labeling
UHPLC	Ultra-high-pressure liquid chromatography
UV	Ultraviolet
VP	Vasopressin
WHO	World Health Organization
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
Yq	Y chromosome microdeletions in the long arm
ZP	Zona pellucida

Abstract

Over recent years a consistent increase in male infertility has been reported. Research into the underlying aetiologies have suggested a reduction in semen quality, possibly related to lifestyle, occupational or environmental exposures, and that sperm DNA strand breaks and base damage might be biomarkers of male infertility. Therefore, the aims of this study were to examine the variations of semen quality parameters and DNA damage in human sperm and associations between these factors and lifestyle factors and environmental exposures.

Twenty participants were initially enrolled in the study and 15 of them completed the entire study. Two hundred and six wide ranging food, lifestyle and occupational questionnaires, 206 semen samples and 59 blood samples were collected from the participants on 12 separate visits during the 6-month study period. The sperm parameters were assessed manually and by computer assisted sperm analysis, Sperm DNA double strand break damaged was visualised by a neutral Comet assay and scored manually to generate a genetic damage indicator (GDI). The levels of the DNA lesion, O^6-alkylguanine (O^6-alkylG) in sperm and buffy coat DNA samples were quantified using a novel assay.

The study showed that total sperm count and sperm concentration were the most variable semen parameters, while the lowest variable parameter was % vital sperm. The Comet assay was shown to have very good reproducibility and repeatability. At baseline, the GDI values of the 20 participants ranged from 29 to 166, and these levels were remarkably consistent over the longitudinal 6 month study period (29+/-3 and 163+/-6 respectively). There was a significant negative correlation between % vital sperm and GDI (R=minus0.57 P=0.01). The novel O^6-alkylG quantitation assay was optimised and showed a range of levels in sperm DNA. These did not correlate with the levels in the corresponding buffy coat DNA nor with the corresponding GDI, the basis of which may be related to the activity of various DNA repair pathways. However, the assay was found to be simple and reproducible and could be used to assess the overall levels of O^6-alkylG in DNA of human sperm or any other tissues in future studies.

Some statistically significant positive or negative correlations were found between a few of the many diet, lifestyle and occupational parameters studied, but the numbers were in all cases too small to have any real confidence. This study is therefore to be seen as a prelude to a much larger study requiring substantially more participants.

Declaration

No portion of the work referred to in the thesis has been submitted in the support of an application for another degree or qualification of this or any other university or other institutes of learning.

Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the "Copyright") and s/he has given the University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs, and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the "Intellectual Property") and any reproductions of copyright works in the thesis, for example, graphs and tables ("Reproductions"), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner (s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication, and commercialization of this thesis, the Copyright, and any Intellectual Property University IP Policy (http://documents.manchester.ac.uk/dsiplay.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library's regulations (see http://www.library.manchester.ac.uk/about/regulations/)

v. and in the University's policy on Presentation of Theses.

Acknowledgments:

The work carried out in this thesis would not have been possible without the invaluable support and assistance of numerous individuals. I am profoundly grateful to my supervisors, Dr. Andrew Povey, Dr. Michael Carrol and Prof Daniel Brison for their support, help, and guidance over the past four years. I would also like to big thank Prof Geoff Margison, for providing CT-DNA, MGMT, and some materials for ASB assay. I am indebted to the staff in MMU especially the Stephane Berneau for their help and support in the process of recruiting participants for the study and collecting both the semen samples and blood from all participants. I would like to thank I gratefully acknowledge the other Ph.D. students, Rasha, Suha, Hatim, Nurul, and special thanks to Hanum for ASB training in my department in the Biomarker lab at the University of Manchester for their kind help and support. And a special big thank Dr. Iqbal Ahmed for his advice and support.

Special thanks to my mom, who is always by my side during the ups and downs. Not forgetting all my family members, who have given me their support and inspiration. Their love, care, and support have brought me this far. And at last but not least, to my beloved wife, Abeer, who is always there for me, thank you for all your support and care.

Chapter 1: Introduction

1.1 Background

In man, infertility is acknowledged to be "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild et al., 2009). If a couple fails to achieve pregnancy a clinical investigation may be required to establish if there are any problems in seminal fluid constituents or fertility-related hormones (Zhu et al., 2006).

Most couples seeking help for infertility are subfertile, which means the ability of the couple to become pregnant is lower than 'normal', but they are in principle still able to conceive, rather than being infertile or sterile (Zhu et al., 2006). Infertility is either primary, when no pregnancy has ever occurred, or secondary, where there has been a pregnancy, regardless of the outcome (Mascarenhas et al., 2012). Agarwa et al., (2015) reported that about one in ten couples are infertile with a male factor being responsible for approximately 40% of the cases (Agarwa et al., 2015).

Male infertility has several possible causes which include testicular failure, infection, obstruction, and sexual dysfunction e.g. impotence, but the most common diagnosis is idiopathic male infertility which is characterized by an unexplained reduction of semen quality, and this accounts for about 60-70% of patients (Nieschlag & Kamischke 2010). For successful conception, the male needs normal spermatogenesis, a normal reproductive system anatomy, and normal sexual function in order to deposit an adequate number of morphologically normal and motile spermatozoa in the upper part of the vagina (Kupka et al., 2014).

The increasing number of couples undertaking assisted reproductive technology (ART) treatment (Joffe 2003) might indicate a possible decline in male fertility in the general population but it could also be because ART treatment is more widely available, and couples can afford it, or because of a decline in female fertility with age since women are increasingly delaying becoming a parent.

The possible decline in male fertility might be due to social changes, occupational and environmental exposure to toxic agents, dietary and lifestyle factors (Schulte et al., 2010). Indeed, an increasing number of studies suggest that environmental exposure to chemicals

such as cadmium and mercury (Wong & Cheng 2011) or physical agents such as heat and radiation (Jayachandra & Srinivasa 2011), introduced and spread by human activity, may have adverse impacts on somatic cells, including sperm, and this may affect male fertility (Jurewicz et al., 2009).

Genotoxin exposures may have a deleterious effect on semen quality and DNA integrity by inducing oxidative DNA damage, DNA alkylation, DNA breaks or other types of damage which, in turn, might reduce male fertility by impairing the spermatozoa's ability to fertilize an egg and produce an undamaged embryo (Schulte et al., 2010).

1.2 Male reproductive physiology

The reproductive functions of the male are regulated by various endocrine hormones (Kopp-Kuhlman 2011) and consist of spermatogenesis, which is the production and maintenance of sperm, the formation the protective fluid (seminal plasma), and the discharge of semen within the female reproductive tract.

1.2.1 Spermatogenesis

Spermatogenesis is a complex process involving the interaction of multiple cells with hormones and growth factors over a relatively long period of time. Spermatogenesis includes the conversion of germ cells into highly specialized sex cells which can be united with an oocyte at fertilization resulting in the production of an embryo (Barrett et al., 2010). Spermatogenesis occurs in the seminiferous tubules during the active sexual life of the male individual, stimulated by the anterior pituitary gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Meehan et al., 2000; Koeppen & Stanton 2009). This stimulation starts at an average age of 13 years and continues throughout the remainder of the man's life (Jungwirth et al., 2014).

Generally, 4.6 cycles are required for a mature sperm to develop from an early spermatogonium (pale type A spermatogonium) to a mature sperm and the duration of each cycle is approximately 16 days. Therefore, the duration of the entire spermatogenic cycle in man is 74±4 days (Hargreave 2000). Each spermatogonium undergoing differentiation after puberty gives rise to 16 primary spermatocytes and each spermatocyte enters meiosis to give rise to four spermatids and ultimately four spermatozoa so that 64 spermatocytes can develop from each spermatogonium (Sharpe 2010). However, 30% to 40% of germ cells are lost,

mainly during the second meiotic division (Mortimer 2005). At male sexual maturity, the rate of sperm production will reach about 200 million sperm per day (Sharpe 2010).

Sperm are released into the lumen of the seminiferous tubules, from which they are carried into epididymis duct for further maturation and storage (Carrera et al., 1994). When the sperm leaves the testis, they are relatively immature and have limited ability to fertilize an ovum (Martin et al., 1982; Strauss & Barbieri 2013). The transport of the sperm through the epididymis and then to the ejaculatory duct requires an additional 12 to 21 days (Sharpe 2010).

1.2.2 Hormonal control of spermatogenesis

The hormonal control of spermatogenesis takes place through the hypothalamic-pituitary - Testicular axis (HPT axis), which involves two axes. The first is the: Hypothalamic-Hypophysial-Seminiferous tubular and the second is the Hypothalamic-Hypophysial-Leydig cell axis (McLachlan et al., 2002; Guyton & Hall 2006).

Neurological activity in the brain regulates the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus into the hypophyseal portal system, which drains into the anterior pituitary gland and stimulates the release of FSH and LH (Guyton & Hall 2006; Strauss & Barbieri 2013).

The role of hormones is not only during the initiation of spermatogenesis at puberty but also in the maintenance of spermatogenesis during adulthood and the re-initiation of spermatogenesis following pathologic or chemically induced hypogonadism (Strauss & Barbieri 2013).

1.2.3 Stages of spermatogenesis

Spermatogenesis can be divided into spermatocytogenesis and spermiogenesis (Guyton & Hall 2006; Barrett et al., 2010). During these processes, the different types of cells are all interconnected by cellular bridges of cytoplasm and surrounded by Sertoli cells, also called sustentacular cells.

These cells are the epithelial supporting cells of the seminiferous tubules and play an important role in protecting and providing nutrients to sperm. They also play a role in the induction of apoptosis which occurs in the meiosis stage of sperm formation. However, this

mechanism is not very efficient because many defective sperm are produced (Meehan et al., 2000; Eddy et al., 2003).

1.2.3.1 Spermatocytogenesis

The basement membrane of each seminiferous tubule is lined with diploid (having the full complement of 46 chromosomes) germ cells called spermatogonia. These can be divided into type A and type B spermatogonia according to the staining properties of their nuclei (Meehan et al., 2000; Mortimer 2005; De Jonge & Barratt 2006).

Spermatogonia are large with large spherical nuclei (Wolf 1989) and undergo continuous mitotic division (Barrett et al., 2010; Sharpe 2010). Type B spermatogonia are interconnected by cytoplasmic bridges and they divide mitotically to form more spermatogonia and preleptotene primary spermatocytes (Hargreave 2000; De Jonge & Barratt 2006).

1.2.3.2 Meiosis

In the meiotic phase of spermatogenesis, the diploid primary spermatocytes undergo a reduction division into secondary spermatocytes, which are haploid in chromosomal number (Martin et al., 1982; Carrera et al., 1994; Strauss & Barbieri 2013). Then the secondary spermatocytes undergo a second meiotic division, which results in the formation of round spermatids which are also haploid (Morales & Clermont 1994).

1.2.3.3 Spermiogenesis

In this phase, morphological changes occur as the spermatid matures and differentiates from a spherical non-motile cell to a linear spermatozoon that is motile (Sharpe 2010). This process takes place while the head of the spermatid remains embedded in the cytoplasm of Sertoli cells (Meehan et al., 2000; Eddy et al., 2003).

In brief summary, spermiogenesis is characterized by the following events (Guyton & Hall 2006). First, the acrosome is generated by a Golgi complex, then the nuclear DNA condenses at one end and the posterior centrioles are polarized at the opposite end and initiate the formation of flagella which provide the structure for motility; during this stage, the mitochondria, which are presumed to supply energy for sperm motility, are assembled in the

spermatid midpiece and finally, the reduction of spermatid cytoplasm occurs and this appears as a residual body (Barrett et al., 2010).

At the end of this phase, the spermatids will elongate to form spermatozoa. An individual spermatozoon can be divided into three components: the head, the midpiece, and the flagellum (tail). The head of a human sperm is about 4.4 μm long and 2.8 μm wide. It is composed of a nucleus that contains highly compact chromatin the DNA of which is organized into loop domains attached at bases to a nuclear matrix (Eddy et al., 2003).

At the chromosomal level, the histones, the predominant chromatin proteins of somatic cells, are replaced by the highly basic transition proteins, which in turn are replaced by the protamines, producing a tightly compacted nucleus with extensive disulfide bridge crosslinking (Carrell 2007; Bungum 2012). Sperm chromatin condensation during spermatogenesis results in DNA taking up about 90% of the total volume of the sperm nucleus. In contrast, in normal somatic cells, the DNA takes up only 5% of the nucleus volume, while in the mitotic division the chromosomal DNA takes up about 15% of the nuclear volume (Eddy et al., 2003).

The anterior portion of the sperm head is surrounded by an acrosomal cap that is a membrane-bound organelle containing the enzymes required for penetration of the egg during the fertilization process (Eddy et al., 2003). These enzymes include hyaluronidase and corona penetrating enzymes which are bound to the outer acrosomal membrane (Yanagimachi 1994), acrosin (as the inactive form proacrosin), neuraminidase, phospholipase, and acid phosphatase (bound to the inner acrosomal membrane) (Eddy et al., 2003). These enzymes are released during the fertilization process to facilitate the penetration of sperm into the cumulus mass, corona radiata, zona pellucida (ZP) and plasma membrane (Yanagimachi 1994; Eddy et al., 2003).

The midpiece is a highly organized segment consisting of helically arranged mitochondria which contain the enzymes required for oxidative metabolism and production of adenosine triphosphate (ATP) (Yanagimachi 1994; De Jonge & Barratt 2006).

The major reorganizational events of the differentiating spermatid are accompanied by significant alterations in the energy suppliers of the cell, by the mitochondria, which exhibit several distinct morphologies as germ cell differentiation proceeds. Spermatogonia and somatic testicular cells contain the ‘cigar-shaped’ mitochondria found in most somatic

tissues. During meiosis, mitochondria with diffuse and vacuolated matrices start replacing the 'somatic' mitochondria. By the beginning of spermiogenesis, the 'somatic' mitochondria have been replaced by 'germ cell' mitochondria, which in turn are replaced by the crescent-shaped mitochondria of spermatozoa (Yanagimachi 1994; Eddy et al., 2003). The midpiece is surrounded by a set of outer dense fibres and, centrally, the microtubule structures of the sperm axoneme which continue into the flagellum. The outer dense fibres, which are rich in disulfide bonds, are thought to provide the sperm flagellum with the rigidity necessary for progressive motility (Eddy et al., 2003). The flagellum of a mature sperm has three major components: the axoneme, a central skeleton comprising 11 microtubules and containing the enzymes and structural proteins necessary for the transduction of the chemical energy of ATP into mechanical movement resulting in sperm motility; a fibrous sheath; and an outer plasma membrane (Stock & Fraser 1987; Carrera et al., 1994; Eddy et al., 2003). Transcription of RNA ceases during mid-spermatogenesis, and translational regulation plays a prominent regulatory role in the extensive protein synthesis that takes place throughout the latter half of spermiogenesis (Morales & Clermont 1994; Carrell 2007).

Spermiogenesis represents an interval of spermatogenesis that may be susceptible to the introduction of both genetic and structural defects in the maturing male gamete because the displacement of the histones from the nucleosomes may leave the DNA of the haploid genome especially sensitive to damage. Although unscheduled DNA synthesis i.e. an indication of DNA repair, has been demonstrated to occur in the early stages of spermatid development, as spermatogenesis proceeds, unscheduled DNA synthesis diminishes, and there is no evidence that any of the sophisticated DNA repair mechanisms that function during meiosis are still operational (Carrell 2007; Bungum 2012).

1.2.4 Seminal plasma

At ejaculation, sperm are transported from their storage site in the epididymis tail through the vas deferens and are mixed with seminal plasma before passage along the penile urethra. Seminal plasma is produced mainly by the seminal vesicles, prostate gland and bulbourethral gland (Strauss & Barbieri 2013). The exact function of many of the components is not known, although most have roles in the maturation, maintenance, and transport of sperm via the male or female reproductive system (Mortimer 2005; Strauss & Barbieri 2013). For example, water and mucus serve as the liquid vehicle and lubricant for the passage of sperm through the male reproductive tract, while buffers (secreted by the prostate gland) neutralize

the acidity in the vagina, and the sugar, fructose (from the seminal vesicles), is a source of energy for the sperm in the seminal fluid or vagina. Seminal plasma also contains carnitine and this is involved in the metabolism of fatty acids (Strauss & Barbieri 2013).

Enzymes that can be found in seminal plasma include fibrinogenase and fibrinolytic enzyme, which are involved in the clotting and subsequently liquefaction of semen in the vagina. Calmodulin is a protein which is believed to play a role in the sperm capacitation process (see below; Strauss & Barbieri 2013).

Seminal plasma also contains a very high concentration of zinc which may have antibacterial and antioxidant activity, and contains prostaglandins which may play a role in sperm transport and suppress the female immune response (Guyton & Hall 2006; Strauss & Barbieri 2013). Seminal plasma also contains trophoblast antigens required to prime the maternal allogenic responses important for placentation and pregnancy (Bujan et al., 1996; Suarez & Pacey 2006; Jones & Lopez 2013).

1.2.5 Sperm capacitation and the acrosome reaction

Freshly ejaculated spermatozoa are unable to fertilize an oocyte (Mortimer et al., 1989; Tesarik et al., 1998). The fertilization ability of spermatozoa develops during their journey through the female reproductive tract (or *in vitro* in a CO_2 incubator), in a process called capacitation (Tesarik et al., 1998).

Capacitation consists of a sequence of subcellular processes that are required for the onset of acrosome mediated hyperactivation of sperm motility (Tesarik et al., 1998). Capacitation is a time-dependent phenomenon, ranging from less than one hour in the mouse to 6 hours in man (Cohen-Dayag et al., 1995), thus, the spermatozoa must reside in the female genital tract for several hours before they can penetrate the oocyte. Capacitation may be caused by a high concentration of cAMP and calcium ions (Tesarik & Testart 1989) and is reversible following a decrease of negative surface charge, an influx of membrane cholesterol, an efflux of calcium ions between the plasma and outer acrosomal membrane and the removal of epididymal and seminal plasma proteins coating the sperm surface (Cohen-Dayag et al., 1995).

The motility of hyperactivated sperm has been correlated with fertilization ability (Boué et at., 1994) presumably by promoting sperm migration from the isthmus to the ampullary

portion of the oviduct and increasing the chances of contact between the spermatozoa and oocyte (Jeulin et al., 1986; Boué et at., 1994; Jones & Lopez 2013).

In a semen sample, approximately 2-12% of sperm are capacitated and only these sperm can undergo the modifications, such as chemotaxis, hyperactivation and the acrosome reaction which is necessary for fertilization (Boué et at., 1994; Cohen-Dayag et al., 1995; Beydola et al., 2013; Jones & Lopez 2013). *In vitro*, capacitation can be accomplished by the removal of spermatozoa from seminal plasma and incubation of the sperm in artificial media (Jeulin et al., 1986; Beydola et al., 2013), but studies of the cellular and extracellular mechanisms, show that the events are different from those occurring in the female genital tract (Jeulin et al., 1986; Beydola et al., 2013).

In vitro, the acrosome reaction can be enhanced by progesterone, peritoneal fluid and follicular fluid (Tournaye et al., 1995; Giojalas 1998; Jones & Lopez 2013; Bravo & Valdivia 2018). The acrosome reaction is an exocytotic, calcium-dependent irreversible essential event after which the life of sperm is very short (Blackmore et al., 1990; Giojalas 1998; Jones & Lopez 2013).

The reaction starts when capacitated sperm are attached to an ovum ZP by the reaction between sperm receptors in the ZP, and a specific oocyte binding protein on the sperm plasma membrane. It involves the release of hydrolytic enzymes through small openings formed by localized fusions of the sperm plasma membrane and the outer acrosomal membrane. These hydrolytic enzymes, including hyaluronidase and acrosin, degrade hyaluronic acid between the cumulus oophorous cells of the ZP forming a tunnel through which the sperm passes, allowing the fusion of the spermatozoa plasma membrane to that of the oolemma (Blackmore et al., 1990; Paulsen et al., 1996; Vierula et al., 1996; Suarez & Pacey 2006).

1.2.6 The fertilization processes

After successful penetration of the ZP, the sperm enters the perivitelline space and reaches the oocyte plasma membrane sideways. At this point, the plasma membrane of sperm head attaches to the oocyte plasma membrane and they fuse to form an opening through which the sperm nucleus, midpiece and most of the tail enter the oocyte cytoplasm (Strauss & Barbieri 2013). This triggers the cortical reaction, a process initiated during fertilization by the release of cortical granules from the egg, which prevents any other sperm from entering

the oocyte. Also, at this point, the metaphase II stage oocyte resumes meiosis II and the female pronucleus is formed. The sperm nucleus decondenses to form the male pronucleus which then fuses with the female pronucleus. The fertilized oocyte at this stage is called a zygote (Mortimer 2005; Strauss & Barbieri 2013).

1.2.7 Reference values for normal semen parameters and fertility

Normal standards for the basic semen features, i.e. volume, motility, sperm concentration, and morphology, have from time to time been published (Ombelet et al., 1997) and reviewed in the World Health Organization (WHO) manuals (WHO 1992; 1999; 2010; Table 1.1).

Table 1.1: Reference values for semen parameters, as published in consecutive WHO manuals

Semen parameters	WHO 1992	WHO 1999	WHO 2010
Volume	≥ 0.2 ml	≥ 0.2 ml	1.5 ml
Sperm concentration	≥ 20 X 10^6/ml	≥ 20 X 10^6/ml	≥ 15 X 10^6/ml
Total sperm count	≥ 40 X 10^6	≥ 40 X 10^6	≥ 39 X 10^6
Total motility (% motile)	≥50%	≥ 50%	≥ 40%
Progressive motility[a]	≥ 25% (grade a)	≥ 25% (grade a)	≥ 32% (grade a+b)
Vitality (% alive)	≥75%	≥75%	≥58%
Normal morphology*	≥30%	≥14%	≥4%
Leukocyte count	<1.0 X 10^6/ml	<1.0 X 10^6/ml	<1.0 X 10^6/ml

[a] Grade a = rapid progressive motility (which refers to sperm that are swimming in a mostly straight line or in very large circles) (25 μm/s); grade b = slow/sluggish progressive motility (5–25 μm /s); Normal = 50% motility (grades a + b) or 25% rapid progressive motility (grade a) within 60 min of ejaculation, * Normal shaped spermatozoa according to the Tygerberg strict criteria (WHO 1999; Lu et al., 2010; Menkveld 2010).

In the 1999 WHO manual, the term ‘normal values’ was changed to ‘reference values’ (Irvine 1998). The values published in the WHO manuals were mostly obtained through studies carried out on so-called ‘normal’ or ‘fertile’ populations and are not the lowest values necessary to achieve ‘normal’ pregnancy, i.e. pregnancies in normal relationships can also be obtained with lower semen parameter values than those indicated in Table 1.1, leading to confusion between terms ‘normality’ and ‘fertility’ (Lu et al., 2010; Menkveld 2010).

Several studies have compared the semen parameter values of males from so-called fertile populations with subfertile populations, to determine minimum cut-off values for the different semen parameters in order to establish a male’s fertility potential (Menkveld 2010;

Jungwirth et al., 2012). Guzick et al., (2001) found that men's fertility potential could be classified into one of three groups based on their semen parameters as: possibly fertile or normal; subfertile; and infertile (Zhu et al., 2006; Jungwirth et al., 2012).

1.2.8 The terminology of semen quality

Three key parameters are assessed during semen analysis: sperm count, sperm morphology and sperm motility. The WHO (2010) has defined terms relating to semen quality as shown in Table 1.2.

Table 1.2: Terminology of semen quality

Term	Refers to
Normozoospermia	The semen sample is normal in concentration, motility, and morphology.
Aspermia	The inability of a patient to give semen samples.
Azoospermia	No sperm in the semen sample (only seminal plasma).
Oligospermia	Total sperm number below the lower reference limit.
Asthenozoospermia	Percentage of progressively motile sperm below the lower reference limit.
Teratozoospermia	Percentage of abnormal sperm morphology higher than normal.
Necrospermia	Spermatozoa in the ejaculated semen are dead.
Leukospermia	Number of white blood cells in the ejaculate more than normal levels.

1.2.8.1 Manual and computer assisted semen analysis (CASA)

Semen analysis is considered as an essential diagnostic assay in the evaluation of male reproductive health and can be undertaken by two methods. One is the manual method and this depends completely on the personal experience of the lab personnel who evaluate the seminal fluid parameters, whereas the second method uses digital imaging and computer software (computer-assisted sperm analysis; CASA) to determine seminal fluid parameters as described below (Keel et al., 2002; Keel 2004; WHO 2010).

In principle, both methods can be performed by simple procedures that do not require complicated equipment. In practice, however, considerable technical expertise and procedural care are needed to obtain reliable and reproducible results. As might be expected, the lack of appropriate standardization protocols has led to wide variability in analytical results (Keel et al., 2002; Keel 2004). Most andrology laboratories and semen processing facilities have a CASA system, but the extent of dependence thereon ranges widely. CASA systems automatically view multiple fields in a shallow specimen chamber to capture and store live images of 500 to >2000 sperm at 50 or 60 frames per second. Potentially CASA

can provide automatic highly accurate measurements of individual sperm motion, allowing the calculation of the velocity and proportion of sperm exhibiting progressive motility, and thereby eliminating the bias of subjective 'estimates' by technicians (Amann & Waberski 2014). Some studies have suggested that CASA estimates of concentration and movement characteristics of progressively motile spermatozoa are significantly related to fertilization rates *in vitro* and *in vivo* (Liu et al., 1991; Irvine et al., 1994; Barratt et al., 1995; Krause 1995; Donnelly et al., 1998; Larsen et al., 2000; Garrett et al., 2003; Shibahara et al., 2004). Practically, the best method to determine the total sperm motility, kind of sperm motility and velocity is by CASA. However, not all CASA systems can evaluate sperm morphology concurrently with motility analysis and CASA is not highly accurate in the determination of sperm count, thus, the best method to determine sperm concentration is manually by using a Haemocytometer (WHO 2010).

1.2.9 Causes of male factor infertility

The factors that affect male fertility can be classified into two groups: firstly, endogenous factors such as genetic, congenital, endocrine and varicocele and secondly, exogenous factors such as radiation, alcohol consumption, febrile reaction, and infection (Wallace et al., 2005; Bansal et al., 2017). Fifty-five-70 % of male factor infertility cases have known causes which include cryptorchidism (maldescended testes), varicocele, genetic causes, hypogonadotropic hypogonadism, autoantibodies against sperm, infections and systematic diseases, while the remaining 30-45% of male infertility cases are considered idiopathic (Jungwirth et al., 2012; Bansal et al., 2017).

1.2.9.1 Endogenous factors

The principal endogenous factors that affect male fertility are genetic factors, cryptorchidism, endocrine disorders and varicocele and these are discussed in more detail below. Most of the factors that reduce semen quality and cause other disturbances of male reproductive functions such as hormones are not fully understood.

1.2.9.1.1 Age

Kidd et al., (2001) reported that a reduction in most sperm parameters such as semen volume, sperm motility, and % of normal sperm morphology but not sperm concentration is correlated with increased male age. It has also been shown that total sperm count, semen

volume and sperm motility was significantly associated with age and abstinence time (Shi et al., 2018). In addition, Abayomi et al., (2018) reported that the semen volume of men aged <45 years was significantly higher than those aged ≥45years. However, Johnson et al., (2015) suggested that the impact of age on semen parameter values is inconclusive.

1.2.9.1.2 Genetic factors

Male infertility can be associated with various genetic factors, including chromosomal aberrations, genetic alterations, and Y chromosome microdeletions. The mean incidence of chromosomal aberrations in infertile men is 5% and these are predominantly sex chromosome abnormalities (Tateno et at., 1998; Ferlin et al., 2007). The most frequent sex chromosome aneuploidy in human males is XXY; Klinefelter's syndrome (KS). Generally, men with KS have small testes, deteriorated function of the Leydig cells and impaired spermatogenesis. KS is found in up to 5% of oligozoospermia and 10% of azoospermia cases. Y chromosome microdeletions in the long arm (Yq) are found in 10–15% of men with non-obstructive azoospermia and 5–10% of men with severe oligozoospermia (Hargreave 2000; Ferlin et al., 2007). The Y chromosome region related to infertility is called the azoospermia factor (AZF) locus and there are three regions, AZF a, b and c, from the proximal to the distal sections of Yq, which have been considered as spermatogenesis loci. The AZFa region deletions usually cause Sertoli cell-only syndrome (SCOS), while complete deletions of AZFb or AZFb and c lead to azoospermia associated with SCOS or pre-meiotic spermatogenic arrest. The AZFc deletion is considered the reason for 3-7% of men having azoospermia or severe oligozoospermia (Tateno et at., 1998; Jungwirth et al., 2012; Hanmayyagari & Guntaka 2015; Bansal et al., 2017). The mechanisms by which these Yq microdeletions disrupt spermatogenesis are unknown (Bansal et al., 2017). Azoospermia can also be caused by other gene mutations including cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations which are considered to be the reason for 60-70% of cases of congenital bilateral absence of the vas deferens (obstructive azoospermia) and 6.6% of cases of non-obstructive azoospermia (Wallace et at., 2005; Bukowska et al., 2013; Bansal et al., 2017).

1.2.9.1.3 Cryptorchidism

Cryptorchidism is a congenital failure of one or both of the testes to descend into the scrotum, occurring in about 3% of full-term and about 30% of premature male infants. It is generally

considered to be associated with decreased sperm count and male infertility in adults (Skakkebaek et al., 2001) and it is the cause of around 8.4% of male factor infertility cases in Europe (Guzick et al., 2001; Jungwirth et al., 2012). Cryptorchidism is also considered to be a risk factor for testicular cancer development (Guzick et al., 2001; Skakkebaek et al., 2001) and it is believed that cryptorchidism, hypospadias (a birth defect of the urethra in the male where the urinary opening is not at the usual location on the head of the penis), impaired semen quality and testicular cancer are all symptoms of testicular dysgenesis syndrome (TDS). TDS is considered to be caused by maternal exposure to environmental endocrine disruptors leading to abnormal embryonic gonadal development during foetal life (Masutomi et al., 2003).

1.2.9.1.4 Endocrine disorders

Male infertility may be associated with hypothyroidism, hyperadrenocorticism hyperprolactinemia or hypogonadotropic hypogonadism (Kacsoh 2000; Strauss & Barbieri 2013). In all of these conditions gonadotropin levels are low because of feedback inhibition or idiopathic hyposecretion (Kacsoh 2000; Barlow & Foster 2003; Weber et al., 2005). Furthermore, hypothyroidism affects both pituitary and testicular function with alterations in the secretion of hormones and increased conversion of androgens to estrogens (Kooy et al.,1990; Barlow & Foster 2003; Weber et al., 2005).

Additionally, other hormones affect spermatogenesis such as norepinephrine, which is stimulated by hypothalamus secretions, serotonin, and dopamine both inhibited by hypothalamus secretion of gonadotropin-releasing hormone (Kacsoh 2000; Weber et al., 2005). However, some studies *in vitro* and *in vivo* have shown that oxytocin (Nicholson et al., 1991) and vasopressin (VP) may affect testicular function. Oxytocin has been reported to affect seminiferous tubule contractility in neonatal rats (Nicholson et al., 1991) and VP regulates steroidogenesis. In addition, some studies mention that VP alters testicular LH and human chorionic gonadotropin receptor content (Valenti et al., 2013).

1.2.9.1.5 Varicocele

Varicocele is an abnormal dilatation in the spermatic veins and it occurs when the valves within the spermatic veins do not work properly. There are two types, subclinical and clinical varicocele. Subclinical varicocele is not visible at rest or during the Valsalva manoeuvre but is demonstrable by scrotal ultrasound. Clinical varicocele is classified into three grades

reflecting the severity of the case; grade 1 is palpable during Valsalva manoeuvre but not otherwise, grade 2 is palpable at rest but not visible, Grade 3 is visible at rest (Weber et al., 2005; Baazeem et al., 2011). Clinical varicocele is found in up to 15% of all adult men, 11.7% of infertile men with normal semen analyses and 25.4% of those with male factor infertility. However, only 20% of men with varicocele seek infertility treatment which means that a varicocele may induce a reduction in semen quality but does not necessarily lead to infertility (Naughton et al., 2001).

It is not clear yet how varicocele impacts on male fertility, but some have suggested that varicocele might cause an increase in testis temperature or cause testicular damage gradually (Naughton et al., 2001). Varicocele is associated with decreased sperm concentration (Baazeem et al., 2011) and causes a progressive decline in fertility due to severe damage, primarily to the seminiferous epithelium associated with a diminution of sperm production. This condition occurs in old individuals and frequently develops without a known cause, but it may follow epididymitis, orchitis injury or neoplasm (Naughton et al., 2001).

1.2.9.1.6 Febrile reaction

The scrotal sac normally keeps the testis at a temperature between 34 and 36^{o}C. At temperatures above 36^{o}C sperm production is greatly inhibited or stops completely (Lue et al., 1999; Guyton & Hall 2006). Hence, fever can inhibit spermatogenesis leading to a state of oligospermia which can appear within three weeks of the febrile reaction and last approximately two months (Lue et al., 1999; Barrett et al., 2010). A prolonged high testicular temperature may also explain infertility associated with varicoceles which involve the reflux of warm blood from the abdominal cavity to the testicular area. (Kandeel & Swerdloff 1988; Kantartzi et al., 2007). Some studies in experimental animals have shown that a small increase in testicular temperature accelerates apoptosis with spermatocytes and spermatids being most susceptible (Kandeel & Swerdloff 1988; Lue et al., 1999).

1.2.9.2 Exogenous factors

Exogenous factors, such as exposure to infective agents, ionising radiation or other environmental exposures such as atmospheric pollution, including pesticides, are mostly not controllable. A number of studies have considered the possible influence of environmental exposures to chemical and physical agents and lifestyle on sperm parameters and fertility

(Irvine 1998; Jurewicz et al., 2009) and reductions in semen quality, such as decreases in semen volume, sperm concentration, motility and normal morphology in relation to environmental exposures have been reported (Carlsen et al., 1992; Auger et al., 1995; Kidd et al., 2001; Rolland et al., 2013; Romero-Otero et al., 2015; Centola et al., 2016; Levine et al., 2017; Olesen et al., 2018; Tiegs et al., 2019).

Exogenous factors that are often termed lifestyle factors, include tobacco smoking, alcohol consumption and nutrition, one aspect of which is obesity. These exogenous factors are considered below.

1.2.9.2.1 Infection

A male urogenital tract infection and inflammation including orchitis and male accessory sex gland infections are among the most important causes of bacteriospermia and male infertility worldwide (Ibadin & Ibeh 2008) being associated with 8-35% of male infertility cases (Adler et al., 2004). Asymptomatic bacteriospermia may also play a major role in male fertility (Diemer et al., 2000; Ibadin & Ibeh 2008). Infective organisms include those responsible for leprosy, tuberculosis, mumps, and sexually transmitted diseases such as chlamydia, gonorrhoea and syphilis and can lead to disturbances in sperm production and/or excurrent ductal obstruction (Morley et al., 1977; Adamopoulos et al., 1978; Köhn et al., 1998; Adler et al., 2004). Viral orchitis, especially mumps, is a well-recognized cause of infertility, but is rare in prepubertal males. Mumps may lead to damage to the seminiferous tubules in patches. In most instances only one testis is severely affected, so that sperm production by the other tests may be sufficient to result in an ejaculate with an adequate sperm concentration (Adamopoulos et al., 1978; Köhn et al., 1998).

1.2.9.2.2 Ionising radiation

Ionising radiation, such as beta or gamma rays can destroy germ cells, especially spermatogonia, while Leydig cells are resistant because of their highly differentiated state (Howell & Shalet 2005; Osterberg et al., 2014). The damage is due to ionizations in DNA that can lead to mutations including lethal mutations that may result in the death of the affected germ cells leading in turn to azoospermia and infertility. A radiation dose in the range of 0.15 Gy may temporarily reduce sperm counts in the testes region, while 2 Gy may result in long-lasting or permanent azoospermia (Vamvakas et al., 1997; Jensen et al., 2006;).

1.2.9.2.3 Air pollution

Air pollution is generally defined as an increased air content of carbon monoxide (CO), nitrogen dioxide (NO_2) and other oxides of nitrogen (NO_X), sulfur dioxide (SO_2), ozone, lead and particulate matter (PM). PM is a mixture of different solid and liquid materials suspended in the air, and they are categorized based on size. Thus, PM_{10} has an aerodynamic diameter between 2.5 µm and 10 µm, and $PM_{2.5}$ has an aerodynamic diameter of less than or equal to 2.5 µm; these are also referred to as coarse and fine particles, respectively (DEFRA, 2011). The exact chemical composition of PM is highly variable depending on their sources and formation mechanisms leading to variations in composition, properties, and potential health impacts (Guttikunda 2008).

$PM_{2.5}$ is considered the most harmful pollutant due to its ability to penetrate deep into the lungs and enter the bloodstreams penetrating the brain and possibly causing DNA damage (Wu et al., 2015). $PM_{2.5}$ can carry trace elements and polycyclic aromatic hydrocarbons (PAHs), a group of compounds that include several endocrine disruptors and these might be able to reach different parts of the body due to their small size (Mi et al., 2001; Kado et al., 2005).

Jeng & Yu (2008) reported that PAH and other endocrine disruptors can affect both the hypothalamic-pituitary axis and testicular spermatogenesis and have the potential for causing sperm alterations in Fisher rats. Another study in rats confirmed the ability of environmental pollution (diesel engine exhaust) to impact on reproductive endocrine function (Watanabe & Oonuki 1999). While exposure to air pollution has been indeed linked to alterations in sperm quality (Adamopoulos et al., 1996; Sram 1999), the relationship between male infertility and pollution remains controversial (Carlsen et al., 1992; Forti & Serio 1993; Jensen et al., 2002; Santi et al., 2018). Nevertheless, it is accepted that human spermatogenesis is impaired by exposure to environmental chemicals such as dioxin, diesel exhaust, and pesticides during fetal life or adulthood (Sharpe & Skakkebaek, 1993; Sharpe 2010).

1.2.9.2.4 Alcohol consumption

Excessive and chronic consumption of alcohol harms spermatogenesis and testicular function through the reduction in serum testosterone, inhibition of gonadotropin secretion and the destruction of the seminiferous epithelium (Kucheria et al., 1985; Guthauser et al.,

2014). In chronic alcoholics, there is a marked reduction in sperm forward motility and an increase in abnormal sperm morphology (Gomathi et al., 1993; La Vignera el at., 2013). The common results of alcohol consumption include spermatogenic arrest and SCOS, and heavy drinkers (more than 80 g/day) may suffer from infertility (La Vignera el at., 2013). While Muthusami & Chinnaswamy (2005) reported a deleterious effect of heavy and chronic alcohol drinking on semen quality, the Chemicals and Pregnancy Study -UK (CHAPS-UK) group reported no correlations between alcohol consumption in the three months before semen analysis and low motile sperm concentration ($< 12\times10^6$/ml progressive motile sperm) (Povey et al., 2012) or sperm morphology (< 4% sperm with normal morphology) (Pacey et al., 2014).

1.2.9.2.5 Cigarette smoking

A study on a large population of men of infertile couples from Switzerland (655 smokers and 1131 non-smokers) showed significant differences in sperm concentration, total sperm count and the total number of motile sperms between smokers and non-smokers (Künzle et al., 2003). A more recent study on idiopathic infertile men (118 smokers and 153 non-smokers) from Italy, reported no differences in sperm parameters between smokers and non-smokers (Collodel et al., 2010). However, after dividing the smokers into mild, moderate and heavy smokers the results showed a significant decrease in sperm concentration in heavy smokers compared with mild smokers and non-smokers. On the other hand, other studies (Denissenko et al., 1996; Zenzes et al., 1999) did not find any effect of cigarette smoking on sperm parameters.

1.2.9.2.6 Nutrition

While testicular function is affected in cases of chronic starvation and malnutrition (Morley et al., 1977), a healthy diet is an important factor associated with good fertility outcomes and the consumption of certain foods has indeed been linked with lower sperm quality (Gaskins et al., 2012; Afeiche et al., 2013; Chiu et al., 2015; Oostingh et al, 2017). Thus Gaskins et al., (2012), demonstrated that a ‘prudent’ dietary pattern (one considered to protect against heart disease, stroke, and other common diseases and denoted by consumption of fruits, vegetables, whole grains, legumes, nuts, fish, and low-fat dairy products) correlated significantly with progressive sperm motility when compared to a ‘western’ diet consisting of processed and red meats, sweets and high energy drinks, pizza, refined grains and snacks.

In support of this, Jurewicz et al., (2018) reported that in a study of 336 men, that there was a positive correlation between the higher consumption of a prudent dietary pattern with higher sperm concentration and higher levels of testosterone (Jurewicz et al., 2018). Furthermore, Afeiche et al., (2013) studied the correlation between semen quality and dairy intake in 189 young men, and they found that a diet containing full-fat dairy foods (absent from vegan diets) adversely affected sperm progressive motility and normal sperm morphology.

In another study, Afeiche et al., (2014) reported in a cohort of 155 men that a diet rich in processed meats resulted in significantly fewer morphologically 'normal' sperm forms, between those in the highest and the lowest quartile of processed meat consumption. Increasing fish consumption was associated with higher total sperm count, rising from $102x10^6$/mL in the lowest quartile of fish consumption, to $168x10^6$/mL in the highest quartile along with higher numbers of morphologically 'normal' sperm forms. Maldonado-Cárceles et al., (2019) studied the association between the consumption of different meat types (red meats, white meats, and fish) with semen quality parameters and reproductive hormone levels in 206 men aged between 18–23 years. They found no correlation between total meat intake and semen quality, but there was a positive correlation between the consumption of shellfish and progressive sperm motility. In addition, men who consumed organ meats (offal) had significantly lower progressive sperm motility compared with men who did not consume this kind of meat (Maldonado-Cárceles et al., 2019).

Chavarro et al., (2010) investigated the effects of isoflavone phytoestrogens present in soya products, widely consumed as dairy alternatives amongst those following a vegan diet, on semen parameters. Higher consumption of foods containing soya and soya isoflavones resulted in $41x10^6$/mL less sperm than those who consumed no soya-containing foods. This is speculated to be in part due to phytoestrogens, which may interfere in the regulation of the HPG (hypothalamic-pituitary-gonadal) axis responsible for controlling reproductive functions including spermatogenesis. Murine models have demonstrated a 25% reduction in epididymal sperm counts in mice fed a high-phytoestrogen diet when compared to mice fed a low-phytoestrogen diet, in addition to a 21% reduction in litter size in the high-phytoestrogen diet group (Cederroth et al, 2010).

Oldereid et al., (1992) reported in a study of 252 men that there was no association between caffeine intake and semen quality and a study on a population of 2554 young Danish men

found that high caffeine intake (>800 mg/day) had no significant effect on sperm concentration. However, the same paper reported that high Cola consumption (>14×0.5L bottles/week) significantly decreased sperm concentration and total sperm count (Jensen et al., 2010). In rats, vitamin A and F deficiency may have an adverse effect in rat seminiferous epithelium, but in man, there is no adequate evidence for such a relationship (Morley et al., 1977; Greco et al., 2005).

1.2.9.2.7 Body mass index

Ma et al., (2018) reported that, in comparison with men with normal body mass index (BMI), being either underweight or overweight was significantly associated with a reduction in sperm concentration, total sperm number, and total motile sperm count, and the latter was significantly associated with a reduction in semen volume. Thus, overweight men aged ≥45 years were approximately 2.5 times more likely to have semen volume <1.5 ml compared with those of normal BMI. In the synergy between age and BMI, age provided a stronger correlation with semen volume than BMI while BMI provided a stronger correlation with sperm count and progressive motility (Abayomi et al., 2018). High BMI has been reported to have negative effects on sperm concentration, motility and normal sperm morphology (Oliveira et al., 2018), but in contrast, the CHAPS-UK group reported no correlations between BMI and the risk of having a low concentration of progressively motile sperm or low percent of normal sperm morphology (Povey et al., 2012; Pacey et al., 2014).

Pacey et al., (2014) reported there was no significant correlation between the percentage of sperm with normal morphology and BMI, type of underwear, smoking or alcohol consumption or having a history of mumps. In the context of BMI, a lack of physical activity has also been shown to have negative effects on fertility (Gaskins et al., 2015), but endurance exercise has also been correlated with a negative effect on sperm quality (Safarinejad et al., 2009). In contrast, Ibañez-Perez et al., (2019) investigated physical activity in relation to semen parameters in 454 men, and they found there was no association between different levels of general physical activity and semen parameters.

1.2.9.2.8 Pesticide and other chemical exposures

Perry (2008) reviewed thirteen studies and reported a significant association between exposure to different kinds of pesticides and semen quality. In addition, there was a significant association between impaired semen quality and occupational risk factors such

as exposure to heavy metals, fumes, and polycyclic aromatic hydrocarbons (PAH), although exposure to pesticides or cement did not reach significance (De Fleurian et al., 2009). Exposure to environmental toxicants is also a risk factor for infertility: consumption of pesticide-contaminated foods has been linked to lower numbers of morphologically normal sperm (Chiu et al., 2015).

Cherry et al., (2008) reported in 874 men that exposure to glycol ethers was related to low motile sperm count in men attending fertility clinics. The same group reported that occupational exposures to lead or glycol ethers was linked to a reduction in motile sperm count and normal sperm morphology (Cherry et al., 2014). Hovatta et al., (1998) reported the semen concentrations of cadmium and lead were low and did not show any correlation with semen quality, but that aluminium may be one of the environmental pollutants causing impaired semen quality.

Kumar et al., (2018), reviewed the potential adverse effects on the male reproductive system of exposure to occupational/environmental agents especially to some organic solvents, pesticides, metals, plasticizers such as phthalates; ionizing and non-ionizing radiations, and extreme heat. Swan et al., (2003) reported that fertile men in an agricultural area of Missouri had sperm counts about 40% lower and higher urinary concentrations of three types of pesticides than men in three urban US areas. In addition, Duty et al., (2003) reported that certain phthalate metabolites were significantly related to reduced semen quality.

Overall, most of these studies involved single semen samples and did not consider the possible correlation of environmental exposures and lifestyle factors, such as alcohol consumption, smoking, BMI, age and diet with semen parameters in the same man sampled repeatedly over a prolonged time period.

1.3 DNA damage in human sperm

1.3.1 Introduction

Fertilization involves the union of the paternal and maternal gamete genomes and as the purpose of spermatozoa is to transfer the genetic information of the father to the child, this should occur without any prior deterioration in DNA quality and quantity (Lewis & Simon 2010). Therefore, sperm DNA integrity is likely to be an important parameter of sperm quality in the prognosis of infertility and the outcome of assisted reproductive procedures

(Bungum et al., 2007; Sharif 2013). Based on this, a conventional semen analysis is insufficient as a diagnostic tool in male infertility, and sperm DNA integrity, which ideally should assess all possible types of DNA damage, should be considered as a complementary diagnostic tool and biological marker of male reproductive health and fertility (Chi et al., 2011).

The chromatin structure of human sperm has special features which are that it is highly organized and extremely condensed, and these characteristics probably provide protection to the sperm genome during transport through the male and female reproductive tracts (Evenson et al., 2002). Indeed, mammalian sperm DNA is the most tightly compacted eukaryotic DNA when compared with the DNA of somatic cells (Aitken et al., 2009; Vaamonde et al., 2016). Notably, in sperm most, histones are replaced by protamines 1 and 2 which facilitates this high order of chromatin packaging which is necessary for normal sperm function and may also be necessary for DNA silencing and imprinting changes within the sperm cell. Decreased concentrations of these protamines may cause defects in packaging and increase susceptibility to DNA damage (Carrell et al., 2007). Protamines 1 and 2 are usually expressed in nearly equal quantities but elevated or reduced protamine ratios are observed in some infertile men (Corzett et al., 2002), and is often associated with severe spermatogenesis defects (Sassone-Corsi, 2002). An important event which occurs during normal spermatogenesis is apoptosis which destroys almost 75% of potential spermatozoa (De Bont & Larebeke 2004; Sakkas et al., 2003). This is essential as Sertoli cells are only able to support a limited number of spermatozoa. It has been suggested that DNA damage (especially DNA strand breaks) in human sperm might result from abortive apoptosis (De Bont & Larebeke 2004; Sakkas et al., 1999), in which apoptosis is initiated in some spermatozoa but, for as yet unknown reasons, is not completed and the cells survive but with damaged DNA (Sakkas et al., 1999).

The structure of DNA purine and pyrimidine bases and the DNA sugar-phosphate backbone can be altered by exposures to chemicals and radiation resulting in DNA damage. DNA damage in the infertility literature, however, largely refers to damage to the DNA backbone and in particular to single-strand breaks (SSB) and double-strand breaks (DSB) (Baldi & Muratori 2014; Ribas-Maynou & Benet 2019). Another term used in the assessment of male fertility is DNA integrity, which is defined as the absence of SSB, DSB or nucleobase modifications in the DNA (Zini & Agarwal 2018). DNA damage can occur in both nuclear and mitochondrial DNA (Aitken & De Iuliis 2010) and at all possible stages during the

formation and transport of sperm cells (Sakkas & Alvarez 2010), although as mentioned earlier, some stages may be more susceptible than others. DNA fragmentation can be higher in the cauda epididymal and ejaculated sperm compared with testicular spermatozoa (Aitken & De Iuliis 2010; Lu et al., 2018), because the latter has the support of Sertoli cells and this tight association is thought to protect against exogenous damaging factors (Milanizadeh et al., 2018).

The origin and mechanisms responsible for sperm DNA damage are not yet fully understood (Latini et al., 2006; Aitken et al., 2009), but an increasing amount of data demonstrate an association between sperm DNA damage and fertility (Harris et al., 1985; Künzle et al., 2003; Baumgartner et al., 2009). Furthermore, several studies in ART have shown that the sperm DNA damage might have adverse effects on the fertilization potential of spermatozoa, the pregnancy rate and live birth rate (Aitken et al., 2009) and for the offspring (Vamvakas et al., 1997; Irvine 1998; Lewis & Simon 2010). Covalent modifications are known as DNA adducts because the normal bases are modified by the addition of a chemical moiety, and if left unrepaired, can produce a mutation or larger genetic change, block DNA replication or cause cell death (Aitken et al., 2009; Baumgartner et al., 2009). Some types of DNA modifications and their associated repair pathways are considered below.

1.3.1.1 Origins and products of oxidative DNA base damage

Oxidative damage to DNA is caused by exogenous free radicals induced by radiation or chemical agents, or endogenous free radicals such as those produced normally during cell respiration (Ollero et al., 2001; Kothari et al., 2010).

There are two kinds of free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS; Kothari et al., 2010). ROS include the superoxide anion (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-) and peroxyl radicals (HO_2-) and these agents can lead to oxidized bases as well as SSB and DSB (Pasqualotto et al., 2000; Kothari et al., 2010). RNS include nitric oxide (NO), nitrous oxide (N_2O), peroxynitrite (NO_3), nitroxyl anion (HNO), and peroxynitrous acid (HNO_3) (Kothari et al., 2010).

Endogenous sources of ROS include round cells with cytoplasmic retention, and leukocytes (neutrophils and macrophages) and these have both been related to increased sperm DNA damage (Gomez et al., 1996; Erenpreiss et al., 2002; Fischer et al., 2003). ROS formation occurs during cellular respiration when 1-5% of oxygen is transformed into superoxide and

then H_2O_2 (Kothari et al., 2010), and this, in turn, is acted upon by catalase and glutathione peroxidase to prevent cellular damage. ROS also have functions in controlling sperm maturation, capacitation, hyperactivation, acrosomal reaction, and sperm-oocyte fusion (Ollero et al., 2001; Kothari et al., 2010). However, the seminal fluid of most infertile men has high levels of ROS (Pasqualotto et al., 2000; Moein et al., 2007).

Exogenous sources of ROS include industrial compounds e.g. phthalates and heavy metals such as lead, and also pesticides, sulfur dioxide, food preservatives, smoking, and alcohol (Kothari et al., 2010). ROS damages the sperm membrane through lipid peroxidation, reducing both sperm motility and sperm-oocyte binding, and also resulting in a loss of ATP which reduces sperm viability (Tremellen, 2008; Agarwal et al., 2014). In addition, increased ROS levels in human semen have been associated with DNA damage (Pasqualotto et al., 2000) and a positive correlation was reported between sperm DNA fragmentation and ROS (Agarwal et al., 2014).

The C4, C5 and C8 positions of guanine are the principal nucleobase targets of OH· -induced DNA damage, the latter reaction producing a C8-OH adduct radical which can end up as either 7, 8-dihydro-8 oxoguanine (8-oxoG) or 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (Fapyguanine) (De Bont & Larebeke 2004; Kothari et al., 2010; Homa et al., 2015). Although neither Fapyguanine nor 8-oxoG significantly block DNA replication, they can form base pairs with adenine which subsequently can lead to GC → TA transversion mutations (Kalam et al., NAR 2006) and are therefore considered promutagenic (Slupphaug et al., 2003). On account of its abundance and promutagenic effect, 8-oxoG is now frequently used as a biomarker for assessing oxidative damage in cell and sperm DNA.

Another type of DNA lesion that can be formed by oxidative stress are etheno-DNA adducts. These maybe a consequence of the sperm membrane containing unsaturated fatty acid residues of phospholipids which upon ROS exposure can result in aldehyde formation (Bartsch & Nair 2004). For example, malondialdehyde produces exocyclic adducts, such as 1, N6-ethenoadenosine and 1, N2-ethenoguanosine (Doerge al., 2000; Badouard et al., 2008; Loeb & Harris 2008), and these have been detected in human sperm DNA. These lesions can lead to transversion mutations and therefore they are classified as mutagenic adducts (Doerge et al., 2000). It has been reported that the level of ethenoguanosine adducts in sperm DNA was similar to that observed in the same man's leukocytes while the level of ethenoadenosine was lower (Doerge et al., 2000; Badouard et al., 2008).

1.3.1.1.1 Repair of oxidative DNA damage

The main repair pathway for DNA base modifications caused by ROS is base excision repair (BER), which also repairs DNA base modifications caused by alkylation (see below), deamination or hydroxylation. There are two BER pathways, short patch and long patch (Kothari et al., 2010). Both of these have four main steps which are: I) recognition and cleavage of the N-glycosidic bond of the damaged base to form an apurinic or apyrimidinic (AP) site, II) cleavage, with resection in long patch repair, of the DNA backbone at the AP site to form a nucleotide gap, III) use of the complementary strand as a template to fill the gap and finally IV) ligation to seal the DNA backbone (Nilsen & Krokan 2001; Slupphaug et al., 2003).

Other pathways can also contribute to repairing oxidative DNA damage including transcription-coupled repair (TCR) and global genome repair (GGR), which are both aspects of the Nucleotide Excision Repair (NER) system, mismatch repair (MMR), translesion synthesis (TLS), homologous recombination (HR) and non-homologous end-joining (NHEJ). NER is the principle mechanism by which the cell recognises bulky deformations in the DNA double helix such as damage induced in DNA by polycyclic aromatic hydrocarbons (PAH) (De Laat et al.,1999; Stojic et al., 2004). After the recognition of the damage, a short single strand that includes the lesion is removed creating a gap that is subsequently filled using the intact strand as a template, as in long patch BER.

MMR corrects the damage by DNA polymerases that escape proofreading activity during DNA replication but it also involved in the repair of some DNA base damage. The recognition of damage is mediated mainly by the heterodimer of the proteins, MSH2 and MSH6 which is called MutSα. In some cases of extra helical loops, another heterodimer of MSH2 and MSH3 (MutSß) can recognize the damage and initiate the repair. After error recognition, MutSα can attract another heterodimer, which consists of MLH1 and PMS2, forming a complex which can translocate in either direction on the newly synthesized strand searching for gaps between Okazaki fragments on the lagging strand, or the free 3'-terminus on the leading strand (Stojic et al.,2004). TLS is a system whereby lesions that block the progress of the normal DNA replication polymerase are by-passed by another polymerase, often leading to misincorporation of bases, but enabling cell survival. HR and NHEJ are the main pathways for the repair of DNA strand breaks and are discussed in that context later.

1.3.1.2 Origins and products of DNA alkylation damage

Alkylating agents are ubiquitous and they arise from exogenous sources such as tobacco smoke, occupational and environmental pollution, diet and anti-cancer drugs such as temozolomide (TMZ) and procarbazine (Povey 2000). The main exogenous alkylating agents are the N-nitroso compounds including nitrosamines, nitrosoureas, and nitrosoguanidines (Bartsch 1984). The contribution of N-nitroso compounds to population exposure is suggested to be 72% from the diet, including processed meat, smoked fish, cheese and beer (Park et al., 2015), 25% from occupational exposure, for example the rubber industry, metalworking, leather tanning, and fish or meat curing (Bartsch & Spiegelhalder 1996), 2% from cigarette smoking and 1% from miscellaneous minor sources, including pharmaceutical products, cosmetics and indoor and outdoor air (Tricker 1997).

The formation of N-nitroso compounds in food occurs by the reaction between nitrogen oxides or nitrous acid and amino compounds in food and the main sources of nitrogen oxides are (I) the addition of nitrite and/or nitrate to food, such as meat, as a preservative; (II) the heating and/or drying food in combustion gases in which molecular nitrogen is oxidized to nitrogen oxides (Tricker & Preussmann 1991).

Alkylating agents can also be generated within the body during metabolism (Beranek 1990; Drabløs et al., 2004) for example S-adenosylmethionine (SAM), which plays an important role in physiological DNA methylation by donating a methyl group (Beranek 1990; De Bont & Larebeke 2004). Alkylating agents can react with DNA at the 4 exocyclic oxygen sites and 7 ring nitrogen atoms of the nucleobases, or the phosphate groups producing 2 possible phosphotriesters (Drabløs et al., 2004).

The simplest and most researched alkyl group is methyl, but 'alkyl' is any substituted methyl group and includes straight chain (e.g. ethyl, propyl, butyl) or branched chain (e.g *iso*propyl), cyclic (e.g. benzyl) or heterocyclic (e.g thenyl) compounds. The DNA adducts formed by alkylation of exocyclic oxygen atoms include O^6-alkylguanine (O^6-alkylG), O^2-alkylthymine, O^4-alkylthymine and O^2-alkycytosine. DNA alkyl adducts due to modification of the ring nitrogen are N1-alkyladenine, N3-alkyladenine, N7-alkyladenine, N3-alkylcytosine, N3-alkylguanine, N7-alkylguanine (N7-alkylG) and N3-alkylthymine (Saffhill et al., 1985; Beranek 1990). The chemical structure and hence reactivity of the alkylating agent reacting with the DNA nucleophilic target determines the relative amounts

of adducts formed in DNA. For the methylating agent, TMZ, this ranges from 70% for the major adduct N7-methylguanine (N7-MeG) to 12% for methylphosphotriesters to 6% for O^6-methylguanine (O^6-MeG) to less than 0.01% for O^4-methylthymine, whereas for an ethylating agent such as ethylnitrosourea, 60% of the adducts are phosphotriesters (Saffhill et al., 1985; Beranek 1990).

The carcinogenicity, mutagenicity and cytotoxicity of alkylating agents has been known for many decades. However, the contribution of DNA adducts to these biological effects has been more difficult to establish, especially for the very minor lesions. By far the most well studied adducts are at the O^6 and N7 positions of guanine, and these have used a range of *in vitro* biochemical, bacterial, mammalian cell culture and animal models (Beranek 1990; Povey et al., 2000; Loeb & Harris 2008).

It has thus been found that N7-MeG is not directly mutagenic or cytotoxic, but loss of this lesion from DNA by spontaneous depurination gives rise to apurinic sites that can be mutagenic and toxic, in some cases via the formation of DNA strand breaks (Saffhill et al., 1985; Povey et al., 2000; Margison et al., 2002; Shrivastav et al., 2009).

On the other hand, O^6-alkylguanine adducts are considered to be the most mutagenic DNA damage, because of their ability to pair with thymine instead of cytosine during DNA replication. Thus, adducts such as O^6-MeG, O^6-n-propylG, O^6-n-butylG and O^6-n-octylguanine (Baumgart et al.,1993) induce GC:AT transition mutations after 2 rounds of replication of DNA containing the lesion (Saffhill et al., 1985; Ellison et al.,1989).

One hypothesis proposes that the mis-insertion of thymine opposite O^6-alkylG by DNA polymerase results in the formation of O^6-alkylG:T pairs that have more stable hydrogen-bonded structures (Singh et al.,1996; Woodside & Guengerich 2002). However, this assumption was contradicted by Gaffney et al., (1984) and later crystallographic studies (Eoff et al.,2007) reporting that O^6-alkylG:T mispairs are not more stable than their O^6-alkylG:C counterparts. In addition to its ability to induce point mutations O^6-MeG also causes recombination events in DNA that could potentially contribute to cell death and malignant transformation. This depends on the action of the MMR system on O^6-MeG:T mispairs, resulting in a futile cycle of removal then reincorporation of T with an associated SSB that may result in a DSB upon further DNA replication. The processing of these breaks

may cause deletions and/or translocations that could lead to loss of a tumour suppressor gene or activation of an oncogene (Margison et al., 2002).

In spite of the possible effects of alkyl-DNA adducts on fertility, there is no published data on the level of O^6-alkylG adducts in human sperm DNA and there is only a single study that investigated the level of N7-MeG in sperm DNA from IVF couples (Stocks et al., 2010), in this study reported a significant negative correlation between N7-MeG level and semen quality and the percentage of oocytes successfully fertilized and N7-MeG levels were significantly higher in men with male factor infertility than in the men with unexplained infertility, this study also showed a significant negative correlation between age and N7-MeG levels in DNA sperm.

1.3.1.2.1 Methods for quantitation of O^6-alkylguanines in DNA

O^6-MeG in DNA can be detected by antibody-based techniques that depend on generating highly specific monoclonal or polyclonal antibodies to the adduct. These can then be used for quantitation using a variety of techniques such as Immuno-slot-blot (ISB) (Povey et al., 2000), immunohistochemistry (Lewin et al., 2006), radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) methodology (Cupid et al., 2004).

Another technique is ^{32}P-post labelling, in which the adduct is detected via the transfer of ^{32}P from ATP to deoxyribonucleotides, obtained by enzymic degradation of DNA, followed by separation and analysis of labelled adducts by thin-layer chromatography (TLC) and autoradiography. This technique has been used widely for O^6-MeG adducts and has a high sensitivity, detecting 1 adduct in 10^{10} normal bases in <10 μg of DNA (Phillips & Arlt 2007; Farmer & Singh, 2008; Klaene et al., 2013; Balbo et al., 2014). It has also been a common method to screen for any adducts in human DNA, but without providing information on the specific chemical structure of the detected DNA adducts.

Mass spectrometry (MS) based assays are being used for detection and quantification, as well as structural identification of O^6-MeG adducts (Kotandeniya et al., 2011). MS is an analytical technique that resolves ions based on their mass to charge ratio, the initial step requiring the alteration of analyte molecules to gas-phase ions (Da Pieve et al., 2013). MS-based assays can detect the O^6-MeG in the base or 2'-deoxynucleoside form and have enabled the identification and quantification of O^6-MeG and O^6- carboxymethylguanine (O^6-CMG) in DNA (Tan et al., 2011; Da Pieve et al., 2013). In addition, ultra-high-performance

liquid chromatography-tandem MS(UHPLC-MS/MS) has been used to quantify O^6-MeG and O^6-CMG in an intestinal cell line DNA (Bussche et al., 2012). However, the sensitivity of the method was not high enough for the analysis of DNA extracted from human tissue (Bussche et al., 2012; Moore et al., 2010). Indeed, only a few studies have been able to detect and/or quantify O^6-MeG adducts in human DNA and these involved colorectal tissue (Povey et al., 2001; Lewin et al., 2006).

1.3.1.2.2 Repair DNA alkylation damage in DNA

In addition to some types of oxidative damage (see 1.3.1.1), short and long patch BER deal with the repair of certain alkyl purine adducts and their imidazole ring-opened breakdown products and this is initiated by the action of base-specific glycosylases or lyases (Jackson et al., 1996; Povey et al., 2000). However, of most relevance to this thesis is the repair of O^6-alkylG adducts by alkyltransferase and alkyltransferase-like proteins.

1.3.1.2.3 O^6-methylguanine-DNA methyltransferase (MGMT)

MGMT, also known as AGT and ATase, is a DNA repair protein that protects against the mutagenic, cytotoxic and carcinogenic effects of O^6-MeG and probably most other O^6-alkylG adducts in DNA (Xu-welliver & Pegg 2002). MGMT functions by the transfer of the alkyl group from the O^6 position of the modified guanine base to a cysteine residue within the MGMT active site pocket in an auto inactivating and stoichiometric process that results in regeneration of the guanine base; as shown in Figure 1.1.

As repair is irreversible, MGMT is known as a ‘suicide enzyme’. MGMT inactivation results in its degradation through ubiquitination pathways by the proteasome (Xu-welliver & Pegg 2002). Hence, the cellular capacity to withstand or avoid the deleterious effects of O^6-alkylG formation in DNA is directly related to both the total number of active MGMT molecules and the rate of its de-novo synthesis (Pegg et al., 1995). Increased MGMT activity increases cellular resistance to alkylating agents genotoxicity whilst depletion of MGMT renders the cell more susceptible. MGMT is thus also a marker of resistance of normal and cancer cells to alkylating chemotherapeutics (Kaina et al., 2007). MGMT has been discovered in 100 different species including archaea, prokaryotes as and eukaryotes. The human version is named MGMT, but in general the protein is often referred to as alkylguanine-DNA alkyltransferase (AGT).

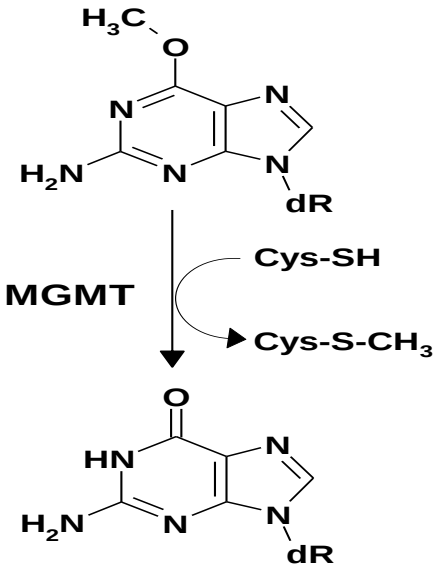


Figure 1.1 The MGMT repair mechanism

MGMT transfers the methyl group from O^6MeG deoxyribonucleosides (upper structure) in DNA to its active site cysteine (Cys-SH) residue to generate S-methylcysteine (Cys-S-CH_3), thus inactivating the MGMT and restoring the guanine deoxyribonucleoside (lower structure). MGMT is also known to repair a wide range of other O^6-alkylguanines in DNA in the same way.

AGTs have shown reactivity towards both duplex and single-stranded DNA substrates; however, repair rates seem to be slower with single strand substrates than duplex (Fried et al., 1996).

It has been suggested that DNA conformational change promotes the interaction between MGMT molecules, resulting in cooperative DNA binding, which is proposed as the main mechanism for the repair of single-stranded DNA (Fried et al., 1996; Tessmer & Fried 2014).

1.3.1.2.4 AGT-like proteins

Alkyltransferase-like (ATL) proteins constitute a repair pathway in many prokaryotic and lower eukaryotic organisms that protects cells against the genotoxic effects specifically of O^6-alkylG in DNA (Margison et al., 2007). ATL proteins differ from AGT proteins in that their alkyl acceptor site contains either tryptophan or alanine but not cysteine and they therefore lack AGT activity, but they bind strongly to a wide range of O^6-alkylG adducts in DNA (Margison et al., 2003; Pearson et al., 2006; Margison et al., 2007).

ATL proteins lack, glycosylase, or endonuclease activities (Margison et al., 2007) but act as signal proteins that bind to O^6-alkyl modified guanines causing nucleotide flipping and

bending of the DNA extensively by 45°, and forming a complex that is recognized by the NER pathway proteins, resulting in the repair of the damage. In those organisms that possess both ATL and AGT proteins, ATL is thought to be responsible for the recognition of bulky O^6-alkyl adducts that are not good substrates for AGT.

ATL proteins are principally found in bacteria and fungi and the most characterized fungal ATL is from the fission yeast, Schizosaccharomyces pombe and is known as Atl1, (Pearson et al., 2006). The very high affinity of Atl1 for O^6-alkylG-DNA adducts has been exploited in developing one of the methods used in this thesis (Chapter 4).

1.3.1.3 Polycyclic aromatic hydrocarbon (PAH) induced DNA damage

PAHs are found in cigarette smoke, air pollution ($PM_{2.5}$), and certain foodstuffs and as a result of combustion (Harris et al., 1985; Kriek et al., 1998). PAH can form adducts in human DNA including sperm DNA. The formation of PAH-DNA adducts in spermatozoa is a potential source of transmissible prezygotic DNA damage because the sperm has no repair mechanism (Harris et al., 1985; Pacey 2010).

One example of PAH is Benzo (a) pyrene which is a highly mutagenic and carcinogenic PAH. It is metabolized to a diol epoxide, diol epoxide-1 (DE-I), which binds to the 2-amino group of guanines to form the BPDE-I-dG–DNA adduct (Gaspari et al., 2003; Kriek et al., 1998). A study on 182 men observed a higher level of PAH-DNA adducts in sperm of infertile men compared to fertile men and the level of PAH-DNA adducts was positively associated with occupational exposure and alcohol drinking but not tobacco smoking (Gaspari et al., 2003). Another study found a significantly higher PAH-DNA adduct level in 94 men with male factor infertility compared to 85 fertile men and a negative correlation between PAH-DNA adduct level and sperm concentration and motility (Zenzes et al., 1999).

Interestingly, PAH adducts have been detected in preimplantation embryos whose fathers smoked, which may suggest that these adducts may be inherited from the paternal sperm to embryo stage (Zenzes et al., 1999). This study also reported that in infertile men PAH-DNA adduct levels were correlated with tobacco smoking but not with alcohol or coffee consumption. A study on 433 men from infertility clinics found a negative association between PAH-DNA adducts and sperm concentration, total sperm number and sperm motility (Denissenko et al., 1996; Pacey 2010). As previously mentioned, PAH adducts are repaired by the NER system.

1.3.1.4 Origin of DNA strand breaks

DNA strand breaks are most commonly formed when the bond between a deoxyribose residue and the phosphate group is broken, resulting in an SSB. When SSB occur in close proximity in opposite DNA strands, this can result in a DSB if the number of intervening nucleotides is not sufficient to hold the two strands together (Eastman & Barry 1992; Vamvakas et al.,1997; Loeb & Harris 2008).

SSB and DSB can be induced during normal cellular processes such as DNA repair (both BER and NER), apoptosis, replication, and recombination and by agents such as ROS, ionizing radiation, chemotherapy drugs, and aromatic compounds (Povey et al.,2000; Stojic et al.,2004).

Hence alkylating agents, for example, those that are used as anticancer drugs, such as bleomycin and TMZ can induce DNA strand breaks (Vamvakas et al.,1997). SSB are not considered dangerous since they can be repaired by using the complementary DNA strand as a template, although studies have reported that SSB may allow the prediction of fertilization potential (Friedberg 2003; Ribas-Maynou et al., 2012 a). DSB are considered one of the most serious types of DNA damage, and may drive the cells to die or mutate (Kaina 2003) and indeed miscarriage has been associated with increasing levels of DSB in sperm DNA (Ribas-Maynou et al., 2012).

There are a number of techniques for the detection and quantitation of SSB and DSB in human sperm and somatic cells. These include the neutral and alkaline Comet assays, the sperm chromatin structure assay (SCASA) and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Friedberg 2003; Ribas-Maynou et al., 2012a): these are described in detail later.

1.3.1.4.1 Repair of DNA strand breaks

SSB are usually easily repaired because the opposite strand of the double helix holds the two ends in close proximity so that if the SSB are simple breaks in the phosphodiester backbone of the DNA, the phosphate and the hydroxyl group can be directly religated (Friedberg 2003). Other break types can be more complex, involving changes in the structure of the terminal deoxyribose residues, and requiring the action of specific repair proteins to produce religatable ends.

There are two main mechanisms involved in repairing DSB namely, HR and NHEJ. As its name implies, HR requires the homologous DNA sequence for repair and this is supplied by the sister chromatid, and takes place very soon after DNA replication when the chromatids are in close proximity. HR is considered to be error-free and is the main pathway for repairing DSB during S-phase and the G2 phase of the cell cycle. In HR there are two major pathways; (I) the DSB repair pathway and (II) the synthesis-dependent strand annealing pathway (Friedberg 2003; Sung & Klein 2006). NHEJ is considered error-prone and occurs usually through the G1 phase of the cell cycle without requiring an intact homologous sequence (Hefferin & Tomkinson 2005; Sung & Klein 2006). NHEJ simply brings the broken ends together and then joins them. A number of proteins contribute to NHEJ including a heterodimer of Ku proteins (Ku70 and Ku80) which binds to the free ends of the DSB to block exonucleolytic degradation. The Ku heterodimer then enrols DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) to form a DNA-dependent kinase complex which in turn recruits DNA ligase IV and its related factors, XRCC4 and polynucleotide kinase. This complex enables the gaps to be repaired by DNA polymerases and the ends to be ligated (Hefferin & Tomkinson 2005).

1.3.1.4.2 Techniques used for analysing DNA integrity in sperm

A number of methods are used for determining the integrity of human sperm DNA, each of which can detect specific aspects of the damage varying from DNA adducts to strand breaks (Gaspari et al., 2003; Ribas-Maynou et al., 2014). Many techniques use DSB and SSB as indicators of DNA integrity as shown in Table 1.3. Currently, the Comet, SCSA and TUNEL assays are the most commonly used assays for measuring DNA fragmentation in sperm (Evenson & Wixon 2006).

Table 1.3: Comparison between techniques to estimate DNA integrity in sperm

Technique	Reference	Measured damage	Detection method	Sample required	Advantages	Limitations
Comet assay	(Ostling & Johanson 1984)	1-DSB under neutral conditions 2-SSB and DSB and alkali labile sites under alkaline conditions	Fluorescence microscopy	1000 to 5000 sperm	1-Very sensitive technique 2- Detects DNA damage in individual cells quantitatively 3- Few cells are required	Alkali labile sites, which are abundant in mammalian cells, can cause over estimate when alkaline Comet assay is used.
Sperm chromatin structure assay (SCSA)	(Evenson et al., 1980)	Percentage of sperm with or without fragmented DNA	Flow cytometry	1 or 2 × 10^6 sperm	1- Less time consuming than the other techniques 2- DNA fragmentation index (DFI) determined by SCSA is a stable parameter overtime	Does not determine the extent of DNA damage in individual sperm.
Acridine orange test (AOT)	(Tejada et al., 1984)	Percentage of sperm containing SSB or DSB	Fluorescence microscopy	0.1- 0.5 ml of the semen sample. 300 cells are analysed.	Inexpensive and easy to perform	1- Rapidly fading fluorescence 2- Non-specific colour 3- Heterogeneous slide staining
TdT-mediated dUTP nick-end labeling (TUNEL)	(Gavrieli et al., 1992)	Percentage of sperm containing SSB and/or DSB	Flow cytometry/ fluorescence microscopy	50 µl of sperm suspension for ART 500 cells sample are observed	1- Flow cytometry increases the sensitivity and reproducibility of the assay 2-Sperm DNA fragmentation determined by TUNEL is a stable parameter over time	1- Estimates the number of cells with DNA damage without quantifying the level of damage 2- Light microscopy decreases the efficiency of the assay 3- Expensive
Sperm chromatin dispersion (SCD)	(Fernández et al., 2005)	Percentage of sperm with or without fragmented DNA	Fluorescence microscopy	0.2ml of the semen sample	Inexpensive and easy to perform	1- New technique and its clinical importance is not proven yet 2- Not accurate in determining the extent of DNA damage

1.3.1.4.2.1 Comet assay

The Comet assay, which is also known as single-cell gel electrophoresis, is a technique for quantitating DNA damage and also DNA repair in cell and tissue samples both *in vivo* and *in vitro*. This assay analyses DNA damage in individual cells and was introduced by Ostling & Johanson in 1984.

It is most commonly applied to animal cells but has also been used to examine the damage in the DNA of plant cells (Collins et al., 1997; Collins et al., 2008): it is both simple and sensitive (Wong et al., 2005; Lovell & Omori 2008) and is able to detect very low levels of DNA damage in small cell numbers including in sperm (Tice et al., 2000; Wong et al., 2005; Baumgartner et al., 2009).

The main principle of the Comet assay is the nuclear decompaction and dissociation of cellular DNA from nuclear components using a lysis solution containing high salt, leaving the DNA strands attached only to the nuclear matrix. This is followed by electrophoresis using a neutral or alkaline pH buffer, depending on the purpose of the test, during which the unattached DNA loops or fragments migrate away from the area of nucleus. Imaging of individual cells uses dyes such as ethidium bromide, SYBR gold or YOYO and visualization uses fluorescence microscopy (Duty et al., 2002; Simon et al., 2011; Ribas-Maynou et al., 2012).

In a typical Comet image, the head contains intact DNA and the tail contains damaged or broken fragments of DNA, as shown in Figure 1.2, (Collins et al., 2008; Lovell & Omori 2008). A unique characteristic of the Comet assay is its ability to distinguish between single and double-stranded DNA breaks when it is performed under alkaline or neutral conditions respectively (Collins et al., 1997), although there are some studies suggesting that the neutral Comet assay measures both double and single-strand DNA breaks (Collins et al., 2008).

However, the alkaline Comet assay also detects alkali labile sites which are defined as DNA structures that are highly sensitive to lysis by incubation in alkaline solution (Collins et al., 1997; Tice et al., 2000; Cortés-Gutiérrez et al.,2009), hence the alkaline Comet assay does not assess SSB plus DSB exclusively.

One of the main advantages of the Comet assay is that it requires only a small number of cells, hence is suitable for the assessment of oligospermia samples that have low sperm count

or small samples left over from clinical use (Hughes et al., 1996; Lewis et al., 2004; Mohammad et al., 2005).

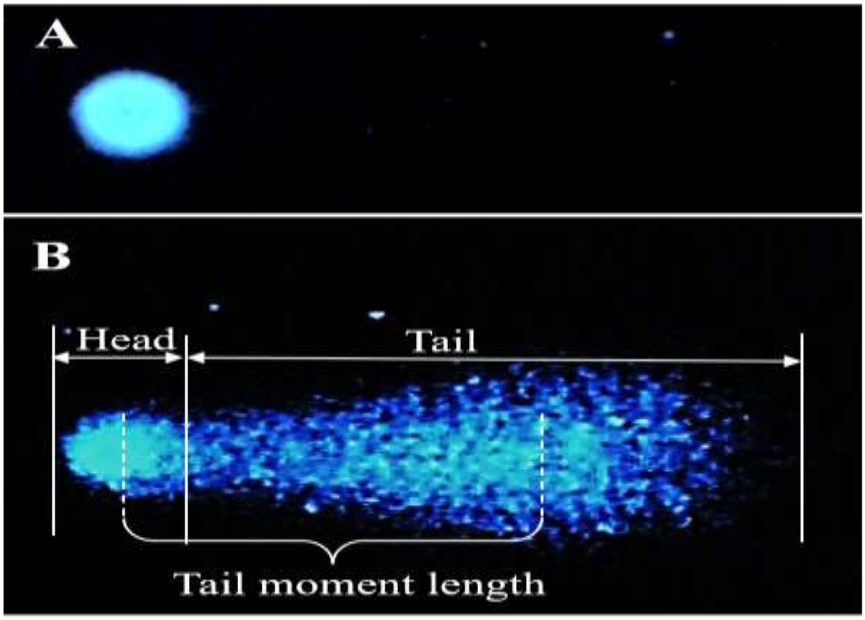


Figure 1.2 Typical neutral Comet images of A: an untreated control cell and B: an H_2O_2 damaged cell.

Algorithms in the Comet software packages define the head and tail regions, determine the DNA content, and calculate the % of DNA in both. The tail moment length (unit; um) is the distance between the centres of gravity of the head and the tail. The Centre of gravity is defined as the midpoint at which there is the same number of pixels either side along the x-axis (Kumaravel et al., 2009). Magnification 20X.

A large number of parameters have been determined from Comet images, and some of these and their definitions are summarised in Table 1.4. Although there is no clear agreement about the best way to analyse Comets to quantify DNA damage (Tice et al., 2000; Wong et al., 2005), the most frequently used measurements are tail length, Comet length, % DNA in the tail, % DNA in the head, tail moment and Olive tail moment. Some studies have shown the reproducibility of some of these parameters (Tomsu et al., 2002; Simon et al., 2010). The primary endpoint as recommended at the International Workshop on Genotoxicity Test Procedures in 2005 is the % DNA in the tail (Lovell & Omori 2008). Tail length is considered to be sensitive to the background intensity, which would affect the precise location of the end of the tail (Bowden et al., 2003; Lovell & Omori 2008). The Comet assay has also been modified to detect oxidative DNA damage. The principle is based on the use of specific enzymes to remove oxidatively damaged bases, and cleave the DNA. The resulting increase in the abundance of strand breaks can then be attributed to the level of

damage (Collins et al., 1997; Smith et al., 2006). Thus, Collins et al., (1996) detected oxidative DNA damage in Human peripheral lymphocytes by using the enzyme endonuclease III (EndoIII) to convert oxidized pyrimidines to strand breaks (Smith et al., 2006). In related studies, there was a dose-dependent increase in DNA damage after exposure of HeLa cells to 0-100 μM H_2O_2 but no increase in control, untreated, cells following incubation with EndoIII, (Collins et al., 1997; Smith et al., 2006).

Table 1.4: Most common Comet assay parameters

Comet parameter	Definition
Comet length	Total length of the Comet
Head length	Distance between the start point of the head and start point of the tail.
Tail length	Distance between the start point of the tail and the end of the tail.
%DNA in head and tail	Percentage of total integrated fluorescence intensity in the head and in the tail
Tail moment	Distance between the centres of gravity of the head and the tail (see Fig 1.2)
Extent tail moment	Percentage of DNA in the tail multiplied by the tail length
Olive tail moment	Percentage of DNA in the tail DNA multiplied by the tail moment length
Tail intensity	Summation of all pixel intensity values in the Comet tail.

Other enzymes have also been applied in the modified Comet assay, such as formamidopyrimidine glycosylase (Fpg) (Collins et al., 1997; Collins et al., 2008), which recognizes oxidized purines including 7,8-dihydro-8-oxoguanine (8-oxoG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (FapyA); and human 8-hydroxyguanine DNA-glycosylase 1 (hOGG1) which specifically recognizes 8-oxoG and 2,6-diamino-4-hydroxy-5-N methylformamido-pyrimidine (methyl-Fapy-G) (Collins et al., 1997; Collins et al., 2008).

1.3.1.4.2.1.1 Comet assay and male fertility

A number of researchers have tried to determine if there is an association between DNA damage as measured by the Comet assay and infertility status. Most of these studies have reported significantly higher DNA damage in men attending infertility clinics in contrast to healthy volunteers (Irvin et al., 2000; Schmid et al., 2003; Singh et al., 2003). Some of these studies used both alkaline and neutral Comet assay (Ribas-Maynou et al., 2012), while others

utilized the neutral Comet assay (Duty et al., 2003a; Singh et al., 2003; Schmid et al., 2007). Using the alkaline Comet assay, it was reported that the number of sperms with high DNA damage levels was significantly higher in men from infertility clinics (Irvine et al., 2000; Simon et al., 2010; Simon et al., 2013).

It has also been reported that % tail DNA and/or Olive tail moment were significantly higher in infertile men (Singh et al., 2003). However, other researchers using the alkaline Comet assay reported that the % DNA in the tail was almost the same in 20 normozoospermic and 20 asthenospermic infertile men and 20 normozoospermic fertile men (Hughes et al., 1996). Interestingly, this study showed also showed that the sperm DNA of asthenospermic infertile men was more susceptible to damage by H_2O_2 or X rays than DNA of normozoospermic infertile men, which in turn was more susceptible than normozoospermic fertile men. This difference in susceptibility might explain the finding in one study (Irvine et al., 2000) that there was a difference in DNA damage but not ROS production or total seminal antioxidant level between normal donors and IVF patients.

Many such studies did not find an association between fertilization percentage and sperm DNA damage in men of both IVF and ICSI couples (Simon et al., 2011; Simon et al., 2013). On the other hand, a relationship between embryo quality and sperm DNA damage has been observed (Simon et al., 2013). Only one group has reported a significant negative impact of DNA fragmentation in neat and prepared sperm, as measured by the alkaline Comet assay, on pregnancy and live birth in IVF couples but not in ICSI couples (Lewis et al., 2004; Simon et al., 2013).

Two studies that used the neutral Comet assay did not find a correlation between % fertilization and sperm DNA damage in men of either IVF or ICSI couples (Morris, et al., 2002; Chi et al., 2011), although the number of participants was small in both of these studies and one of them also used frozen neat sperm samples to measure DNA damage levels (Morris, et al., 2002; Chi et al., 2011). However, using the Comet assay to measure DNA damage in cryopreserved sperm has been reported as being reliable (Duty et al., 2002; Jiang et al., 2006). Ribas-Maynou et al., (2012) used both the alkaline and neutral Comet assay and showed more extensive DNA strand breaks in Asthenoteratozoospermic (ATZ) patients with or without varicocele and Oligoasthenoteratozoospermic (OATZ) patients in comparison with normal fertile donors.

1.3.1.4.2.1.2 Comet assay and lifestyle and environmental exposures

No significant correlation was found between cigarette smoking and DNA damage as assessed by neutral or alkaline Comet assay in the sperm of men attending infertility clinics (Duty et al., 2003a; Ribas-Maynou et al., 2012). Other studies investigated the effect of some environmental estrogenic substances including β-Estradiol, diethylstilbestrol, and nonylphenol and reported that these compounds were associated with an increase in DNA damage measured by the alkaline Comet assay (Irvine et al., 2000; Bennetts et al., 2008). Başaran et al., (2019) showed there was no significant impact on sperm DNA damage occupationally exposed boron workers. On other hand, it has been reported that sperm DNA might be more sensitive than peripheral lymphocytes DNA to damage induced by ethylene glycol monomethyl ether (Anderson et al., 1997).

Both the alkaline and the neutral Comet assay have been used to study the relation between BMI and sperm DNA damage (Lewis et al., 2004; Mohammad et al., 2005). Both studies reported elevated DNA damage in obese men (BMI ≥ 30) but not in overweight men (BMI ≥ 25) compared to men with normal BMI < 25. Additionally, one study reported that there was no correlation between high dietary intake of vitamin C, vitamin E, folate or zinc (an anti-oxidant factor) and sperm DNA damage in older men (>44 years) (Hughes et al., 1996).

1.3.1.4.2.1.3 Comet assay and sperm parameters

Morris, et al., (2002) found a negative relationship between Comet tail moment and sperm concentration and morphology. Another study reported negative associations between DSB and sperm motility and morphology but not sperm concentration (Chi et al., 2011) and a third study noted that the number of sperms with high levels of DSB (Comet length> 300μm) was associated negatively with sperm parameters (Trisini et al., 2004). While Duydu et al., (2012) reported a statistically significant correlation between DNA-strand breaks and motility/morphology parameters of sperm samples, Schmid et al., (2007) reported there was no significant association between sperm parameters and the DNA damage: both reports used the neutral Comet assay.

1.3.1.4.2.2 Sperm chromatin structure assay (SCSA)

The SCSA test was first described over 30 years ago by Evenson et al., (1980). Sperm are diluted in buffer, treated with an acidic solution to cause partial DNA denaturation in situ,

then stained with the fluorescent dye, acridine orange and the sample is analysed by flow cytometry (Bungum et al., 2011). Green fluorescence is obtained if the dye intercalates into double-stranded DNA, and red fluorescence if it is attached to fragmented DNA (breaks in DNA or single-stranded DNA), (Evenson et al., 1980). This allows the determination of the extent of DNA denaturation in terms of a DNA fragmentation index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity, or an abnormally high DNA stainability (HDS) (Lin et al.,2008). These provide a measure of the percentage of immature spermatozoa within the semen sample, which can also be considered in the assessment of male infertility (Evenson & Wixon 2006).

1.3.1.4.2.3 Acridine orange test (AOT)

This test is carried out in the same way as SCSA but is simpler and less expensive since it employs fluorescent microscopy rather than flow cytometry. However, unlike flow cytometry, vague colours, rapid fading and heterogeneous staining can cause problems when visual analysis is used (Claassens et al., 1992).

1.3.1.4.2.4 Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay

This method depends on attaching biotinylated dUTP to the 3` ends of fragmented DNA using terminal deoxynucleotidyl transferase (TdT), provided that these ends terminate with an OH group. This creates a signal that can be detected by flow cytometry, fluorescence or light microscopy (Gavrieli et al., 1992; Sun et al., 1997; see Table 1.3). The TUNEL assay can determine only the percentage of spermatozoa with detectable DNA fragmentation but does not quantify the level of damage in each sperm. In addition, the dense DNA packaging in sperm can limit the number of sperms that can be stained and this reduces the accuracy of the assay (Gavrieli et al., 1992). Furthermore, using light microscopy instead of flow cytometry to observe and count the stained cells decreases the sensitivity and reproducibility of the assay. The expense of this technique also reduces its application in clinics (Sun et al., 1997; Evenson & Wixon 2006).

1.3.1.4.2.5 Sperm chromatin dispersion (SCD) assay

In this method, intact sperm are immersed in an agarose matrix on a slide, denaturated by treatment with an acid solution, and then treated with a lysis buffer to remove sperm

membranes and proteins. This results in nucleoids with a central core and a peripheral halo of dispersed DNA loops (Fernández et al., 2005).

Sperm with fragmented DNA release their DNA loops forming large halos. Sperm that produce a very small halo or no halo at all contain non-fragmented DNA. Sperm can be stained with Wright's stain (which contains a mixture of eosin (red) and methylene blue dyes) for visualization under bright field microscopy or an appropriate fluorescent dye such as DAPI (Fernández et al., 2003) for visualization under fluorescent microscopy (Evenson & Wixon 2006).

1.3.1.4.2.6 Toluidine blue assay

This test evaluates the sperm chromatin integrity by using toluidine blue which is a basic stain that is more likely to bind with phosphate residues of sperm DNA in nuclei with loosely packed chromatin and/or damaged DNA than densely packed ‘intact’ DNA. Observed using light microscopy, damaged sperm will be stained blue while normal sperm will remain colourless (Erenpreisa, et al., 2003; Trisini et al., 2004).

1.3.1.4.2.7 Aniline blue assay

Aniline blue is an acidic stain that is used to detect sperm chromatin integrity. Sperm with damaged DNA can be associated with residual histones which lead to looser chromatin packaging. This allows increased accessibility to the basic groups of the nucleoprotein and the binding acidic dyes such as aniline blue (Auger et al., 1990).

Again, under light microscopy, damaged sperm will be stained blue while normal sperm will remain colourless (Auger et al., 1990).

1.4 Rationale and hypothesis of the thesis studies

None of the studies described above considered the possible correlations between lifestyle exposures, such as alcohol consumption, smoking, and diet, sperm parameters and DNA damage measured by the neutral Comet assay in the same men over a defined period of time.

Any data presented are therefore almost universally based on a single sample and if there is a natural variation in the sperm parameters over a relevant time period, any conclusions reached may be flawed. In addition, there are no studies on correlations between neutral

Comet parameters, sperm parameters and the levels of O^6-alkylguanines in DNA, because routinely applicable assays for the latter are not available or practical.

Based on this, the hypotheses in this thesis are that there are variations in sperm qualities, DNA strand breaks as determined by the neutral Comet assay, and the levels of O^6-alkylG in DNA through a defined study period and that these parameters are more sensitive to environmental exposure and lifestyle and thus, in combination, likely to be better indicators of fertility.

The specific objectives of this thesis are therefore to:

1. Determine the inter and intra-individual variation in semen quality and examine associations between occupational, environmental and lifestyle exposures and semen quality (Chapter 2).

2. Determine the inter and intra-individual variation in DNA damage measured by the neutral Comet assay and examine associations between occupational, environmental and lifestyle exposures and Comet assay parameters (Chapter 3).

3. Optimise novel methodology using Atl1 to determine if O^6-alkylguanines are detectable in human sperm DNA and if so to examine associations between occupational and environmental exposure and O^6-alkylG levels in sperm and white blood cell DNA, and their relationship to DNA damage in sperm measured by the neutral Comet assay (Chapter 4).

Chapter 2: Assessment of variations in semen quality in a longitudinal study of young men and correlation with occupational and environmental influences

2.1 Introduction

Although there are a large number of studies on the relationship between semen parameters and fertility, their results are not necessarily consistent. Some but not all studies have suggested that potential risk factors for poor semen quality include age, cigarette smoking, heavy alcohol drinking, diet, obesity, and exposure to some physical and chemical agents (Oliva et al., 2001; Sharpe 2010; Gaskins et al., 2012; Afeiche et al., 2013; Chiu et al., 2015; Bergamo et al., 2016; Gabrielsen & Tanrikut 2016; Sifakis et al., 2017). In addition, the quality of sperm in an ejaculate, for example, sperm concentration, can vary considerably over time within individuals (WHO 1992; Xie et al., 2018). Keel (2006) reported that the most variable sperm parameter was sperm count in both 74 infertile and 65 normal donors to a clinical andrology laboratory. However, Chia et al., (2001) reported no significant variation in the month to month fluctuation in semen volume and sperm concentration following the analysis of 7656 semen samples between 1991 to 1995 in a Singapore fertility clinic. In support of this, Carlsen et al., (2005) studied the longitudinal changes in semen quality in 158 Danish young men and reported that sperm concentration, total sperm count and sperm morphology did not change significantly during 4 years of follow-up. The overall objective of the thesis studies was to analyze semen parameters in relation to a range of potential impaired fertility risk factors including DNA damage, of which O^6alkylG was of considerable interest. This section of the thesis focuses on two aspects of this objective.

2.1.1 Aims and objectives

The aim of the work in this chapter was to study the possible impact of occupational, environmental and lifestyle exposures on inter and intra-individual variation in semen quality at the study baseline and over a 6 month time period. Therefore, the specific objectives of this chapter were to:

1) Characterize the study population.
2) Assess inter and intra-individual variation in semen quality using 7 parameters: semen

volume (ml), % progressively motile sperm, % non-motile sperm, sperm concentration, total sperm count and % vital sperm and % morphologically normal sperm.

3) Examine the potential effects of occupational, environmental and lifestyle exposures on semen quality.

2.2 Materials and methods

2.2.1 Materials

All chemical reagents were purchased from Sigma-Aldrich UK unless otherwise stated. Dulbecco's phosphate buffered saline (DPBS) without Mg++ and Ca++ was from SeaKem®LE. Two-chamber microscope slides were supplied by CellVision Technologies UK. Sterile semen sample collection pots were from Sterilin, UK. Ethanol (95%) for fixation of slides for sperm morphology assessment, and vacutainers containing EDTA or heparin for whole blood collection were supplied by Thermo Fischer Scientific.

2.2.2 Methods

2.2.2.1 Recruitment of participants and sampling regime

This study was approved by the ethical committee at Manchester Metropolitan University (MMU: EthOS Reference Number: 0482; Appendix 1). The study was advertised at locations on both the MMU campus and the University of Manchester (UoM) campus to recruit participants (See Appendix 2).

Subsequently, each man who was interested in this study was given the participant information sheet (PIS), a consent form and a questionnaire (See Appendix 3). Participants were asked to attend every 2 weeks for a total of 12 times, at the MMU laboratory, donate a semen sample and complete the questionnaire (see below). In addition, participants were asked to donate blood at the first, fourth, sixth, ninth and twelfth visits. The study started on the 11th of April 2017 and ended on the 15th of December 2017.

2.2.2.2 Questionnaire structure

The questionnaire was based upon one used for a previous study investigating male infertility (Altakroni 2015) with some minor modifications. There were four sections in the baseline

questionnaire. The first contained questions on age, ethnicity, height and weight, job, education, marital status, and sexual activity. Participants were also asked about the possible hazards in their job and use of personal protective equipment during work or in-home over the last three months before treatment. Men were also asked whether they were exposed or not to 15 agents including metal dust and fumes, pesticides, fertilizers, oils or greases, detergents, paints, solvents, x-rays, chemotherapy, and heavily vibrating machinery. The participants were also asked about their general health and if they had had fever or flu, varicocele or illness due to their job or taken any medication in the last 3 months.

The second section was concerned with their lifestyles such as smoking habits, type of smoking, alcohol and drug consumption. Each participant recorded the type and number of alcoholic beverages they consumed per week and the type of drugs consumed, if any, such as methamphetamines, cannabis, inhalants, tranquilizers, cocaine, narcotics, and hallucinogens. The participants were asked how many times in the week they engaged in mild, moderate and strenuous physical activity and about their type and style of underwear.

The third section asked about diet and dietary supplements including the consumption of meat (red, white and fish), nuts, coffee and nutrition supplement intake such as whey, pea and soy protein, zinc, selenium, and vitamin. The participants were asked how they consumed 91 food items on a scale of 1-7 with 1) being never consumed, 2) less than once a month, 3) once or twice a month, 4) once per week, 5) 2-3 times per week, 6) 4-6 times per week and 7) every day.

In the final section, the participants were asked if they had smoked any kind of cigarette or had been exposed to smoking or had worked in the previous 24 hours and whether they had eaten any food items such as chicken or other poultry, red meat for example (beef, lamb, ham, sausages) and fish through the previous 24 hours. Participants were also asked about travelling in the last three months. At the second and subsequent visits, men were asked the same questions except for those such as age and ethnicity.

2.2.2.3 Procurement of semen and blood samples

All the semen samples were produced by masturbation after a recommended 2-5 days of abstinence. The participants were instructed on how to produce and collect the sample and were given a sterile collection pot. Semen samples were produced on the MMU laboratory site in a designated, secure room but if the participants preferred to produce the semen

sample at home, they were provided with the collection pot and then asked to fill in a form after they had brought in the samples (Appendix 4). Participants signed a semen procurement form each time they donated a sample (Appendix 4) and were asked to complete the questionnaire after the semen sample was produced. Up to 32 ml of blood was taken at the same visit by Dr. Stephane Berneau.

2.2.2.4 Semen analysis

All the semen samples underwent standard semen analysis according to WHO guidelines (WHO 2010). Firstly, the semen sample was liquefied at 37°C for 30 minutes and the total volume of semen was measured using a graduated pipette. After taking aliquots for analysis by Computer Assisted Semen Analysis (CASA), manual sperm counting, and sperm morphology and vitality assays, semen samples were centrifuged at 800 x g for 10 minutes at room temperature (RT). The supernatant (seminal plasma) was transferred to labelled 1.5 ml Eppendorf tubes and the sperm pellet to a labelled cryogenic tube. The tubes were snap-frozen in liquid nitrogen before transfer to long term storage at -80ºC.

2.2.2.4.1 Computer Assisted Semen Analysis (CASA)

To measure sperm motility and concentration, 5 µl of the liquefied semen sample was applied to a CASA slide for analysis by CASA. The CASA software (Sperminator; Figure 2.1) is able to track at least 200 motile sperm per specimen in each millisecond and assess the concentration, motility, and progressive velocity of sperm and also allows the user to select/de-select other cells that are present.

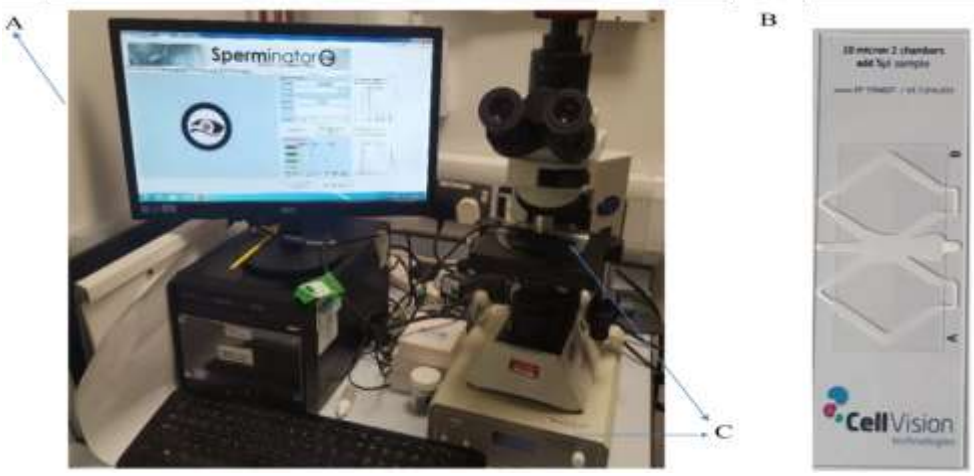


Figure 2.1 Computer Assisted sperm (CASA)
(A) Computer Assisted Sperm Analyser with Sperminator programme page displayed on monitor; (B) CASA slide and (C) The slide heater stage.

The following semen quality categories were determined by CASA: sperm concentration, motility types A, B, C, and D, and sperm velocity. Summation of A and B provided % progressive motile sperm and D provided, % non-motile sperm.

2.2.2.4.2 Manual counting of sperm

Sperm were counted manually using a haemocytometer after diluting 5-fold by mixing 12.5 µl of the sperm sample with 50 µL DPBS, vortexing and loading 10 µl of the dilution onto a haemocytometer slide (Figure 2.2A). Only whole sperm i.e. sperm with the complete sperm shape with head and tail were counted. Counting started in each row going from left to right as shown in Figure 2.2B. In each row, the number of sperms needed to be more than or equal to 100 but if the number of sperms was lower than 100 then the next row was also counted, the sperm concentration was determined using the equation below: -

$$C = (N/n) / D$$

C = concentration, N = total count in chamber, n = total number of rows counted and
D = the dilution factor.

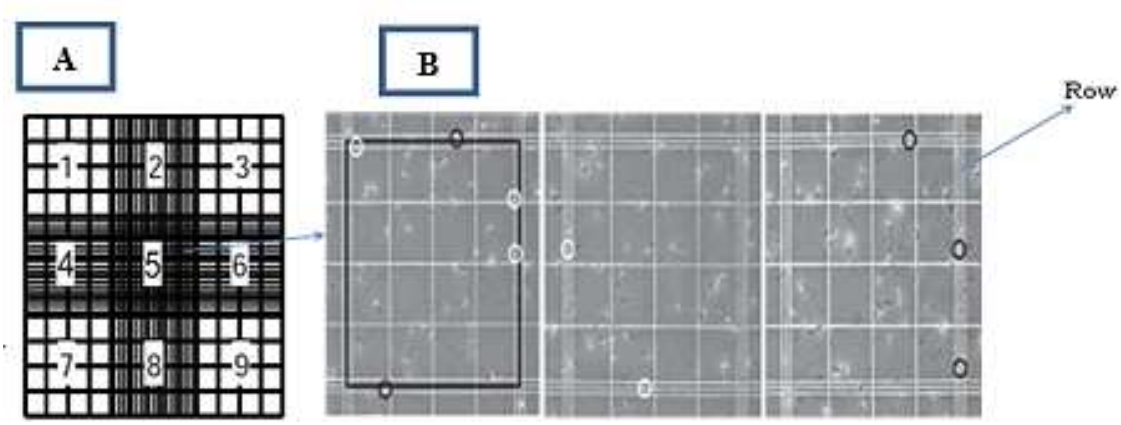


Figure 2.2 Haemocytometer used for sperm count
A: The Haemocytometer grid: 1, 3, 7 and 9 are large squares for leucocyte counts; 5 is for sperm counts; 2,4,6 and 8 are spacers; B: close up of part of grid number 5.

2.2.2.4.3 Sperm morphology and vitality assays

To determine sperm morphology (WHO 2010) 10 µl of the liquefied semen sample was pipetted and smeared onto two slides as shown in Figure 2.3A, dried for one minute, fixed in 95 % ethanol for five minutes at RT and then stored at 4^{o}C. Papanicolaou stain was used for sperm morphology at St Mary's Hospital (Manchester) and semen samples were analysed at baseline, six and twelve weeks during the study period.

For sperm vitality analysis, 20µl eosin-nigrosin stain was added to 20 µl of liquefied semen in a 500 µl Eppendorf tube and mixed using a pipette to ensure homogeneity. Two microscope slides were produced by smearing 10µl of the stained sperm sample onto a slide using the feathering technique recommended for undiluted samples (WHO 2010) as shown in Figure 2.3A. The slides were examined under 63 X/1.4 objectives with oil immersion. Membrane-damaged dead sperm are stained pink or purple, while live membrane-intact sperm exclude the dye and appear white as shown in Figure 2.3B. A total of 200 sperm were counted and the numbers of live and dead sperm were recorded, % of live sperm were calculated using the equation below: -

*% of live sperm = (Number of live sperm / total number of sperms counted) *100*

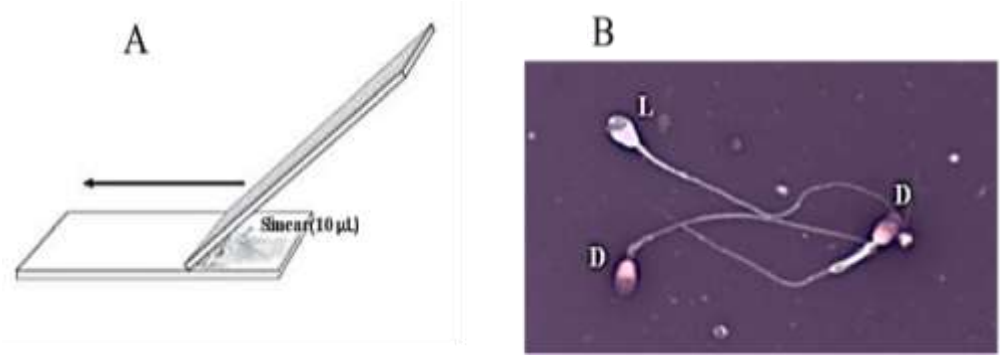


Figure 2.3 Slide of sperm preparation for vitality staining and vitality images
A: The stained sperm was smeared onto a glass slide by feathering [(WHO 2010)]; B: The sperm were visualized as live (L; white) or dead D: (pink) and the images were taken by light microscope (magnification 63x with oil immersion).

2.2.2.5 Processing of blood samples

Blood was taken from participants into red-top vacutainers and the sample was left to clot for 15–30 minutes undisturbed at RT. The clot was removed by centrifugation at 1,000–2,000 xg for 10 minutes in a refrigerated centrifuge and the serum was transferred to labelled 1.5 ml Eppendorf tubes and stored at -80^{o}C. Blood was additionally collected in anticoagulant vacutainer tubes containing heparin (green-top) or EDTA (purple top). Both heparin and EDTA tubes were centrifuged for 10 minutes at 1,000–2,000 xg and three layers were distinguished: a layer of clear fluid (the plasma), a layer of red fluid containing most of the red blood cells, and a thin layer in between (buffy coat). The plasma layer and the buffy coat were transferred to separate labelled 1.5 ml Eppendorf tubes and stored at -80^{o}C, while the red blood cells were discarded.

2.2.2.5.1 Manual leucocyte counting

A haemocytometer was used to manually determine the number of leucocytes in each buffy coat sample after 1:20 dilution in DPBS, and 10µl added to a haemocytometer chamber. As leucocytes are bigger than sperm, the four corner squares were used to count them (Figure 2.2A) and the total determined using the equation below: -

Total Cell number = no. of cells in 1 large square x Dilution factor/ volume factor

Dilution factor = reciprocal of dilution (20)
Volume factor = (width x length x height) = 0.1

2.2.2.6 Air pollutant exposure data

As most of the participants were students at UoM or MMU, it was intended that the air pollutant data for NO_2, NOx, SO_4, O_3, $PM_{10,}$ and $PM_{2.5}$ levels between 1/4/2017 and 31/12/2017 would be collected from stationary monitoring stations at Manchester University, Manchester Piccadilly and Sharston from publicly available data on the DEFRA website (DEFRA 2019). However, only $PM_{2.5}$ data were available for inclusion in this study.

2.2.2.7 Statistical analysis

The coefficient of variation was applied to find what was the most variable parameter among each sperm parameter (Alvarez et al., 2003) as in the equation below: -

*Coefficient of Variation = (Standard Deviation / Mean) * 100*

A paired T-test was used to examine the differences between sperm concentrations determined by CASA and Haemocytometer, and the Pearson correlation coefficient was applied to find the correlation between CASA and haemocytometer methods. As the data were non-parametric, the Spearman's rho value was determined to find if there was any correlation between the semen parameters and the other parameters obtained, divided into exposed and unexposed groups, as described in the text. One-way ANOVA was conducted to find if there was any significant difference in sperm parameters between ethnicity, sexual activity, marital status, BMI and Leisure score index (LSI) groups or any exposed or non-exposed groups. However, for some exposure factors such as BMI and exercise, specific criteria were used. Thus, participants were divided according to their BMI into two groups, participants with BMI < 25 kg/m^2 and participants with BMI ≥ 25 kg/m^2 as 25 kg/m^2 is the

limit between normal BMI and overweight (WHO 2012). Also, the participants were divided into four smaller BMI groups according to Povey et al., (2012); low normal BMI (18.5-22.99 kg/m^2), high normal BMI (23-24.99 kg/m^2), overweight (BMI= 25-29.99 kg/m^2), obese (BMI > 30 kg/m^2). Values given for mild, moderate and strenuous exercises were input into the following equation to give Total LSI for each participant: -

$$LSI = (9 \times strenuous) + (5 \times moderate) + (3 \times mild)$$

The participants that scored ≤23 were classed as insufficiently active and those that scored ≥24 were classed as active (Amireault & Godin, 2015).

A paired T-test was initially used to compare between the hazardous agent exposed and non-exposed groups. Hazardous exposures were then classified into five groups depending on the number of physical and chemical agents that each participant was exposed at the baseline of the study. Hence if the participants were not exposed to any of these agents, they were classified as zero, the participants who were exposed to any one, two, three or four of these agents were categorized as one, two, three or four, respectively, and one-way ANOVA was used for analysis.

Concerning general health, the participants were firstly considered to have a health issue or not so A paired T-test was used. Then they were categorized into five groups depending on the number of any health issues. Thus, the participants who had no health issues were categorized as zero, the participants who had one health issue as one, the participants who had 2 or 3 health issues as two, the participants who had 4 issues as three and the participants who had 5 or more health problems as four. A one-way ANOVA was used for comparison among these groups. Smoking habits were considered on a binary scale as either non- or ex-smokers as no participants self-reported as current smokers, and also on a continuous scale as the number of years the participant had smoked. The type of cigarettes smoked was not included in the analysis. A paired T-test was used to compare between these two groups.

Regarding alcohol consumption, the participants were categorized depending on the type and number of alcoholic beverages consumed per week. Units of alcohol per drink were recorded at baseline of study as one pint of 4% beer, 2.3 units; one pint of 4.5% lager or cider, 2.6 units; one 175ml glass of 13% wine, 2.3 units; one 25 ml measure of 40% spirits, 1 unit (Drinkaware 2016). As well as dividing participants by whether or not they drank alcohol, the participants who were drinkers were divided into two other equal groups

depending on the number of alcohol units they consumed. A one-way ANOVA was used and Spearman's rho was used to find if there was any correlation between the alcohol units consumed and sperm parameters. The classification of jobs into manual / non-manual was based on the SOC2010 coding (Institute for Social & Economic Research 2014) and a paired T-test was used to compare between these two groups. One-way ANOVA was used to compare the mean values of sperm parameters in relation to diet. The food items were grouped into 28 categories depended on the general kind of diet and the mean frequency of consumption was calculated for each of these by summation of each item and multiplying it by the total serving of each item. It should be noted that the present study was intended to determine the practicality and feasibility of a much larger future study which would involve many more participants and have much greater power.

2.3 Results

Twenty participants were initially enrolled in the study and 15 of them (75%) completed the entire study.

2.3.1 Procurement of questionnaires

All 20 participants completed the baseline questionnaire, and one participant left the study at the 2nd visit, therefore 19 men completed the questionnaire at this visit. Another participant left the study at the 5th visit, so 18 participants completed the questionnaire at this visit. Another participant left the study on the 7th and another at the 8th visit and finally another participant left the study at the 9th visit so only 15 participants completed all the questionnaires to the end, from the first to the 12th visit.

2.3.2 Procurement of semen and blood samples

The CASA method was used on all 206 semen samples to determine % progressive sperm motility, % non-motile sperm and sperm count. Two slides per semen sample (412 slides in total) were prepared to determine % vital sperm, and similarly, 412 slides were fixed in 95% ethanol although only 53 slides from the baseline, sixth and the twelfth visits were assessed using a Papanicolaou stain. All 206 semen samples were analysed in the neutral Comet assay (Chapter 3). A total of 52 sperm pellets (from the baseline, sixth and the twelve visits) were processed using an 80 % density gradient to remove round cells from the sperm pellet,

and then the sperm concentration was determined manually. DNA was extracted from 41 sperm pellet samples and O^6 alkylG levels were determined in 28 of these (Chapter 4).

In total, 73 blood samples were taken throughout the study period, 18 at baseline, 15 at the 2nd visit,16 at the 6th visit 11 at the 9th visit and 13 at the 12th visit. Each blood sample was divided into several tubes for processing. Of a total of 128 buffy coat samples obtained, duplicates from 40 participants were pooled and DNA extracted for quantitation of O^6-alkylG adducts (Chapter 4).

2.3.3 Baseline analyses

Data for all 20 participants were obtained at the start of the study and defined as the baseline values. Appropriate statistical analyses were carried out in relation to the study parameters obtained from the semen analyses, pollution data and questionnaires, as described in detail in 2.2.2.5.

2.3.3.1 General demographics

A number of the baseline demographics are summarized in Table 2.1. Of note is that the average age of the 20 participants was 24.3± 4.8, which is a very narrow range and clearly does not include even middle-aged men. Only two men were smokers one of them was ex-smoker and the second one was currently smoking e-cigarettes.

Table 2.1: Demographics of the study population at baseline

Variable		Baseline value (n=20)
Age (years) (Mean ± SD)		24.3 ± 4.8
BMI(Kg/m^2) (Mean ± SD)		24.4 ± 3.5
Abstinence (days) (Mean ± SD)		2.7 ± 1
Exposed to different kinds of smoke (%)		43.5
Regular alcohol consumption (%)		73.5
Drug consumption (%)		27
LSI (%)	**Active**	80
	Insufficiently active	20
Wore underwear (%)		90
Participants percentage travelled (%)		46.5
Travel (%)	**Car**	40
	Bus	23.5
	Train	26.5
	Plane	30

SD: standard deviation.

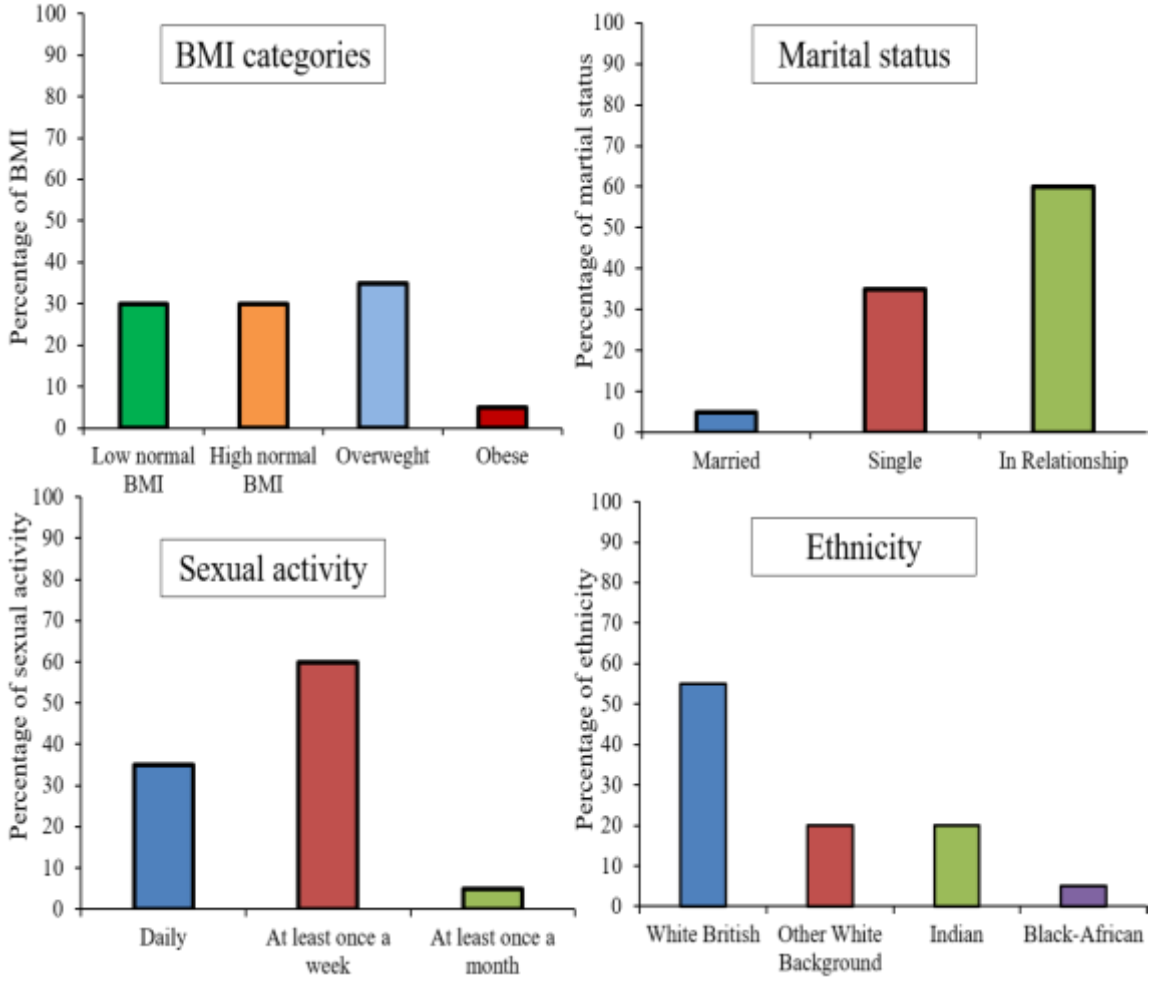


Figure 2.4 General demographics of study population at baseline

Concerning the occupation of the participants, 60 % of them were students, the rest included a waiter, administration officer, and a bar supervisor. Figure 2.4 above shows the BMI categories, marital status, sexual activity, and ethnicity, of the study population at baseline.

2.3.3.2 Occupational exposures

Baseline occupational exposures are summarised in Figure 2.5. There was only one participant who was not exposed to different chemicals or physical agents at work or home in the 3 months prior to baseline.

In addition, the exposure to X-rays was as high as 80% and this was followed by exposure to detergents or soap in the workplace (55%). No participant self-reported exposure to pesticides, herbicides, or chemotherapy.

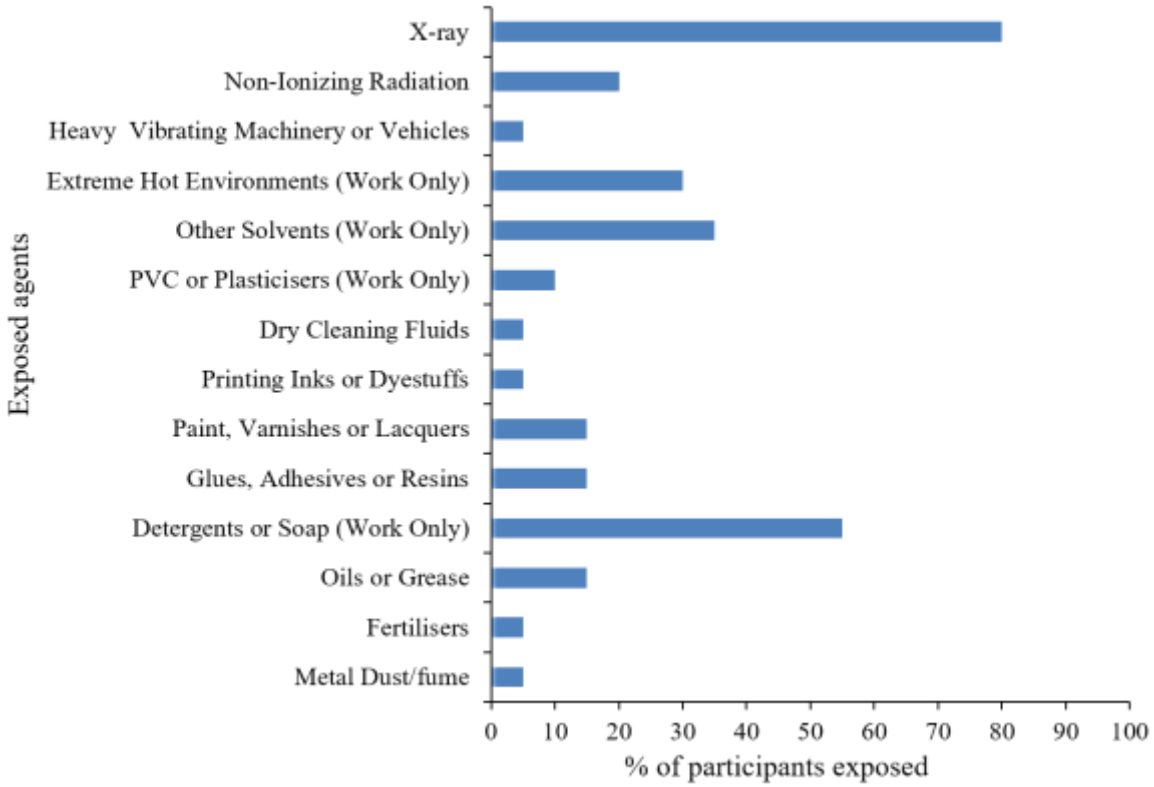


Figure 2.5 Participants exposed to hazardous agents at work or home 3 months before the start of the study at a baseline

2.3.3.3 Health

General illness was reported by 55% of participants, 40% reporting fever or flu (Figure 2.6).

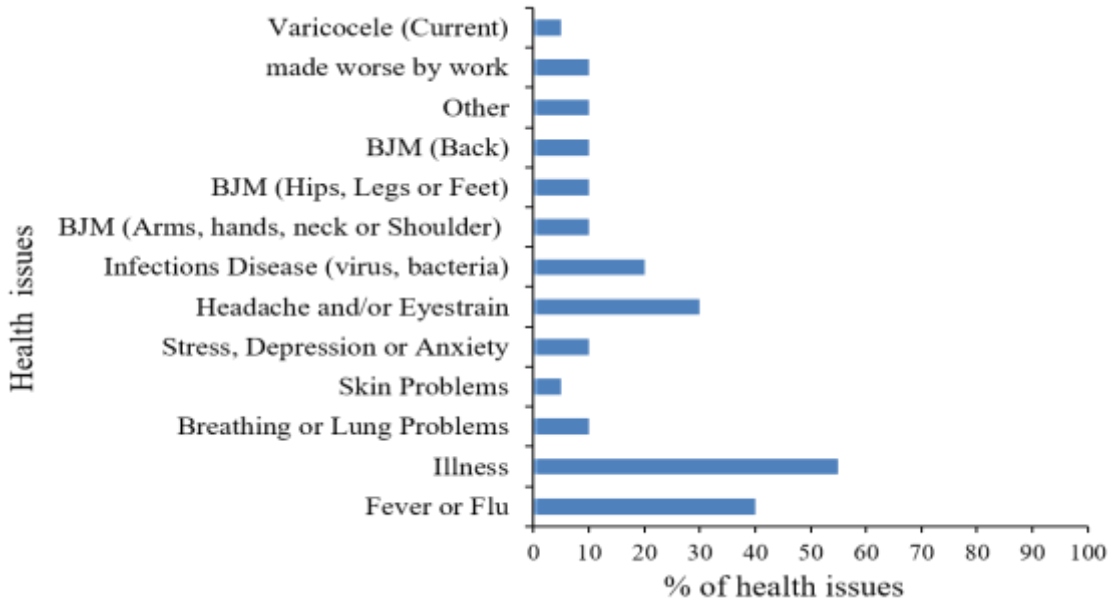


Figure 2.6 General health of the participants at study baseline
BJM: refers to bone join muscle problems

Most other health issues were reported by 5-10% of the participants but headache (30%) and infections (20%) were more commonly reported. None of the participants had pelvic region problems, hearing problems, heart disease/attack or other circulatory system problems or previous varicocele although 5% reported current varicocele.

2.3.3.4 Diet and dietary supplements

Figure 2.7 shows that 80% of participants were only fish eaters while only 5% were vegan.

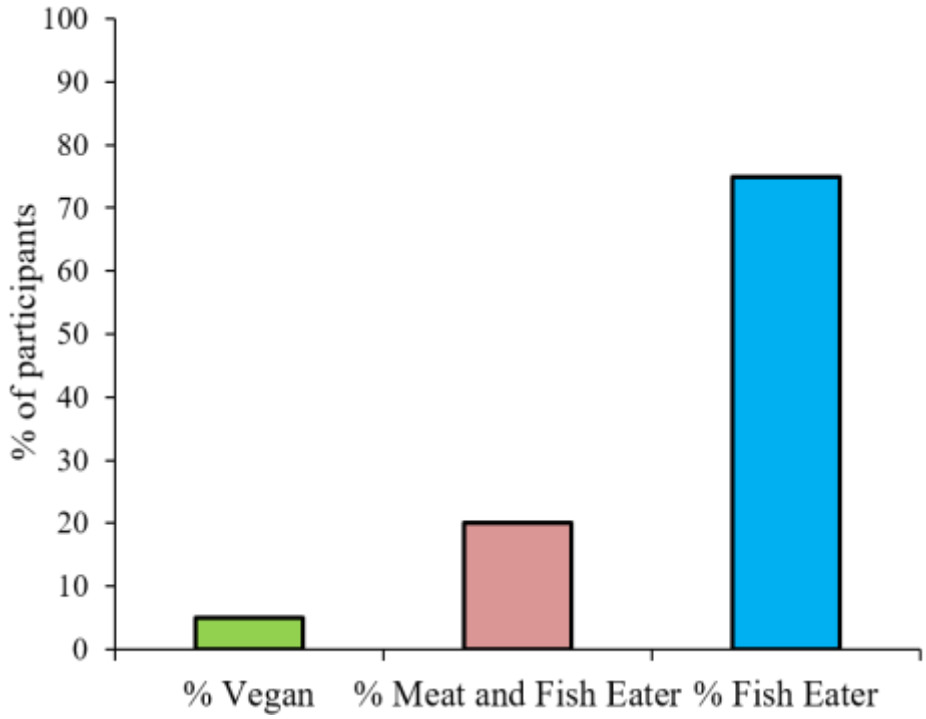


Figure 2.7 Diet categories of all participants at study baseline

Table 2.2 shows the consumption of different food items at baseline. The food item consumed by the highest percentage of participants was cereals and the lowest was pulses.

Table 2.2: Frequency of food item consumption by the study population at baseline

Food item	% of population
Chicken	14.5
Red Meat	8.5
Processed Meat	6
Fish	6.5
Vegetable oil	6.5
HF Dairy	23.5
LF Dairy	16.5
Eggs	27.5

Table 2.2: Continued

Food item	% of population
Fruits	14.5
Other Veg	25.5
Leafy Green	10
Crips/Chips	9
Pulses	4
Species	9.5
Cereals	76
Sugar and confectionary	40.5
Caffeinated non-sugar beverages	41
Nuts & Seeds	10

HF and LF: indicate high and low fat

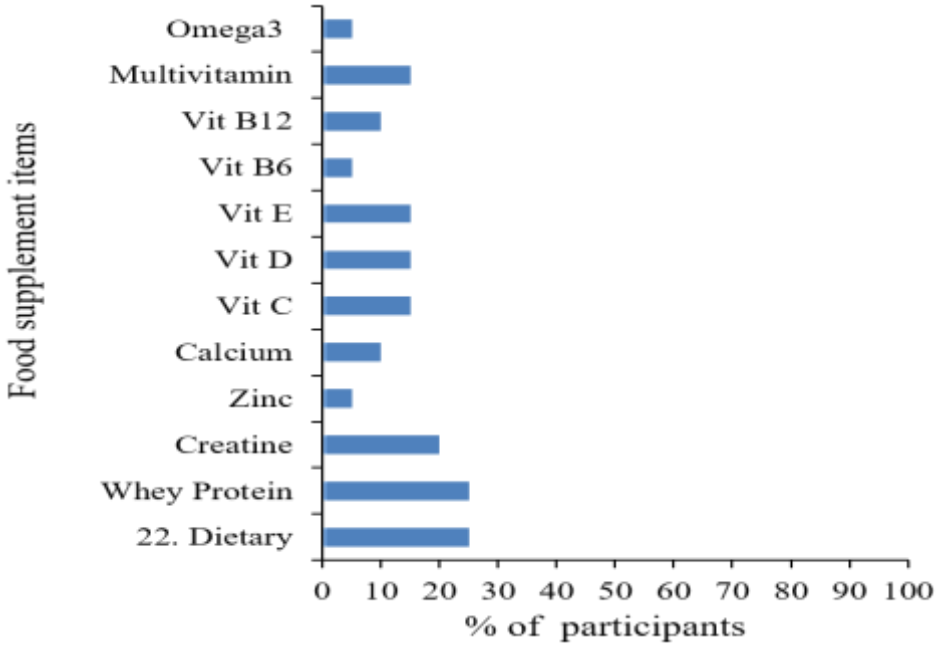


Figure 2.8 Consumption of different food supplement items by the study population at baseline

22 Dietary: general food supplement consumption.

Figure 2.8 above shows the consumption of different types of food supplements. The consumption of whey protein and creatine were the highest among the different types of food supplements at baseline in all 20 men.

2.3.3.5 Semen analyses

2.3.3.6 Correlation between CASA and manual sperm counting

There was a close correlation between CASA and manual sperm counting (R^2= 0.92; P <0.001) as shown in Figure 2.9.

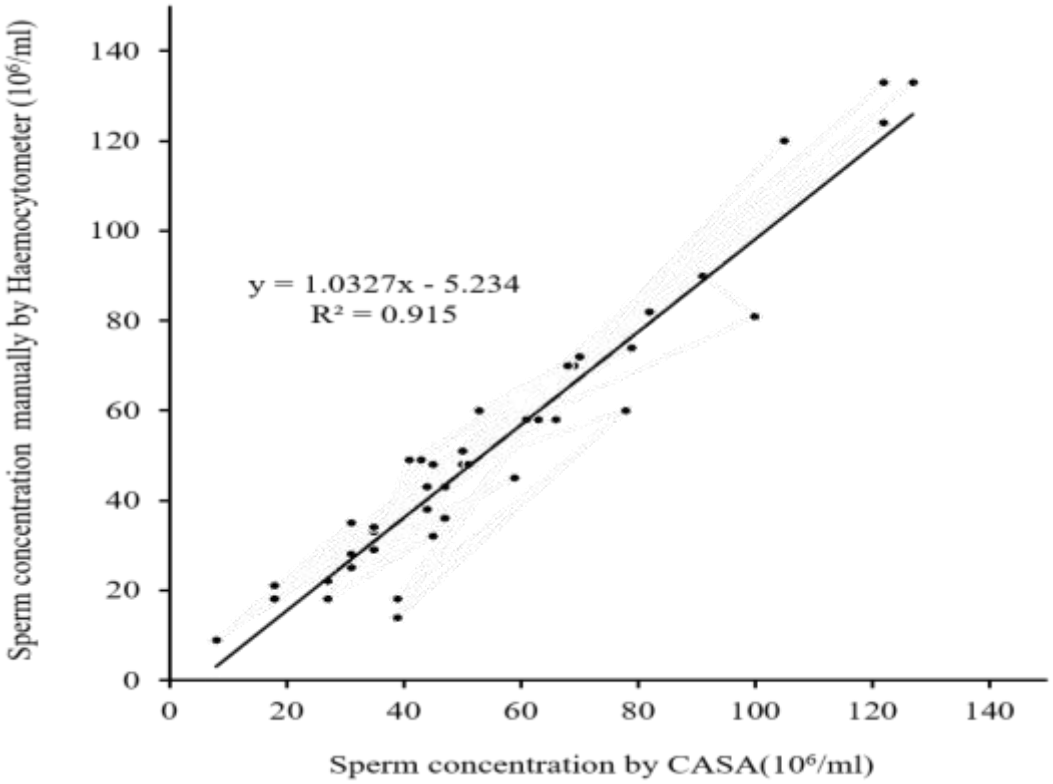


Figure 2.9 Correlation of sperm concentration as determined by CASA and manually by Haemocytometer

Number of semen samples =206

2.3.3.7 Semen quality at baseline

The semen characteristics of all men in the study were within WHO reference values Table 2.3.

Table 2.3: Semen parameters of the study population at baseline

Semen parameter	Value +/- SD (n=20)	Reference value (WHO 2010)
Volume (ml)	3.0 ± 1.5 (↔)	1.5- 7.6
Sperm concentration ($X10^6$ /ml)	45.5 ± 29.5 (↔)	≥ 15
% progressively motile sperm	57.6 ± 17.0 (↔)	≥ 32%
% non-motile sperm	32.2 ± 15.8 (↔)	< 40
Sperm count ($X10^6$)	42.2 ± 118.5(↔)	≥ 39
% vital sperm	86.4 ± 9.2(↔)	≥58%
% morphologically normal sperm	4.6 ± 2.2 (↔)	≥4%

SD: standard deviation; n: number of participants; ↔: in the range of the WHO references value.

The correlations between the various sperm parameters are presented in Table 2.4. The most significant correlations (P<0.01) were between sperm concentration and % progressively motile sperm and % vital sperm. Additionally, there was a significant (P<0.05) positive

correlation between the % morphologically normal sperm and sperm concentration, % progressively motile sperm and % vital sperm.

Table 2.4: Correlations between sperm parameters at study baseline

Sperm parameters	Volume (ml)	Total sperm count ($X10^6$)	Sperm concentration ($X10^6$/ml)	% progressively motile sperm	% non-motile sperm	% vital sperm	% morphologically normal sperm
	Spearman's rho (n=20)						
Abstinence (day)	0.23	0.44	0.35	0.21	-0.25	0.23	0.31
Volume (ml)		**0.77****	0.35	0.29	-0.31	0.21	-0.15
Sperm count ($X10^6$)			**0.81****	**0.63****	**-0.67****	**0.51***	0.24
Sperm concentration ($X10^6$/ml)				**0.75****	**-0.80****	**0.57****	**0.48***
% progressively motile sperm					**-0.98****	**0.80****	**0.62****
% non-motile sperm						**-0.82****	**-0.59****
% vital sperm							**0.52***

Significant correlations are shown in bold; *p<0.05; Significant correlations are shown in bold; **p<0.01.

2.3.4 Association of sperm quality and general demographic parameters at baseline of the study

There were no significant correlations between sperm parameters and age, BMI and LSI at the baseline of study (Table 2.5).

Table 2.5: Semen parameters and male age, BMI and LSI at study baseline

Sperm parameters	Spearman's rho correlation (n=20)		
	Age (years)	BMI(Kg/m2)	LSI (%)
Volume (ml)	-0.18	-0.29	0.13
% progressively motile sperm	-0.10	0.18	-0.25
% non-motile sperm	0.12	-0.17	0.21
Sperm concentration($X10^6$/ml)	-0.33	0.13	0.08
Sperm count ($X10^6$)	-0.37	-0.10	-0.04
% vital sperm	-0.10	0.15	-0.13
% morphologically normal sperm	-0.12	0.12	-0.14

BMI: body mass index; n: number of participants.

The data presented in Table 2.6 shows that there were no significant differences in sperm quality by ethnicity (one African was not included in this comparison) or between the BMI categorized groups (one man was categorized as obese and was excluded from this comparison) or by marital status or frequency of sexual activity (one man was married and another had a frequency of sexual activity which was at least once a month and both of these were excluded from this comparison) or between the LSI groups or between travelled and non-travelled status or between the wearing or not of underwear.

Table 2.7 deals with exposures to physical and chemical agents. There was no significant difference in any sperm parameters between exposed and non-exposed to all of these agents at baseline. Exposures were then subcategorised as described in Table 2.8.

There were significant differences in the semen volume between those men exposed to no agents and those exposed to one or to four or more agents. Other significant differences are described in the Table legends but in all cases, it must be noted that the number of participants in each category is very small.

Table 2.6: Semen parameters, demographic and lifestyle factors at study baseline

Variables		Sperm parameters (Mean ± SD)						
		Volume (ml)	% progressive sperm motility	% non-motile sperm	Sperm concentration ($x10^6$/ml)	Total Sperm count ($x10^6$)	% vital sperm	% morphologically normal sperm
Ethnicity (n=19)	White British (n=11)	2.6 ± 1.1	59.5 ± 17.8	30.6 ± 17.7	53.8 ± 35.5	155.0 ± 124.4	86.0 ± 10.4	4.7 ± 2.5
	Indian (n=4)	3.8 ± 2.7	43.6 ± 13.2	43.5 ± 12.2	37.2 ± 15.6	154.1 ± 161.2	82.1 ± 8.1	3.8 ± 2.0
	Other White (n=4)	3.2 ± 1.0	64.7 ± 9.8	26.7 ± 7.3	35.3 ± 8.0	115.2 ± 48.4	91.3 ± 2.7	4.5 ± 0.6
[A]BMI categories (n=19)	Low normal BMI (n=6)	3.0 ± 0.7	56.2 ± 23.0	32.8 ± 20.3	44.8 ± 27.0	142.0 ± 110.4	83.4 ± 13.2	5 ± 3
	High normal BMI (n=6)	3.4 ± 2.1	50.6 ± 10.3	39.1 ± 11.0	38 ± 17	151.0 ± 129.5	86.3 ± 5.6	3.7 ± 1.2
	Overweight (n=7)	2.7 ± 1.2	65.4 ± 12.0	25.5 ± 12.7	55.0 ± 39.3	148.8 ± 117.5	90.4 ± 4.6	5 ± 2
Marital Status (n=19)	Singular (n=7)	3.5 ± 2.2	52.5 ± 19.2	35.3 ± 18.0	49.0 ± 36.7	190.8 ± 163.6	83.5 ± 9.0	4.1 ± 3.1
	In Relationship (n=12)	2.6 ± 1.0	60.1 ± 16.2	30.8 ± 15.6	45 ± 27	120.4 ± 84.1	87.7 ± 9.6	4.7 ± 1.6
Sexual activity (n=19)	Daily (n=7)	3.0 ± 2.4	52 ± 16	38.1 ± 15.7	33.6 ± 17.6	111.2 ± 131.1	81.8 ± 12.1	4.4 ± 1.7
	At least once a week (n=12)	3 ± 1	58.6 ± 16.0	30.8 ± 14.8	50.7 ± 34.2	155 ± 117	88.5 ± 6.4	4.5 ± 2.5
LSI categories groups (n=20)	Active (n=4)	3 ± 1	68.3 ± 11.6	23.1 ± 9.6	51.7 ± 36.8	151.0 ± 95.3	92.3 ± 3.0	4.8 ± 1.0
	Insufficiently active (n=16)	3 ± 6	55.0 ± 17.2	34.5 ± 16.4	44.0 ± 28.6	140.1 ± 126.2	85.0 ± 9.6	4.6 ± 2.4
Under wear (n=20)	Worn(n=18)	3.0 ± .1.6	58.2 ± 17.5	31.7 ± 16.2	45.4 ± 30.5	141.2 ± 122.0	86.0 ± 9.6	4.7 ± 2.2
	Not worn(n=2)	3 ± 1	52.8 ± 11.4	37.0 ± 14.8	46.8 ± 25.7	151.4 ± 117.0	90.6 ± 1.0	3.5 ± 2.1
[B] Travel	Travel (n=10)	3.1 ± 1.0	61 ± 20	29.0 ± 17.4	47.0 ± 31.6	156.4 ± 113.5	88.4 ± 7.7	4.4 ± 2.6
	Not travel (n=10)	2.7 ± 2.0	54.5 ± 13.4	35.4 ± 14.2	44.1 ± 28.8	128.1 ± 127.7	84.4 ± 10.4	4.8 ± 1.8

SD: standard deviation; n: number of participants; [A] BMI Low normal = 18.5-22.99 kg/m2; high normal =23-24.99 kg/m2 overweight= 25-29.99 kg/m2; [B]: if participants travelled or not 3months prior to study start;

Table 2.7: Semen parameters and exposure to physical and chemical agents at study baseline

Hazardous agents exposed (No) /(Yes)	Semen parameters for unexposed / exposed (Mean ± SD)						
	Volume (ml)	% progressively motile sperm	% non-motile sperm	Sperm concentration ($X10^6$/ml)	Sperm count ($X10^6$)	% vital sperm	% morphologically normal sperm
[A] Exposed (2/18)	5.2±3.7 2.7±1.0	64.2±19.1/ 57±17.1	25.8±17.2/ 33±16	78.5±39/ 42±27.2	335.2±85/ 120.8±102	87.4±10.4/ 86.3±9.4	5.0±1.4/ 4.6±2.3
Oils or Grease (17/3)	2.8±1.6/ 3.6±0.6	56.4±17.7/ 64.6±11	33.8±16.3/ 23±9	41.5±27/ 68.6±38.4	126.5±117.3/ 231.5±95.5	85.6±9.7/ 91±1	4.9±2.2/ 2.7±1.2
Detergents or Soap (Work Only) (9/11)	2.8±2.0/ 3±1	55.1±20.7/ 59.7±13.7	35.2±18.8/ 29.8±13.3	42.6±31.4/ 48±29.1	128.8±132.8/ 153.2±110.7	82.6±11.8/ 89.5±5	5.0±2.2/ 4.4±2.2
Glues, Adhesives or Resins (17/3)	2.9±1.6/ 2.8±1.0	56.5±17.7/ 64±11.6	33.8±16.3/ 23.1±9.2	40.4±27/ 74.5±30.5	129.1±117/ 216.5±119	86±9.8/ 88.9±3.5	4.8±2.3/ 3.7±1.5
Paint, Varnishes or Lacquers (17/3)	3.0±1.6/ 2.4±0.6	57.5±17.4/ 58.6±16.5	32.6±16/ 30.1±17.4	42.6±27.5/ 62.4±41.4	138.8±118.5/ 161.4±142.7	86±9.8/ 89.3±3.8	4.6±2.4/ 4.7±0.6
PVC or Plasticisers (Work Only) (18/2)	2.9±1.6/ 3.3±0.4	56±16.8/ 73.4±5	34±15.7/ 17.4±6.6	43.1±26.6/ 67.5±58.1	134.8±116.2/ 209.1±165	85.7±9.3/ 93.4±2.3	4.7±2.3/ 4.0±0.0
Other Solvents (Work Only) (13/7)	3.0±1.8/ 3±1	56.8±19.7/ 59.2±11.1	33.8±18.1/ 29.3±11	43.8±30.3/ 48.8±29.8	141.3±131.5/ 144±99.3	84.2±10.6/ 90.7±3	5.1±2.5/ 3.7±1.3
Extreme hot environments (Work Only) (14/6)	3.0±1.8/ 2.8±1.1	60±13.8/ 53±12.4	30.3±12.8/ 35±13.2	40.5±22.1/ 39±17	131.3±118.7/ 112.7±72	87.8±6.4/ 82.2±12	5.1±2.2/ 3.8±1.7
Non-Ionizing Radiation (16/4)	3.0±1.6/ 2.6±1.5	60.3±17/ 50.8±12.6	30±15.3/ 37.1±14.2	47±31.5/ 34.8±22.1	150.7±126.1/ 106.8±99.3	87.7±7.5/ 84±7.7	4.8±2.3/ 3±2
X-ray (4/16)	3.8±2.7/ 2.7±1.1	59.5±14.7/ 57.2±17.8	31.5±14.6/ 32.4±16.5	53.5±36.3/ 43.5±28.5	200.8±162.8/ 127.6±106.5	89.3±6.4/ 85.7±9.8	5.5±1.3/ 4.4±2.3

SD: standard deviation; [A]: Exposed: the participants who had been exposed or not exposed to any of these agents.

Table 2.8: Impact of Physical and chemical exposure on semen parameters at baseline

Sperm parameters (Mean ± SD)	Hazardous agents				
	Zero (n=2)	One (n=5)	Two (n=5)	Three (n=4)	Four (n=4)
Volume (ml)	**5.2[a]±3.7**	2.5±1.1	3±1	3.5±0.5	2.2±1
% progressively motile sperm	64.2±19.1	55.8±12.8	66.4±8.8	59.3±26.3	44.0±15.8
% non-motile sperm	25.8±17.2	36.0±13.2	**23.0[b]±7.8**	29.6±21.1	45.1±17.2
Sperm concentration (10^6/ml)	78.5±39.0	33.7±20.0	63.5±34.1	36.3±21.2	30.8±24.1
Sperm count (10^6)	**335.2*±85.0**	74.7±60.3	194.1±138.0	124.4±63.0	83.2±101.6
% vital sperm	87.4±10.4	89.0±4.8	90.4±3.6	88.0±10.7	76.2±12.3
% morphologically normal sperm	5.0±1.4	4.8±1.5	6.2±2.4	3.5±2.6	3.3±2.0

n: number of participants; SD: standard deviation; Zero: participants not exposed to any agents; One: participants exposed to any one of these agents; Two: participants exposed to any two of these agents; Three: participants exposed to any three of these agents; Four: participants exposed to four or more of these agents; [a]: Significant increase between zero group vs one and four groups $p<0.05$; [b]: Significant decrease between two group vs four group shown in bold, $p<0.05$; * Significant increase between zero group vs one; three and four groups shown in bold; *$p<0.05$.

Regarding general health status, there were no significant difference in sperm parameters between the participants who did or did not suffer from different health issues at baseline as shown in Table 2.9. Health issues were then sub-categorized as described in Table 2.10. There were significant differences in: semen volume between men with no health issues and those with 3 or 4 health issues; % progressively motile sperm when men with 1 or 2 health issues were compared with those with 4 or 5 issues and in % of non-motile sperm between men with 4 or more issues and those with 2 issues.

There were no significant differences in sperm parameters between groups categorized by combined exposure to cigarette smoking, alcohol, and drug consumption at baseline as shown in Table 2.11. Alcohol consumption was then sub-categorized as described in Table 2.12. There were no significant correlations between sperm parameters and alcohol consumption categories, except that there was a significant negative correlation (P=0.02) between semen volume and alcohol consumption categories.

Table 2.9: Semen parameters and general health issues in the three months prior to sample collection at study baseline

Health issues (No) /(Yes)	Sperm parameters of those men with no health issues/health issues (Mean ± SD)						
	Volume (ml)	% progressively motile sperm	% non-motile sperm	Sperm concentration ($X10^6$/ml)	Sperm count ($X10^6$)	% vital sperm	% morphologically normal sperm
[A] Health issues (2/18)	5±4/ 2.7±1.0	57.8±10.0/ 57.6±17.7	32.6±7.6/ 32.2±16.6	40.0±15.3/ 46.2±31.0	229.6±234.3/ 132.5±107.1	85.7±8.0/ 86.5±9.5	5.5±2.1/ 4.5±2.2
Fever or flu (12/8)	3.1±1.7/ 2.6±1.2	58±16/ 57.2±19.3	32.5±15.7/ 31.7±17.0	44.1±30.2/ 47.7±30.2	153.7±135.8/ 125.1±92.4	86±10/ 87.1±8.5	5.0±2.2/ 4.0±2.1
Illness (9/11)	3.5±2.0 /2.5±0.8	65±13/ 51.8±18.0	26.3±12.2/ 37.0±17.3	39.5±22.4/ 46.1±33.8	166.2±134.3/ 122.7±106.3	90.0±5.7/ 83.0±10.7	5.4±2.5/ 4.0±1.7
Breathing or lung problems (18/2)	3.0±1.6/ 2.8±0.4	58.2±15.7/ 53±34	31.7±14.3/ 37.0±34.5	43.7±26.1/ 62.4±65.3	137.7±114.3/ 183.0±201.7	87.6±7.0/ 76.0±22.5	4.7±2.3/ 3.5±0.7
Stress, depression or anxiety (18/2)	3.0±1.5/ 2±1	58±16/ 55±32	31.0±14.8/ 35.7±31.3	44.0±26.2/ 60.0±66.4	142±117/ 144.0±185.3	86.5±9.2/ 86.0±12.6	4.7±2.2/ 4.0±2.8
Headache and/or eyestrain (14/6)	3.2±1.6/ 2.3±1.0	63.2±15.2/ 44.7±13.8	27.4±14.6/ 43.5±13.0	51.7±31.7/ 31.1±18.2	168.5±125.4/ 81.0±77.7	88.0±9.4/ 83.0±8.4	5.1±2.0/ 3.3±2.0
Infections disease (virus, bacteria) (16/4)	3.0±1.6/ 2.2±1.0	60.0±17.3/ 48.0±12.4	30.0±15.8/ 40.5±14.7	47.2±31.2/ 39.0±23.3	152.4±124.3/ 101.4±94.2	86.6±10.0/ 85.8±6.5	5.0±2.3/ 3.5±1.7
BJMA (18/2)	3.0±1.6/ 2.5±0.1	60±16/ 36.8±11.2	29.7±14.4/ 54.5±10.0	48.1±30.0/ 22.4±8.8	152±121/ 54.6±20.0	87.6±7.0/ 75.6±22.0	4.7±2.3/ 4.0±1.4
BJMH (hips, legs or feet) (18/2)	3.0±1.5/ 2.0±0.6	58.6±17.6/ 49.4±6.5	31.2±16.2/ 41.0±9.3	47.3±30.6/ 29.4±1.0	151.7±121.3/ 57.0±16.7	86.7±9.4/ 84.5±9.5	4.5±2.3/ 5.5±0.7
BJM (back) (18/2)	3.0±1.6/ 3.0±0.6	58.0±17.5/ 54.0±13.2	31.6±16.2/ 37.8±13.8	46.8±30.8/ 34.4±8.1	147±124/ 100.5±44.8	86.0±9.6/ 90.3±1.3	4.6±2.3/ 5.0±0.0
Other (18/2)	3.0±1.6/ 2.3±0.4	59.6±16.2/ 40.2±16.0	30.3±15.0/ 49.0±17.7	48 ±30/ 23.7±10.6	152.3±120.8/ 51.4±15.5	87.7±7.0/ 75.2±21.5	4.7±2.3/ 3.5±0.7
Made worse by work (18/2)	3.0±1.5/ 1.8±1.0	59.8±16.3/ 38.6±8.8	30±15/ 52.7±7.3	48.3 ±29.6/ 20.3±11.8	153.5±119.4/ 41.0±39.4	86.7±9.3/ 84±10	4.7±2.2/ 3.5±2.0
Any medication (12/8)	3.4±1.6/ 2.2±1.0	60.6±16.4/ 53.3±17.7	29.4±14.4/ 36.4±17.7	46.8 ±29.8/ 43.7±30.8	167.6±126.5/ 104.2±101.0	87.6±7.8/ 84.7±11.3	4.8±2.6/ 4.4±1.5

SD: standard deviation; [A] Health issues: indicate if the participants had or did not have any of these health issue., BJMA (arms, hands, neck or shoulder and BJMH (hips, legs or feet).

Table 2.10: Semen parameters differences by categorized health issues at baseline of the study

Sperm parameters (Mean ± SD)	Health issues				
	Zero (n=2)	One (n=4)	Two (n=5)	Three (n=5)	Four (n=4)
Volume (ml)	**5.0[a]±3.9**	3.2±1.2	3.0 ±1.2	2.3±0.8	2.4±1.0
% progressively motile sperm	57.8±10.0	65.1±18.5	69.8±6.3	52.2±18.5	**41.7[b]±14.5**
% non-motile sperm	32.6±7.6	26.2±18.2	21.8±5.4	34.4±16.4	**48.4[c]±15.7**
Sperm concentration (10^6/ml)	40.0±15.3	48.6±37.2	55.1±30.7	48.0±35.7	30.5±24.1
Sperm count (10^6)	229.6±234.3	177.6±146	146.6±83.6	117.0±117.7	89.0±99.3
% vital sperm	85.7±8.0	90.0±6.4	**92.4[d]±3.0**	83.4±8.4	79.5±14.5
% morphologically normal sperm	5.5±2.0	5.5±3.7	5.4±1.0	4±2	3.0±1.4

n: number of participants; SD: standard deviation; Zero: participants with no health issues; One: men with one health issues; Two: men with two or three health problem; Three: men with four health issues; Four: men with five or more health issues; [a] Significant increase between zero issue group vs three issue group shown in bold $p<0.05$; [b]: Significant decrease between four issue group vs one and two issue groups shown in bold $p<0.05$; [c]: Significant increase between four issue group vs one and two issue groups shown in bold $p<0.05$; [d]

Table 2.11: Semen parameters and exposure to cigarette smoking, alcohol and drugs at study baseline

Sperm parameters (Mean ± SD)	Smoking habit, alcohol and drugs consumption (No) /(Yes)		
	Exposed to cigarette smoking (11/9)	Regular alcohol consumption (7/13)	Drug consumption (15/5)
Volume (ml)	3.0±1.8/ 3.0±1.1	3.2±0.8/ 2.8±1.8	3.2±1.5/ 2.1±1.2
% progressively motile sperm	60.4±17.7/ 54.3±16.1	55.3±20.8/ 59.0±15.1	58±18/ 56.7±14.7
% non-motile sperm	31.0±17.2/ 33.7±14.7	34.0±18.6/ 31.3±14.8	31.8±16.7/ 33.4±14.4
Sperm concentration (10^6/ml)	49.7±33.7/ 40.6±24.4	44.4±31.7/ 46.2±29.6	49.6±32.0/ 33.6±18.0
Sperm count (10^6)	153.0±131.2/ 129.2±107.0	140.7±91.3/ 143.1±134.4	166.8±126.0/ 68.5±45.6
% vital sperm	87.0±10.2/ 85.8±8.3	84.1±13.0/ 87.7±6.7	86.4±9.6/ 86.5±8.6
% morphologically normal sperm	5.0±1.5/ 4.1±2.8	3.7±2.3/ 5.1±2.1	4.5±2.3/ 4.8±2.0

Table 2.12: Semen parameters and alcohol consumption categories at study baseline

Sperm parameters (Mean ± SD)	Alcohol consumption and Spearman correlation test			
	Non- or light-drinkers (n=7)	Moderate drinkers (n=10)	Heavy drinkers (n=3)	Spearman correlation R (n=20)
Volume (ml)	3.2±0.8	3±2	2±1	**-0.5***
% progressively motile sperm	55.3±20.8	57.1±16.1	65±11.4	0.1
% non-motile sperm	34±18.6	34±15.5	22.6±9	-0.13
Sperm concentration (10^6/ml)	44.4±31.7	38.2±25.3	73±31.6	0.17
Sperm count (10^6)	140.7±91.3	138.5±138.8	158.4±145.7	-0.18
% vital sperm	84.1±13	87±7.2	90.1±4.5	0.12
% morphologically normal sperm	3.7±2.3	5±2.3	5.3±1.5	0.26

n: number of participants; SD: standard deviation; Non- or light-drinkers: refers to participants who consumed <1 unit of alcohol per week; Moderate drinkers: refers to participants who consumed 1-20 units per week; Heavy drinkers: refers to participants who consumed >20 units per week; *Bold correlation is significant at the 0.05 level (2-tailed).

Table 2.13: Semen parameters differences in four exposure habit categorized issues at baseline

Sperm parameters (Mean ± SD)	Alcohol consumption and Spearman correlation test			
	(i) (n=3)	(ii) (n=9)	(iii) (n=4)	(iv) (n=4)
Volume (ml)	3±1	3.4±1.8	2.3 ±1.3	2.4±1.2
% progressively motile sperm	72.2±6.6	53.5±20.1	59.0±12.1	55.0±16.4
% non-motile sperm	20.4±6.8	35.8±18.7	29.8±13.1	35.5±15.7
Sperm concentration (10^6/ml)	59.3±41.0	44.1±32.4	57.1±25.5	27.1±11.6
Sperm count (10^6)	171.0±105.6	163.5±135.7	144.3±143.6	70.7±52.3
% vital sperm	91.8±2.7	83.3±11.3	91.1±4.3	84.8 ±9.0
% morphologically normal sperm	6±1	**3.3*±1.7**	6.8±2.4	4.3±1.7

n: number of participants; SD: standard deviation; (i): refers to those men who were not exposed to any of the three items (smoking , alcohol, and drug); (ii): refers to men who were exposed to any of the three; (iii): refers to men who were exposed to any two of the three; (iv): refers to men who were exposed to all three; * Significant decrease versus I and iii groups $p<0.05$.

Exposures were then sub-categorized as described above in Table 2.13, there were no significant differences between the categories except there was a significant reduction in % normal sperm morphology in the ii group when compared with i and iii groups.

2.3.4.1 Correlation between semen parameters and diet and dietary supplements at study baseline

The data presented in Table 2.14 shows that there were no significant differences between meat and fish eaters and fish eaters in any sperm parameters.

Table 2.14: Semen parameters and diet at study baseline

Sperm parameters (Mean ± SD)	Diet	
	Meat and fish eater (n=4)	Fish eater (n=15)
Volume (ml)	2.7±1.4	3.0±1.6
% progressively motile sperm	54.0±6.7	57.3±18.5
% non-motile sperm	35.2±10.5	32.7±17.0
Sperm concentration (10^6/ml)	33.3±24.2	45.0±27.4
Sperm count (10^6)	105.8±100.6	143.1±123.4
% vital sperm	85.0±6.7	86.3±10.0
% morphologically normal sperm	3.3±2.0	5.0±2.2

n: number of participants; SD: standard deviation

There were no significant correlations between the standard semen parameters and the consumption of five types of meat. However, there was a significant negative correlation between % progressive sperm motility and chicken consumption, and a significant negative correlation between red meat consumption and sperm count (P= 0.05). There were no significant correlations between the standard semen *p*arameters and the consumption of any vegetable type or nuts and seeds. However, the consumption of dried fruits showed a significant negative correlation with % non-motile sperm and a significant positive correlation with % morphologically normal sperm (P= 0.05). There were no significant correlations between the standard semen parameters and the consumption of the various world-wide foods: crisps/chips, pulses, spices, cereals and pizza. However, there were significant positive correlations between the consumption of crisps/chips and sperm concentration and between and the consumption of cereals and the % morphologically normal sperm (P= 0.05).

There were no significant correlations between the standard semen parameters and the consumption of: sugar and confectionary; caffeinated sugar, low sugar or non-sugar beverages or non-caffeinated sugar or non-sugar beverages. However, there was a significant positive correlation between the consumption of caffeinated low sugar beverages and % vital

sperm (P= 0.05). Five of the participants consumed dietary supplements, while fifteen men did not take any kind of food supplement. There was no significant difference in sperm parameters between consumers and non-consumers as shown in Table 2.15.

Table 2.15: Semen parameters and consumption of dietary supplements at study baseline

Sperm parameters (Mean ± SD)	Dietary supplement items	
	Not consumed (n=15)	Consumed (n=5)
Volume (ml)	3.3±1.5	1.7±1
% progressively motile sperm	59.6±17.8	53.4±14.7
% non-motile sperm	30.1.4±16.3	36.3±15
Sperm concentration (10^6/ml)	49±32	59.6±17.8
Sperm count (10^6)	168.4±124.8	62.6±50.4
% vital sperm	86.7±9.8	84.4±8.3
% morphologically normal sperm	4.5±2.3	5±2.2

n: number of participants; SD: standard deviation; Not consumed: refers to participants that did not take any dietary supplement items; Consumed: refers to the participants that had taken any kind of dietary supplement items.

2.3.4.2 Semen quality and participant exposures twenty-four hours prior to sample collection at baseline

There were no significant differences in semen quality between the sub-categories described in Table 2.16.

Table 2.16:Semen parameters and exposures 24 hours prior to semen collection

24 hours of exposure factors (No/Yes)	Sperm parameters not exposed/exposed (Mean ± SD)						
	Volume (ml)	% progressively motile sperm	% non-motile sperm	Sperm concentration ($X10^6$/ml)	Total sperm count ($X10^6$)	% vital sperm	% morphologically normal sperm
Exposure to smoke (16/4)	2.7±1.6/ 3.8±0.4	56.3±18.4/ 63.3±7.0	34±17/ 25.2±6.0	46.0±30.4/ 57.2±26.1	124±117/ 215±107	85.2±10.0/ 91.3±2.4	4.6±1.8/ 4.8±3.8
Job attendance (10/9)[M]	2.8±1.0/ 3±2	58.6±20.4/ 54.3±12.0	31.0±18.7/ 35.6±12.0	51.3±33.7/ 42.0±18.3	143.8±115.0/ 126±126	86.6±11.6/ 85.3±6.2	5.0±2.5/ 4.0±1.8
Red meat consumed (11/9)	3±1/ 3±2	58.5±17.5/ 56.6±17.0	30±16/ 35.0±15.8	54±31/ 40±17	161.5±116.2/ 118.6±124	86.7±11.0/ 86±7	4.7±2.7/ 4.4±1.5
Chicken or poultry consumed (8/12)	2.7±1.0/ 3.0±1.7	66.4±12.2/ 51.8±17.4	24.6±12.8/ 37.3±16.0	63±39/ 41±18	169.6±134.5/ 124±109	91.3±4.7/ 83.2±10.0	5.4±2.3/ 4±2
Fish consumed (15/5)	3.0±1.6/ 2.3±1.3	56.8±15.6/ 60.3±22.0	32.3±14.8/ 31.8±20.3	52±33/ 46±19	154.8±128.2/ 104.7±82.4	85.6±10.0/ 89±7	4.4±2.3/ 5.2±2.0

[M] refers to one missing set of data, SD: standard deviation

2.3.5 Longitudinal analyses

2.3.5.1 Questionnaire answers throughout the study period

Only two participants showed no differences in any of the answers to the question about - general health in the past three months. At all times that the questionnaires were answered, of the participants 75% reported that they never smoked during the study, 55% reported that they consumed alcohol,5% reported no drug consumption and 16 % did not travel. In addition, there were no large variations in the answers concerning physical, chemical exposure agents and health issues.

2.3.5.2 Levels of PM2.5 throughout the study period

The average $PM_{2.5}$ concentration recorded at Manchester Piccadilly station was 7.4±2.6µg/m,^{3} the variation throughout the study period is shown in Figure 2.10.

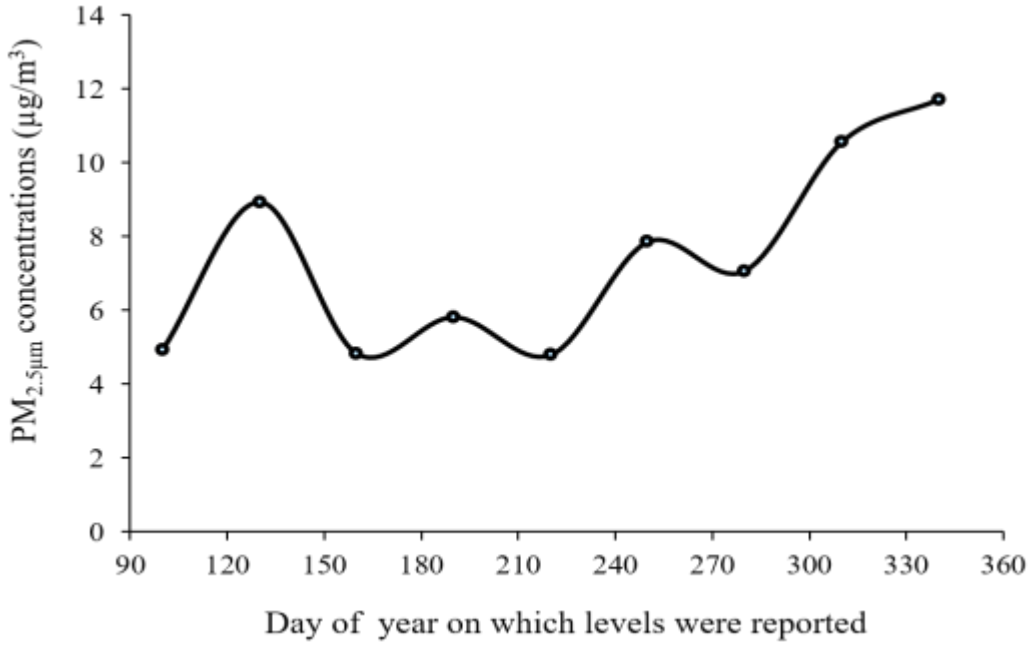


Figure 2.10 Variabilities of $PM_{2.5}$ throughout the study period

2.3.5.3 Food items consumed throughout the study period

Table 2.17 lists the food items that were consumed throughout the study period. Excluded from the table are items that were reported to have been consumed less by than 5% of the participants. These were: non-caffeinated sugar beverages, non-caffeinated non-sugar beverages, caffeinated lower sugar beverages, dried fruits, Soy, seafood, and offal. Sixty

eight percent of participants did not consume any kind of dietary supplement at all times while two participants consumed supplementary food substances at all times.

Table 2.17:The frequency of consumption of different food items through the study period

Food items	Q2/n19	Q3/n19	Q4/n19	Q5/n18	Q6/n18	Q7/n17	Q8/n/16	Q9/n15	Q10/n15	Q11/n15	Q12/n15
	% of the participants consuming the indicated food items										
Chicken	13	10	9	7	10	9	11	13	13	16	14
Red meat	11	5	5	7	7	6	5	8	9	8	8
Processed meat	8	7	7	9	9	9	10	13	10	9	12
Fish	8	12	5	5	3	5	5	6	8	5	8
Oil	6	7	7	8	6	8	9	9	10	11	12
HFDairy	24	26	22	24	21	24	22	22	19	24	26
LFDairy	11	11	12	8	12	12	7	13	14	13	13
Eggs	23	22	24	26	9	18	14	15	19	15	13
Fruits	5	10	10	7	5	9	6	6	5	7	8
Cruciferous	4	4	4	4	3	3	4	4	5	5	5
Leafy green	6	5	5	5	4	6	5	7	7	7	8
Crips/chips	10	10	11	11	12	11	11	13	9	12	10
Pulses	5	6	4	5	5	5	3	4	5	4	8
Other veg	20	22	18	22	20	23	25	26	26	33	38
Spicy	7	7	5	5	4	7	4	6	6	9	7
Cereals	52	43	37	35	38	42	36	40	45	43	44
Pizza	4	3	3	5	5	6	4	4	4	5	4
Sugar and confectionary	27	34	27	28	26	28	33	24	27	29	30
Caffeinated non-sugar beverages	30	31	27	30	28	32	38	33	32	34	40
Caffeinated sugar beverages	4	5	5	5	5	5	6	7	7	8	7
Nuts & Seeds	6	8	7	5	5	7	5	9	9	9	11

Q: the visit number at which the food frequency questionnaire was completed from 2nd to 12th; n: the number of participants completing each questionnaire.

2.3.5.4 Lifestyle and environmental exposures throughout the study period

There were no substantial differences in the lifestyle and environmental exposures through the six months of study (data not shown). This might reflect the short time periods of the study and between each completed questionnaire.

2.3.5.5 Variability of semen parameters throughout the study period

Appendix 5 lists all of the results for all 7 semen parameters for all participants throughout the study and Table 2.18 lists the mean values. Most mean values are based on 12 semen

samples but others involved 1, 4, 6, 7, or 8 samples as shown. The % normal sperm morphology was not included in this analysis as it was determined only three times.

The % vital sperm was in the range of WHO 2010 reference values for all participants. The semen volumes were in the range of WHO 2010 reference values except for three participants who had lower values (Table 2.18). There was only one participant whose % progressive sperm motility was lower and % non-motile sperm was higher than the WHO 2010 reference values. Two participants had lower sperm concentrations than the WHO 2010 reference values, and one of these had a lower sperm count than the WHO 2010 reference values (Table 2.18).

The data in Table 2.18 was used to determine the mean and CV of the semen parameters throughout the study period. Figure 2.11 shows that % vital sperm was the parameter with the smallest variation (CV 3- 18.5%), and the total sperm count was the parameter with the highest variation (CV 34-107.2%).

The CV of % non-motile sperm was higher than the CV of total sperm count for two participants, and another participant his CV of sperm concentration was higher than that for total sperm count. The CV of sperm concentrations ranged between 27- 71.5% which was higher than the CV of % progressive sperm motility, except for one man. Seven men had CVs for the % non-motile sperm that were higher than those for their sperm concentration and three men had CVs for semen volume higher than those for their sperm concentration (Figure 2.11).

In addition, some participants showed particularly high variations in % progressive motile sperm, % non-motile sperm and semen volume and the values for these participants are presented in Figure 2.12.

Table 2.18: Semen parameters throughout the study period

Participant ID (n)	Sperm parameters mean ± SD					
	Semen volume (ml)	% progressively motile sperm	% non-motile sperm	Sperm concentration ($X10^6$/ml)	Total sperm count ($X10^6$)	% vital sperm
P01 (6)	6.7±2.4(↔)	52.5±5.8(↔)	37.0±3.2(↔)	34.5±17.3(↔)	216.3±133.2(↔)	79.7±4.4(↔)
P02 (12)	5.8±2.3(↔)	60.6±21.0(↔)	31±22(↔)	75.7±44.2(↔)	415.0±232.8(↔)	83.4±11.2(↔)
P03 (1)	3.5	70(↔)	22(↔)	26.4(↔)	92.4(↔)	95(↔)
P04 (12)	1.2±0.5(↓)	55.8±9.5(↔)	36.5±10.0(↔)	28.5±10.1(↔)	35.0±16.6(↔)	83.3±5.0(↔)
P05 (12)	2.5± 0.6(↔)	45±13(↔)	43.4±16.6(↔)	33.5±12.2(↔)	82.8±37.7(↔)	79.8±4.6(↔)
P06 (12)	1.8±0.5(↔)	64.0±8.3(↔)	26.7±8.4(↔)	56.8±24.5(↔)	98.5±49.5(↔)	86.3±3.8(↔)
P07 (12)	2.8±0.3(↔)	59.7±10.0(↔)	30.3.10.5(↔)	53.8±25.2(↔)	154.5±77.0(↔)	88.0 ±5.7(↔)
P08 (7)	2.0±0.9(↔)	34.6±21.4(↔)	58.1±24.0(↔)	35.1±24.5(↔)	81.4±87.2(↔)	78.4±14.5(↔)
P09 (12)	2.5±0.7(↔)	71.4±7.5(↔)	19.8±6.6(↔)	60.0±22.3(↔)	147.0±51.7(↔)	90.0±3.2(↔)
P10 (12)	1.0±0.2(↓)	79±8 (↔)	13.4±5.4(↔)	86.4±26.0(↔)	89.0±30.5(↔)	92.0±2.8(↔)
P11 (12)	2.0±0.6(↔)	70.6±7.3(↔)	21±7(↔)	61.0±19.8(↔)	116.7±48.0(↔)	91.2±2.4(↔)
P12 (12)	2.4±1.0(↔)	31.5±5.5(↔)	58.0±6.6(↔)	18.5±11.7(↓)	40±26(↔)	77±5(↔)
P13 (12)	4.0±0.4(↔)	59.6±10.7(↔)	32.5±10.5(↔)	37.5±14.5(↔)	153.0±64.7(↔)	86.2±5.0(↔)
P14 (4)	3.6±0.4(↔)	49.9±6.3(↔)	41.7±11.1(↔)	30.3±15.6(↔)	110.2±58.2(↔)	85.5±3.9(↔)
P15 (12)	3±1(↔)	39.8±14.0(↔)	50.4±15.8(↔)	30.6±9.4(↔)	91.4±47.0(↔)	82.0±5.8(↔)
P16 (12)	2.2±0.8(↔)	70±7(↔)	19.6±6.3(↔)	95.6±31.1(↔)	195.6±88.0(↔)	90.5±3.8(↔)
P17 (12)	3±1(↔)	26±11(↓)	64.2±13.3(↑)	26.1±12.8(↔)	73.3±37.4(↔)	69.0±8.7(↔)
P18 (12)	1.7±0.4(↔)	66.0±15.8(↔)	24.2±15.6(↔)	76.3±35.5(↔)	138.2±88.2(↔)	84.5±5.3(↔)
P19 (8)	1.4±0.4(↓)	49.3±14.1(↔)	44.9±15.3(↔)	8.0±5.7(↓)	10.2±7.3(↓)	85.3±2.7(↔)
P20 (12)	3.0±1.3(↔)	60.8±8.0 (↔)	29.7±8.2(↔)	68.3±18.5(↔)	186±92(↔)	82.2±5.5(↔)

P1 to P20: the participants from 1 to 20; SD: standard deviation; n: number of semen samples from each participant; ↔: within the range of WHO reference value; ↓: lower than WHO reference value and ↑ higher than WHO reference value.

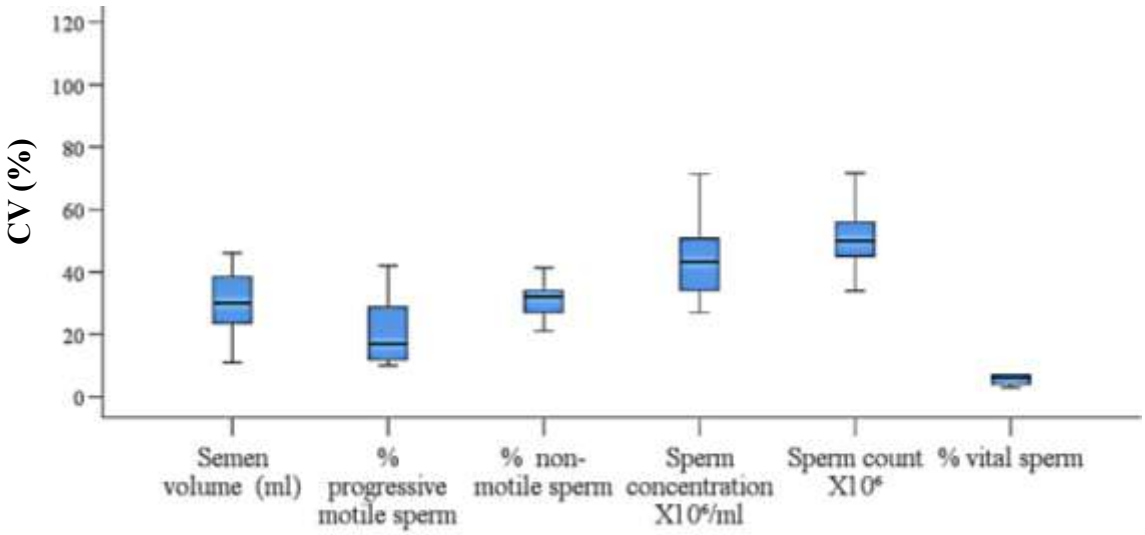


Figure 2.11 Variability in sperm parameters throughout the study period

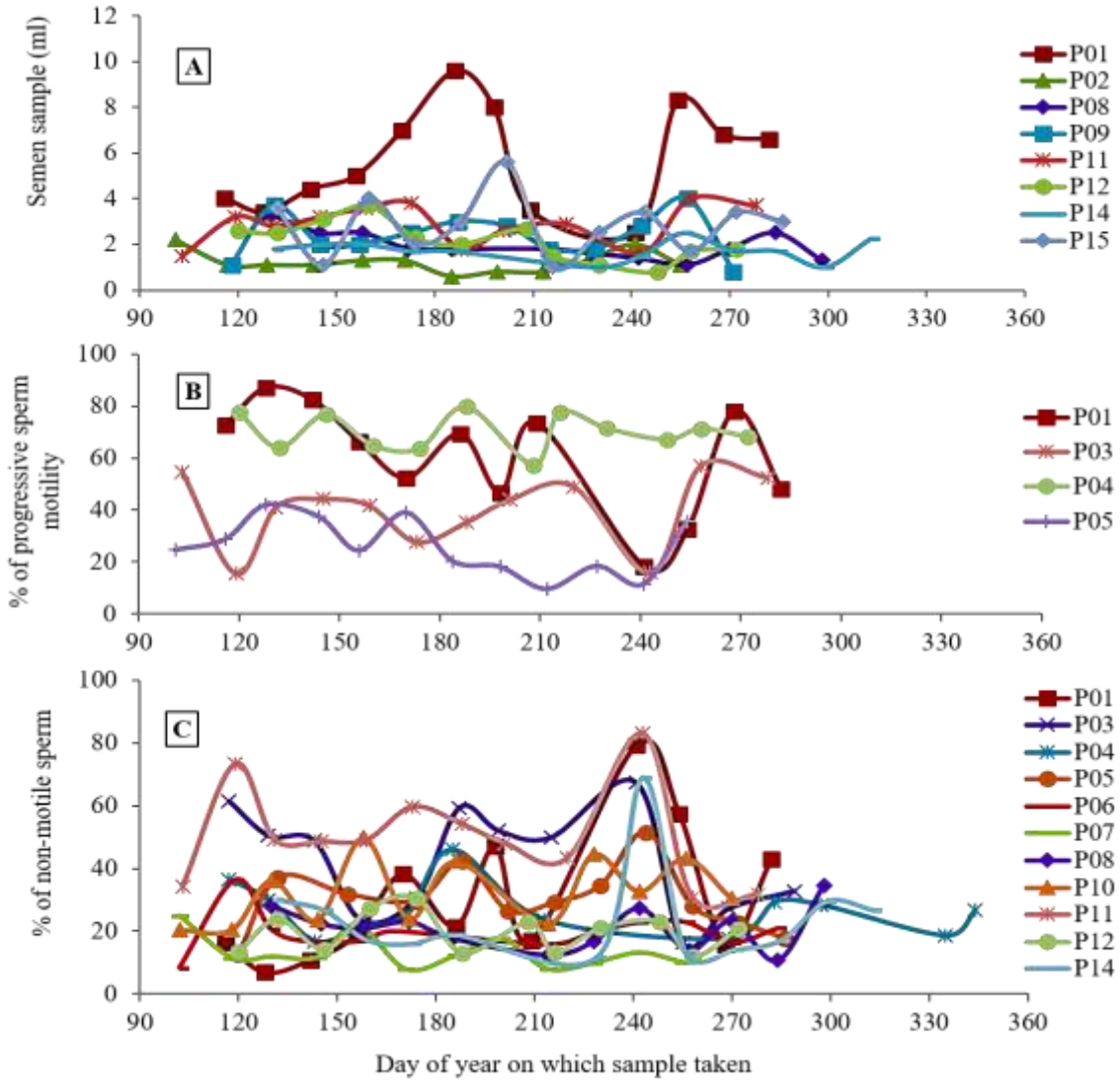


Figure 2.12 The participants showing the highest variabilities in semen parameters

A: Semen volume (ml), B: % progressive sperm motility, C: of non-motile sperm, P1 to P15 indicates participant number.

2.4 Discussion

In this chapter an examination of the correlation between sperm concentration determined by CASA and haemocytometer was undertaken and it was concluded that there was a significant positive correlation between these two methods. This was compatible with previous findings (Larsen et al., 2000; Wijchman et al., 2001; Rajashri et al., 2016).

In addition, the possible impact of various lifestyles and environmental exposures on sperm parameters was investigated firstly at baseline, when data was available for all 20 participants and then in a longitudinal study spanning a period of approximately three spermatogenesis cycles in the same individuals. Of the initial participants in this study, 15 completed the whole study and 5 did not, these dropping out after the 2nd. 5th, 7th, 8th and 9th visits.

In the baseline analyses, after exposure to various agents and health issues were categorized, there was a significant difference in the semen volume between those men exposed to none, one or four or more agents. There was a significant difference in the % of non-motile sperm across the groups and also there was a significant difference in sperm count between those unexposed and those exposed to any one, three or four or more agents. There was a clear decrease in all sperm parameters except for the % non-motile sperm when the unexposed groups were compared to the four exposed groups. In terms of sperm parameter variations over the longitudinal study, % vital sperm had the lowest whilst the total sperm count had the highest. However, some participants also had high variabilities in % progressive motility, non-motile sperm, and semen volume.

Most of the sperm parameters for most the participants were within the range of WHO 2010 reference values, and therefore those findings were compatible with Alvarez et al., (2003), but not with those reported by Carlsen et al., (2005). The latter study of 158 Danish young men reported that sperm concentration, total sperm count and sperm morphology did not change significantly during 4 years of follow-up. But the present study involved fewer participants and was over a much shorter time interval.

2.5 Conclusion

The small number of participants in this study did not enable confirmation of any association of environmental exposures or lifestyle factors with any of the sperm parameter examined. The possible size of any future studies are considered in Chapter 5.

Chapter 3: DNA damage in human sperm and its association with semen quality and environmental influences

3.1 Introduction

The integrity of sperm DNA is critically important for the accurate transmission of paternal genetic information as damage to the DNA can potentially affect the next and future generations (Shamsi et al., 2009). In sperm, as in somatic cells, DNA damage can be caused by endogenous agents, which are produced as normal metabolic by products, such as ROS and alkylating agents, and by exogenous factors including exposure to radiation, environmental and occupational toxicants, smoking and chemotherapy.

More recently, the integrity of sperm DNA has emerged as a parameter for understanding male infertility and many studies have investigated the association between environmental and lifestyle factors and conventional sperm parameters (Simon et al., 2010; Bungum et al., 2011).

Recent studies have started to measure the levels of DNA damage in human sperm and correlate them with sperm quality and exposures to identify whether human-made chemicals and pollutants and changes in diet and lifestyle can reduce male fertility (Pacey 2010).

A number of techniques have been used to detect DNA damage in human sperm such as SCSA, the TUNEL assay, and the Comet assay, as described in Chapter 1. Although both the SCSA and TUNEL assays can only indicate the percentage of sperm with fragmented DNA, which might be either SSB or DSB, in contrast, the Comet assay is a sensitive and simple assay that reportedly measures different types of DNA damage including strand breaks and alkali labile sites, providing quantitation of DNA damage in individual cells and enabling differences in response within a cell population to be studied. Additionally, it is an inexpensive technique and requires only a small number of cells or sperm (Tice et al., 2000; Wong et al., 2005; Baumgartner et al., 2009). Many of the published studies did not consider the impact of lifestyle and environmental exposures on DNA damage variability, and this is addressed in the present chapter.

3.1.1 Aims and objectives

The overall aim was to study the influences of lifestyle and environmental exposure on DNA damage of human sperm, as determine using the neutral Comet assay, through a six-month study period. Specifically, the objectives of this chapter were to:

1. Assess the temporal variation in sperm DNA damage.

2. Determine associations between sperm DNA damage and semen quality.

3. Examine correlations between lifestyle and environmental exposures and sperm DNA damage.

3.2 Materials and methods

3.2.1 Materials

All reagents were supplied by Sigma-Aldrich unless otherwise stated.

3.2.1.1 Cell culture

The cell line, HepG2, derived from the liver tissue of a fifteen-year-old male with differentiated hepatocellular carcinoma was purchased from Sigma-Aldrich UK. Cells were cultured in culture medium, i.e. minimum essential medium MEM (Gibco) containing 10% Foetal Bovine Serum, 2 mM l-glutamine, 0.105% sodium bicarbonate, 1% penicillin (10,000 units/ml), streptomycin (10 mg/ml) and 29.2 mg/ml L-glutamine in a 10 mM citrate buffer (PSG 100X, Gibco, UK). The final pH was 7-7.6. Trypsin-EDTA comprised 0.5 g trypsin and 0.2 g EDTA in 100ml of water.

3.2.1.2 Neutral Comet assay procedure

Normal melting point (NMP) agarose (SeaKem®LE Agarose, Lonza) was dissolved at 1% (w/v) in DPBS without Mg++ and Ca++ Low melting point (LMP) agarose (SAFC Biosciences ordered through Sigma) was dissolved at 0.7% (w/v) in the same buffer.

Lysis stock buffer contained 2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-base, pH 10 and was freshly prepared and stored at 4°C. Dimethyl sulphoxide

and Triton X-100 to final concentrations of 10% and 1% respectively, were added 10 min before use to make complete lysis buffer. For the lysis of sperm, proteinase K (DNase free) (100 μg/ml) and 10 mM DTT were added to portions of the complete lysis buffer. Electrophoresis buffer contained 300mM anhydrous sodium acetate and 100mM Tris-base adjusted to pH 9 using NaOH and was prepared fresh and stored at 4°C before use.

Neutralization buffer contained 0.4 M Tris-base adjusted to pH 7.5 by adding HCl and was stored at 4°C before use. SYBR gold (Invitrogen, UK) was diluted 1:10,000 in 10 mM Tris-HCl, 1mM EDTA, pH 7.5 and stored at 4°C.

3.2.1.3 Study population and sample collection

As mentioned previously 206 semen samples were obtained from all participants in the study, processed and stored as described in Chapter 2.

3.2.2 Methods

3.2.2.1 HepG2 cell culture

Approximately 1.5 x 10^6 HepG2 cells were seeded in a 75 cm^3 flask, in culture medium (see above) and incubated at 37ºC, 5% CO_2 and 3% O_2 in N_2. Every 5-7 days, when the cells had reached confluence, the cells were subcultured by washing twice with 6 ml DPBS without Mg++ and Ca++and then trypsinized using 750 μl of 0.05% w/v trypsin, at 37ºC, 5% CO_2 and 10 % O_2 in N_2 for 5 min an adjustment that was applied only at the time cells were harvested. Culture medium (6 ml) was then added to neutralize the action of trypsin. The HepG2 suspension was then transferred to a disposable 15 ml plastic tube and centrifuged at 1000 rpm, 4ºC for 5 min in a Sorvall RCB3 centrifuge. The supernatant was discarded and the HepG2 pellet was resuspended in 1 ml of culture medium and divided into two 75 cm^3 cell culture flasks each containing 10 ml culture medium and incubated at 37°C, 5% CO_2 and 3% O_2 in N_2. For HepG2 preservation, around 1-2 x 10^6 cells were dispersed in 1ml of culture medium containing 10% v/v DMSO, then chilled at 4°C for 30 min, and finally stored in a cryogenic vial at -80ºC. Two methods were used to harvest HepG2 cells from flasks. In the first, cells were harvested by trypsinization as described above and were suspended in 200 μl of DPBS in disposable 2ml microtubes. Alternatively, HepG2 cells were collected by scraping and then mixed with 6ml DPBS in disposable 15 ml Corning tubes and centrifuged

at 1,000 rpm, 4ºC for 5 min. The supernatant was discarded and the HepG2 pellet was suspended in 200 µl of PBS in 2ml microtubes.

3.2.2.1.1 Cell counting

Cell concentrations were determined by using a haemocytometer (Section 2.2.2.3.2). Briefly, 10µl of HepG2 cell suspension was added to each haemocytometer chamber, the number of cells in each chamber was counted, and the total cell concentration in 1 ml medium was calculated using the equation below: -

$$\textit{Total cell number (in 1 ml) = average cell number x } 10^4/\textit{ml}$$

3.2.2.1.2 Cell treatment

Cells were treated with H_2O_2 (5, 10, 20 and 40 µM) or TMZ (5 µM) in DPBS for one hour at 37ºC. They were then centrifuged for 5 min at 2400 rpm to remove the treatment agent and the supernatant was discarded and the cells resuspended in 200 µl of DPBS.

3.2.2.1.3 Neutral Comet assay of HepG2 cells and sperm

Slides were prepared by placing duplicate 175µl aliquots of melted 1% NMP agarose on the two areas of non-frosted side of microscope slides and then covered with coverslips (two 22×22 mm) and left at RT for 35 min to solidify. The coverslips were then removed, and the slides were kept in a box at RT overnight. All subsequent operations were carried out under low light or dark conditions wherever possible.

Approximately 10^6 HepG2 cells which had been harvested by either trypsinization or by scraping were suspended in 200uL DPBS. Approximately 2-3x10^5 (in 20uL) of treated and untreated cells were added to 230µl of DPBS and placed on ice for 10 min. The suspensions were then added to 1 ml aliquots of 0.7% LMP agarose at 37˚C and 100 µl of the mixtures added in duplicate to the NMP agarose-coated slides then covered with coverslips. After 15 min at RT, the covers were carefully removed and the slides immersed in complete lysis buffer and left for 100 min at 4°C. Then the slides were removed from the lysis buffer washed once with dH_2O for 5 min and left for 5 min to dry. Semen samples were centrifuged for 10 min at 900g and 4˚C to separate sperm and seminal plasma and then the sperm samples were washed twice with DPBS and suspended in DPBS at a concentration of 2×10^3 sperm/µl.

Aliquots (30 µl) of the sperm suspension were then added to 220µl of 0.7% LMP agarose at 37˚C and duplicate 75 µl aliquots processed in the same way as the HepG2 cell slides up to the lysis stage. For sperm lysis, the slides were immersed in cold complete lysis buffer, left for 1 hour at 4˚C, transferred to fresh lysis buffer containing 10 mM DTT, incubated for another hour at 4˚C, transferred to fresh lysis buffer containing proteinase K (100 µg/ml) and incubated for 1.5 hours at 37˚C. The slides were removed from the lysis buffer and washed once with dH_2O for 5 min.

For electrophoresis, the slides were placed horizontally in a black plastic electrophoresis tank containing freshly prepared cold electrophoresis buffer (the level of the buffer was approximately 0.25cm above the slide) and left for 20 min before the electric current was applied. Electrophoresis for HepG2 cells was one hour at 13V and 120-125 mA and for sperm was 25 min at 25 V and 300 mA. After electrophoresis, the slides were placed in cold neutralization buffer for 5 min twice and left to dry overnight.

The next day, slides were immersed in dH_2O for 30 min, left to dry for 5 min, stained by addition of 100 µl of diluted SYBR gold, covered with coverslips and left for a further 30 min at RT. The coverslips were removed, and the slides washed twice in dH_2O for 5-10 min to remove excess staining, covered again with a coverslip and left 2-3 hours.

3.2.2.1.3.1 Reproducibility and repeatability of the neutral Comet assay in human sperm

One semen sample was not from the longitudinal study and was used as a control to check reproducibility and repeatability of the neutral Comet assay. A Haemocytometer count indicated 24×10^6 sperm/ml. The sample was divided into 120 aliquots each containing approximately $3x10^5$ sperm and stored at -80^{o}C. The neutral Comet assay was carried out on aliquots on five different days and the rest of the aliquots were used as controls on each day that longitudinal sperm samples were analysed.

3.2.2.1.3.2 Analysis of Comets

The slides were examined at 20 X magnification using a NIKON Fluorescence Microscope with FITC filter (EX: 465-495, DM505 and BA: 515-555) provided with a HITACHI, HV, BCCD camera which was used to capture images for analysis. The images were analysed

either manually or automatically using OpenComet_imagej_v1.3 (http://www.cometbio.org/) and TriTeK CometScore™ Freeware version 1.5 (TriTeK Corp, US), (http://rexhoover.com/index.php?id=cometscoreas).

Both software packages can measure several parameters (Gyori et al., 2014; Altakroni 2015) as described in detail in chapter 1 (section 1.3.2.2.1.) Table 1.4. Neither of these automated analyses methods were able to analyze cells with very high levels of DNA damage because the head is far away from the tail (Shangula et al., 2019). Therefore, DNA damage was also evaluated by visual scoring of the Comets and assigning them to one of five categories according to the extent of DNA migration and tail length (Figure 3.1). The final score was the Genetic Damage Indicator (GDI), obtained by multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to the formula below (Collins 2004; Marques et al., 2016):

$$GDI = (\%\ grade\ 0\ cells \times 0) + (\%\ grade\ 1\ cells \times 1) + (\%\ grade\ 2\ cells \times 2) + (\%grade\ 3\ cells \times 3) + (\%\ grade\ 4\ cells \times 4)$$

The GDI was calculated from the grade scores of 200–250 cells and GDI values were expressed as "arbitrary units"

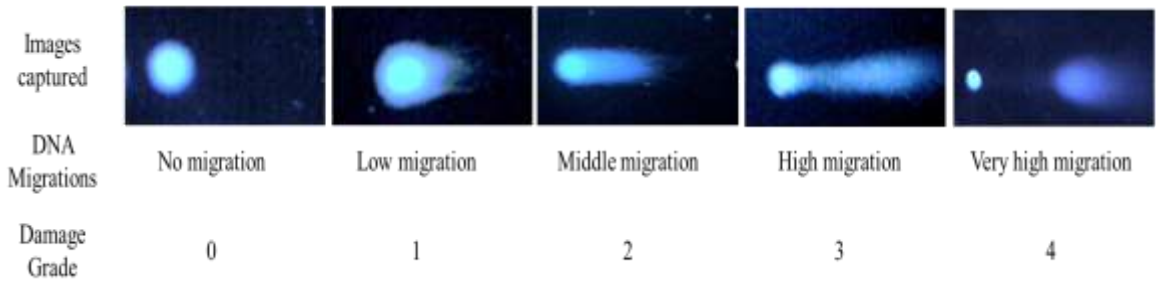


Figure 3.1 Grades of DNA Damage
HepG2 cells were harvested by scraping and processed as described in the text, and examined using a NIKON Fluorescence Microscope, with FITC filter, (EX: 465-495, DM505 and BA: 515-555) and images captured with a HITACHI, HV, BCCD camera. Magnification 200X.

3.2.2.2 Statistical analyses

The Spearman's rho was used to investigate the correlations between Comet tail parameters GDI and sperm parameters. The results using aliquots of the sperm sample that was not from the longitudinal study and were analysed by neutral Comet assay and the images were

analysed manually to calculate the GDI and the Comet tail parameters were obtained automatically by two software packages (Image J and Tritek). To examine the possible effect of the exposure to occupational and environmental factors on Comet tail parameters and GDI, the men were divided into exposed/unexposed groups.

3.3 Results

The HepG2 cell line was initially used to establish and optimize the neutral Comet assay before performing the assay in human sperm samples since the latter samples are precious. Subsequently, the neutral Comet was applied to the aliquots of the off-study sample and then the 206 semen samples collected during the longitudinal study.

3.3.1 Effects of treatment of HepG2 cells with H_2O_2 or temozolomide (TMZ)

HepG2 cells (0.125×10^6) were treated with H_2O_2 (0, 5, 10 and 20 μM in DPBS) for one hour at 37°C and processed as described above. Representative Comet images are shown in Figure 3.2.

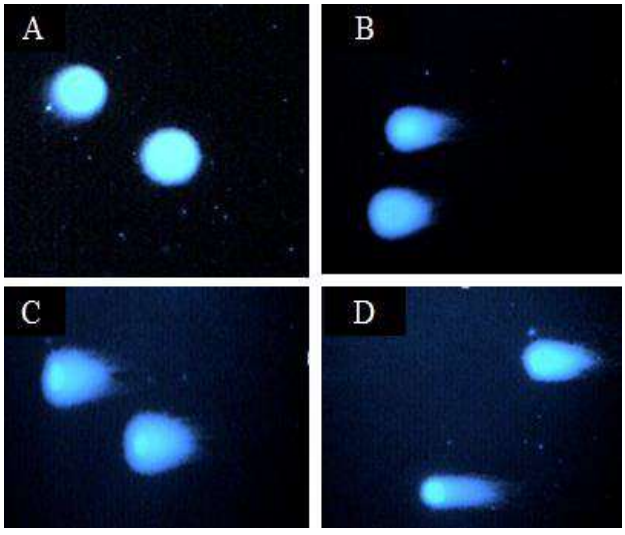


Figure 3.2 Neutral Comet assay images of HepG2 cells treated with H_2O_2
HepG2 cells were harvested by scraping, treated with H_2O_2 and processed as described in the text. A: control cells; B; C; D: 5, 10 and 20 μM. H_2O_2 Magnification 200X.

Cells (200-250) were scored on each slide and the mean (± SD) for tail length, % of DNA in the tail, tail moment and Olive tail moment were calculated by Open Comet_imagej_v1.3

software as displayed in Figure 3.3. All of the tail parameters increased linearly with increasing H_2O_2 concentration.

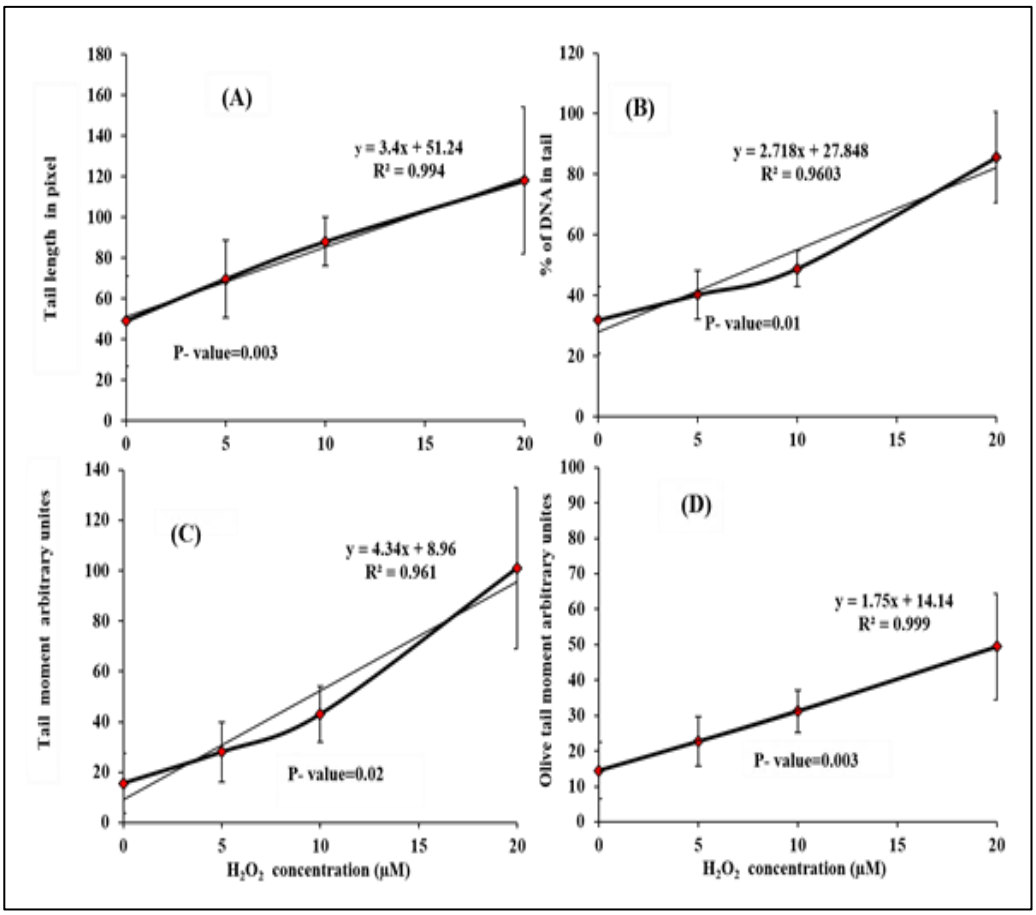


Figure 3.3 Dose dependent DNA damage induced by H_2O_2

HepG2 cells were harvested by scraping, treated with H_2O_2 and processed as described in the text. Images were analysed by OpenComet_imagej_v1.3 software to give: (A) Tail Length, (B) % DNA in Tail (C) Tail moment and (D) Olive tail moment.

3.3.1.1 Comparison of DNA damage between using scraping and trypsin to harvest the cells.

Two methods (trypsinization and scraping: Section 3.2.2.1) were used to harvest the cells after treatment with H_2O_2. After processing and image capture, tail length, % DNA in the tail, tail moment and Olive tail moment were determined by Open Comet_imagej_v1.3 software (Figure 3.4).

All tail parameters generally increased with increased H_2O_2 concentration with either harvesting method but damage levels at 20uM H_2O_2 as determined by tail length, tail

moment and Olive tail moment, but not % DNA in Tail were somewhat lower in cells harvested by scraping as shown in Figure 3.4.

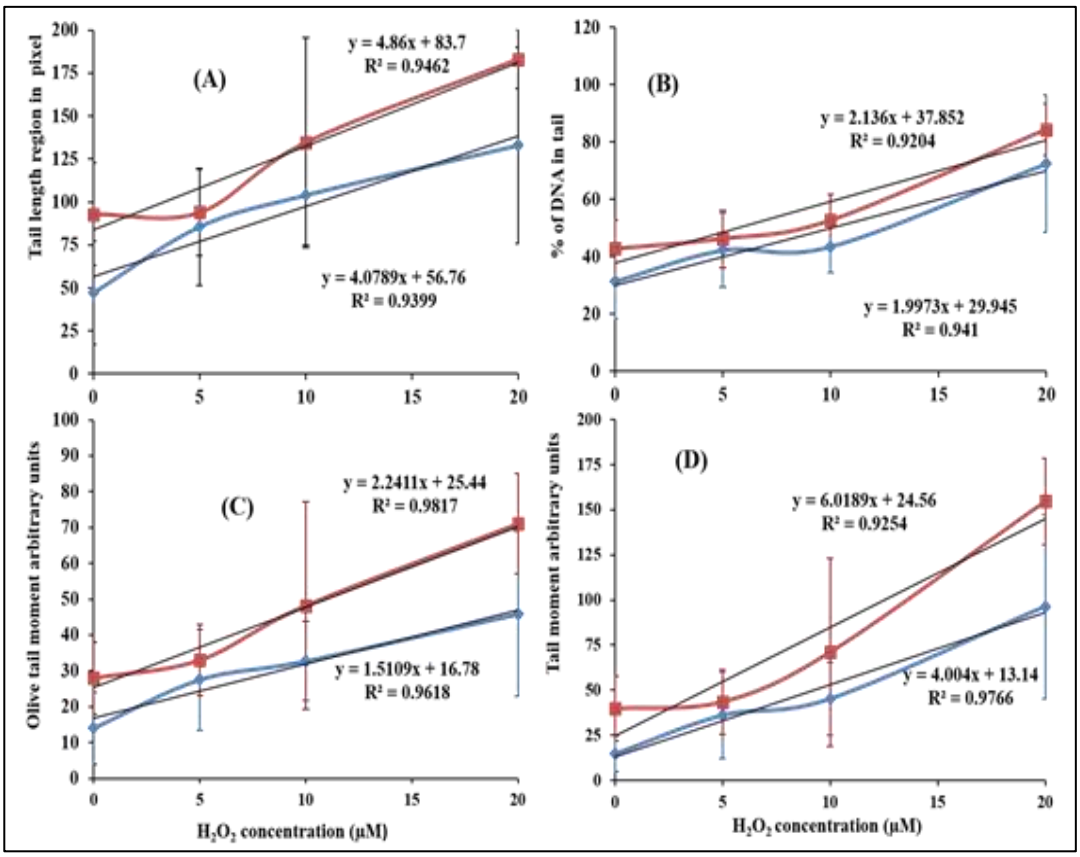


Figure 3.4 DNA damage in HepG2 cells assessed by neutral Comet assay
HepG2 cells were harvested by scraping, treated with H_2O_2 ls harvested by scraping (blue lines) or trypsinisation (red lines) and processed as described in the text. Images were analyzed by OpenComet_imagej_v1.3 software to give: (A) Tail Length, (B) % DNA in Tail (C) Tail moment and (D) Olive tail moment.

In further studies, damage induced in HepG2 cells by the methylating agent, TMZ H_2O_2 was compared with that induced by H_2O_2 as shown in Figure 3.5.

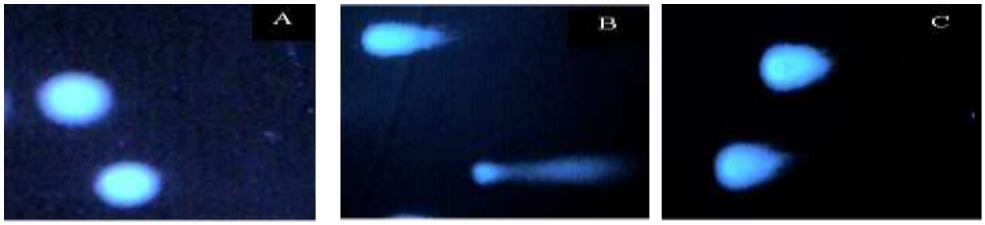


Figure 3.5 Neutral Comet assay images for HepG2 cells treated with TMZ and H_2O_2
HepG2 cells were harvested by scraping, treated with H_2O_2 or TMZ harvested by scraping and processed as described in the text. A: control cells; B: cells treated with TMZ (5 µM); C: cells treated H_2O_2 (20µM). (magnification 200X).

Manual visual grading for 200-250 cells showed there were statistically significant increases in grades 2, 3 and 4 levels of DNA damage after cells were treated with H_2O_2 and TMZ when compared with untreated cells. Correspondingly, there was a significant decrease in grade 0 and 1 levels of DNA damage in treated cells when compared with untreated cells as shown in Figure 3.6.

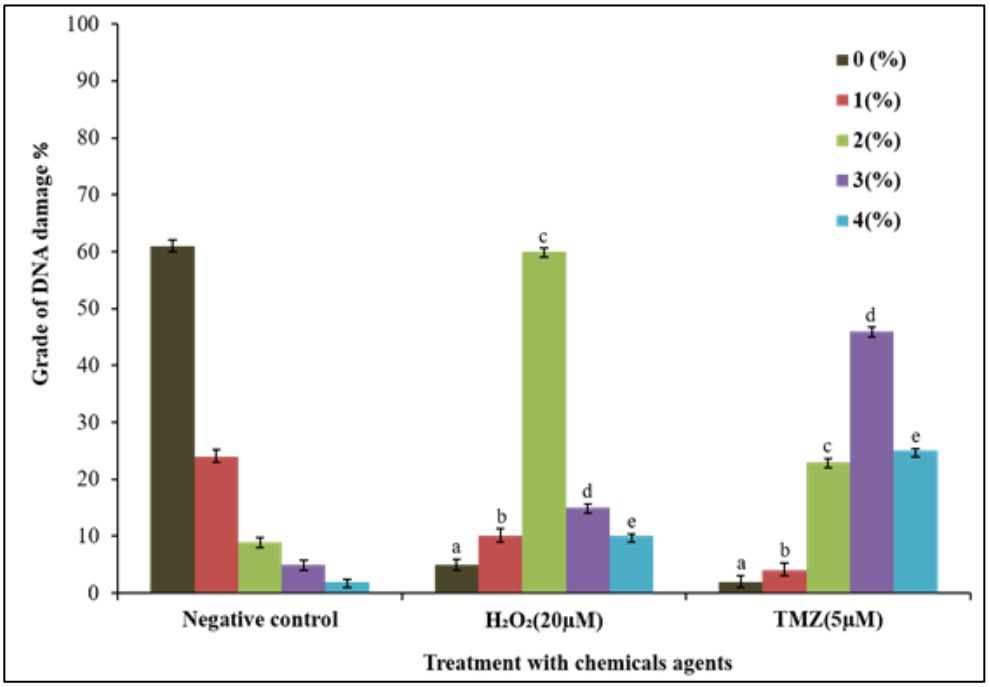


Figure 3.6 Mean values of individual DNA damage levels (0, 1, 2, 3 and 4), measured by the Comet assay in HepG2 cells after 1-hour exposure to H_2O_2 and TMZ
Bars represent standard error; statistically significant differences ($p<0.05$) are: a vs grade 0 in the negative control (untreated cells); b vs grade 1 in negative control; c vs grade 2 in negative control; d vs grade 3 in negative control; e vs grade 4 in the negative control.

Comparison of the GDI values with the tail length, % of DNA in the tail, tail moment and Olive tail moment as analysed by both software packages is shown in Table 3.1. There was a statistically significant increase in Comet tail DNA damage after cells were exposed to H_2O_2 and TMZ when compared with the negative control group. Comet software showed that TMZ has significantly greater ability to increase tail length, tail moment and Olive tail moment when compared to the negative control group or the H_2O_2 treated group suggesting that TMZ had greater ability to induce strand breaks than H_2O_2 at the doses used in these studies. The results for the two software packages were not statistically different, however, neither package was able to determine grades 3 and 4 of DNA damage, as the Comet tail was too far away from the head.

Table 3.1: Comparison of Comet parameters in HepG2 cells treated with H_2O_2 and TMZ

Chemical agents	Comet tail parameters (Mean± SD) [α]								GDI (Mean ± SD)
	Tail length (a)	% of DNA in tail (a)	TM (a)	OTM (a)	Tail Length (b)	% of DNA in tail (b)	TM (b)	OTM (b)	
Negative control	22±1	**54.5±8.3***	12±1	10±1	16±1	**66.3±3.2***	**11±1***	**8±0.7***	**67±3***
H_2O_2 (40µM)	**62±5***	82.3±5.4	**51±2.3***	**37±2.1***	**44±2***	91±2	40±1.2	**56±1.4*#**	250±3
TMZ (5 µM)	**69±2*#**	91.±6	**63±1.8*#**	**47±1.1*#**	**50±1.4*#**	94±1.6	47±1.2	37±1	286.7±2

HepG2 cells were harvested by scraping after treatment with H_2O_2 or TMZ, for 1 hour at 37oC, processed and analysed by (a) Image J or (b) Tritek software. Values shown are the to the mean and SD for three experiments TM: Tail moment, OTM: Olive tail moment; Statistically significant differences*vs control and #vs H_2O_2 in bold ($p<0.05$).

3.3.2 Reproducibility and repeatability of the neutral Comet assay in human sperm

Single aliquots randomly selected from the 120 aliquots of one human sperm sample, which was stored at -80ºC (section 3.2.2.1.3.1) were analysed on five different days using the neutral Comet assay and the images are shown in Figure 3.7.

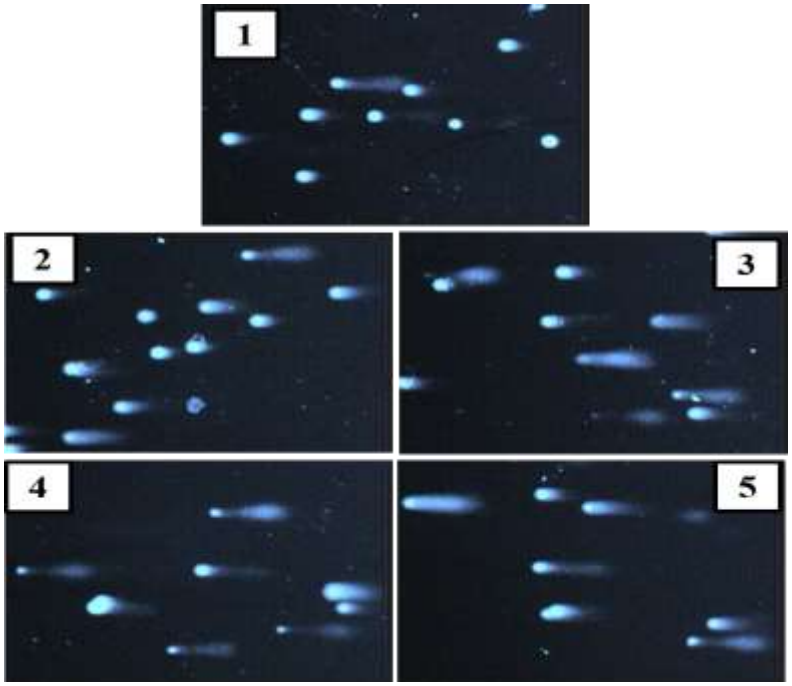


Figure 3.7 Images of neutral Comets of aliquots of the same human sperm sample measured on five different days (magnification 100X)

Analysis of the images showed there were no significant variations in all Comet tail parameters using both of the software packages and the manually obtained GDI over the five analyses as shown in Table 3.2.

In addition, these experiments thus indicated that the neutral Comet assay was reproducible. Therefore, one of the aliquots was used as a control each of the 16 times the Comet assay was undertaken for the longitudinal semen samples and Table 3.3 shows the results for these analyses.

Table 3.2: Tail parameters and GDI in aliquots from the same sample measured on five different days

Aliquot *	Image j				Tritik				GDI
	Tail length	% DNA in tail	TM	OTM	Tail Length	% DNA in tail	TM	OTM	
1	89.4	73	65.5	54.4	52	48	38	38	99
2	89.2	75	67	54.6	44	46	23	31	95
3	87	80	69.5	56.4	41	48	32	31	101
4	79	79	62.5	55.8	46	53	35	37	103
5	100	76	76	47	56	55	43	32	100
(Mean ± SD)	89±7.5	76.6 ±3	68 ± 5	53.6 ± 3.8	47.8 ±6.1	50 ±3.8	34.2 ±7.5	33.8 ±3.4	99.6±3
CV	8	4	7	7	13	8	22	10	3

∗ Each aliquot was processed on different days. TM: Tail moment, OTM: Olive tail moment.

There were no statistically significant differences in all Comet tail parameters from both software packages and GDI over the 16 Comet assays. The CV was higher for all Comet parameters using Tritek software when compared with Image j software.

The highest CVs were those for in Tail length, TM and OTM (22.8%, 19.5% and 19.8% respectively) using the Tritek software, which indicates its higher variance as shown below in Table 3.3. In conclusion, the neutral Comet assay was reproducible and repeatable.

Table 3.3: Tail parameters and GDI in aliquots from the same sample measured on sixteen different days during the longitudinal study

Aliquot * (n=16)	Image j				Tritek				GDI
	Tail length	% DNA in tail	TM	OTM	Tail Length	% DNA in tail	TM	OTM	
1	91	72	66	54	52	48	38	38	100
2	90	74	67	55	44	46	23	31	96
3	87	80	70	56	41	48	32	31	102
4	80	78	63	56	46	53	35	37	100
5	81	83	67	57	76	40	47	27	113
6	84	83	70	60	74	35	46	31	92
7	102	94	96	54	79	48	40	35	100
8	99	82	81	53	49	41	51	32	113
9	96	85	82	55	78	61	39	25	95
10	91	67	61	40	64	37	46	47	90
11	98	80	78	50	62	34	36	25	100
12	100	76	76	47	56	55	43	32	100
13	98	66	65	44	71	36	44	27	80
14	90	83	75	48	51	38	33	21	83
15	83	80	66	33	86	41	55	37	76
16	80	69	55	32	66	41	40	31	75
(Mean±SD)	90.6 ±7.6	78.3 ±7.3	71.1 ± 9.9	49.6 ± 8.5	62.2± 14.2	43.9± 7.8	40.5 ±7.9	31.7 ±6.3	94.7± 11.5
CV	8.4	9.4	13.9	17	22.8	17.8	19.5	19.8	12.2

∗ Each individual aliquot was processed on a day on which some of the longitudinal samples were processed for a total of 16 days; TM: Tail moment and OTM: Olive tail moment.

3.3.2.1 Correlations between Comet software package data and GDI in human sperm

There was a highly significant positive correlation between all the tail profiles obtained using either Image J or Tritek software packages and the GDI values as shown in Figure 3.8.

There were also a significant highly positive correlations between the % DNA in tail and OTM and between tail length and TM in both software packages as shown in Figure 3.9. Given these results, the tail length using Image J software might be considered the better sperm DNA damage indicator.

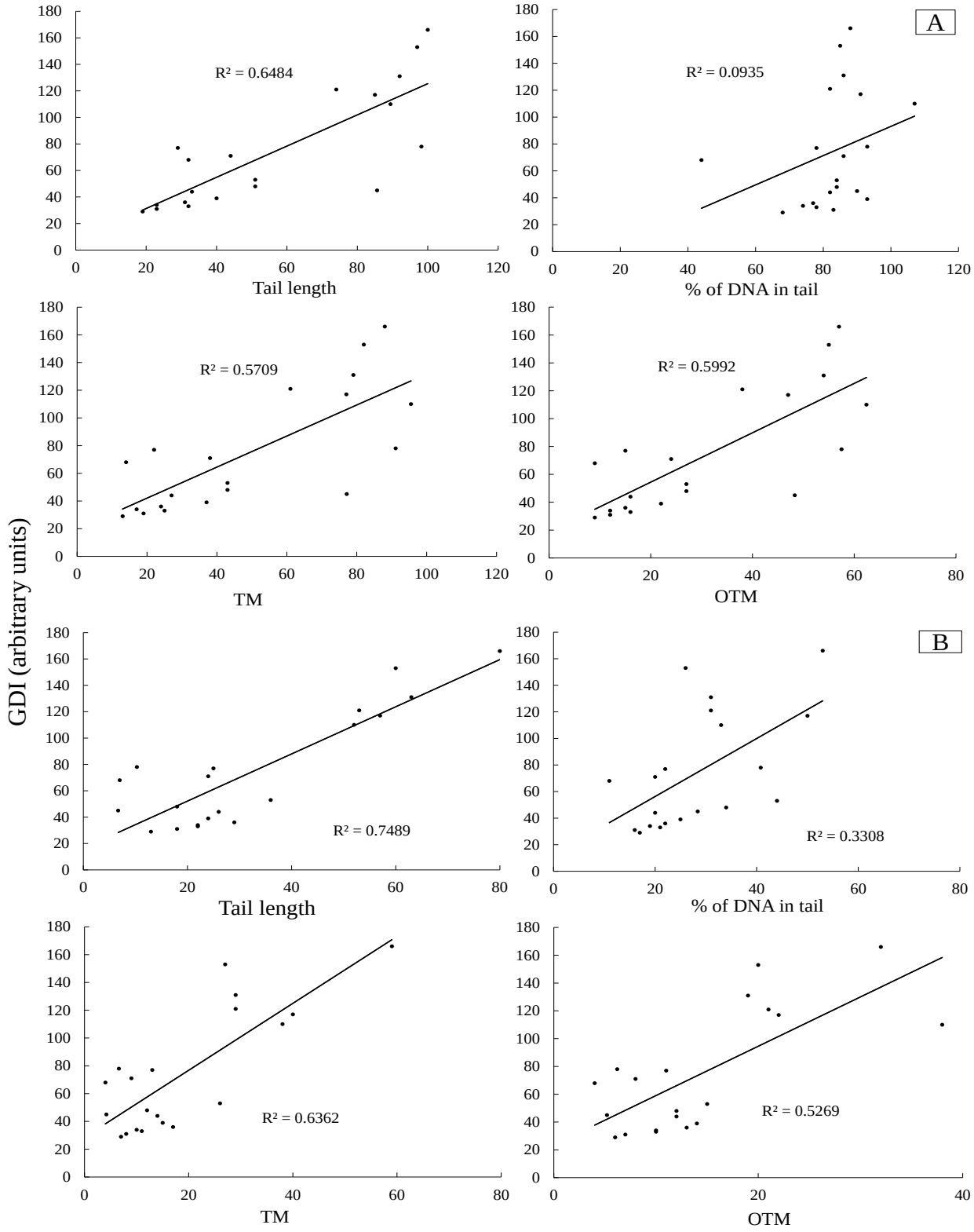


Figure 3.8 The correlation between GDI and Comet parameters using the two software packages at study baseline

The number of sperm samples included in this correlation was 20; Spearman's rho correlation (R^2); A: The correlation between GDI and Comet parameters using Image J software; B: the correlation between GDI and Comet parameters using Tritek software; TM: Tail moment, OTM: Olive tail moment.

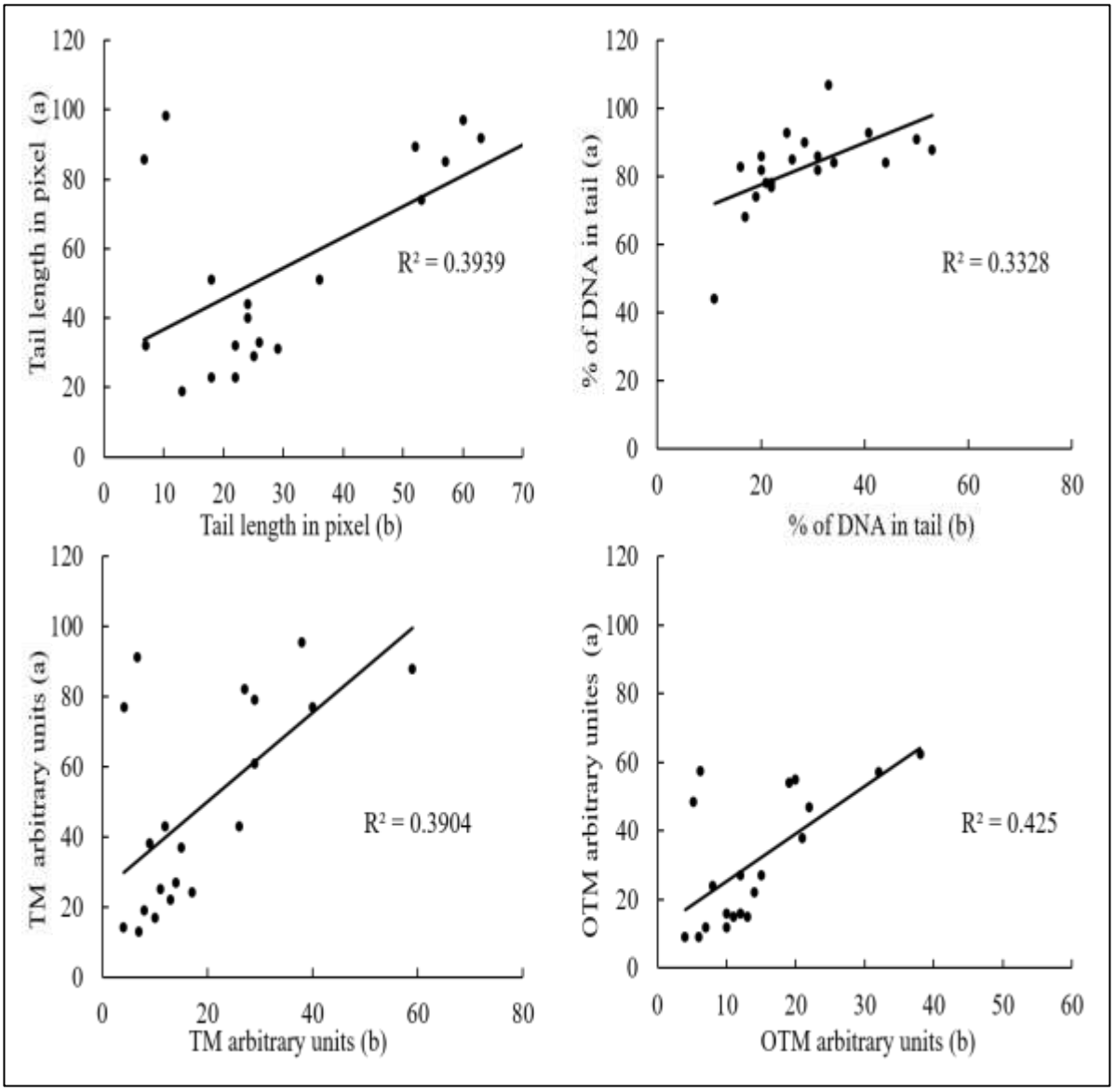


Figure 3.9 The correlation between Comet parameters in two software packages at study baseline

The number of sperm samples included in this correlation was 20; Spearmans'rho correlation (R^2); a: Comet parameters using Image J software; b: Comet parameters using Tritek software; TM: Tail moment and OTM: Olive tail moment.

3.3.3 Sperm DNA damage in the longitudinal study

All 206 semen samples from the longitudinal study were processed to determine sperm DNA damage by the neutral Comet assay. Each image was analyzed manually and the GDI was calculated. The images were also analyzed by both software packages to determine the tail characteristics. At baseline, the GDI value of the 20 men ranged between 29 and 166 with a mean of 74.2±43.6 and the CV was 58.7% which indicated that the GDI was highly variable.

There were six men from the 20 who had a GDI of more than 100 at baseline, while nine men had GDI lower than 50 and five men between 50 and 100 (Figure 3.10).

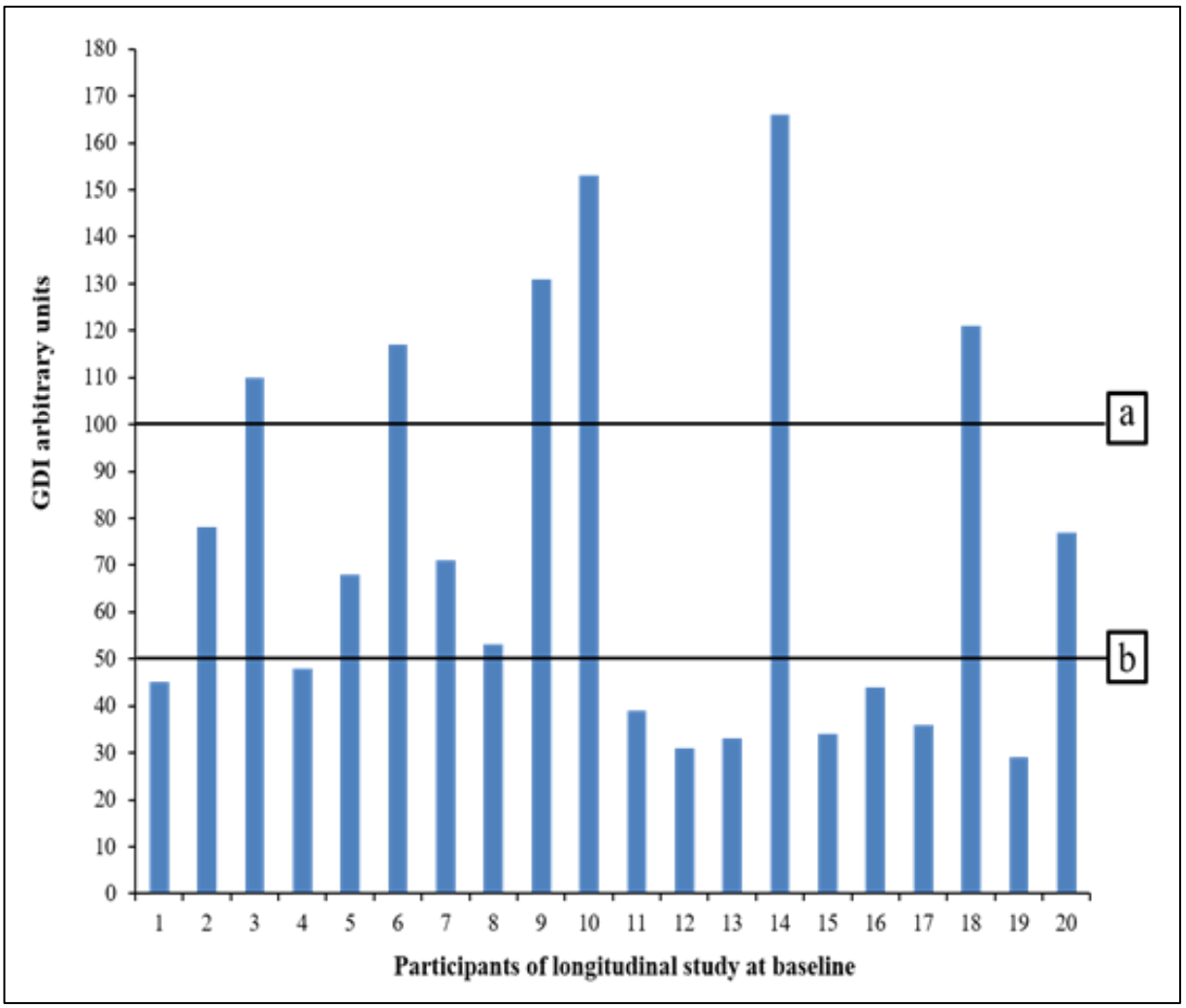


Figure 3.10 GDI levels of all participants at study baseline
a: HDG (GDI>100) six men; b: LDG (<50) nine men

Hence, the participants could be categorized depending upon their GDI level as either highly DNA damaged HDG (GDI>100), medium DNA damage MDG (GDI 50-100) and low DNA damage LDG (<50).The manual grading of LGD, MGD and HGD showed that the dominant grades were 0, 1 and 2 in the LGD, MGD and HGD groups respectively. Grade 3 was not recorded in LGD samples and Grade 4 was not seen in any group as the shown below in Figure 3.11.

Figure 3.12 below shows the variabilities Comet in tail length analysed by both Image J and Tritek software for all 20 sperm samples at baseline.

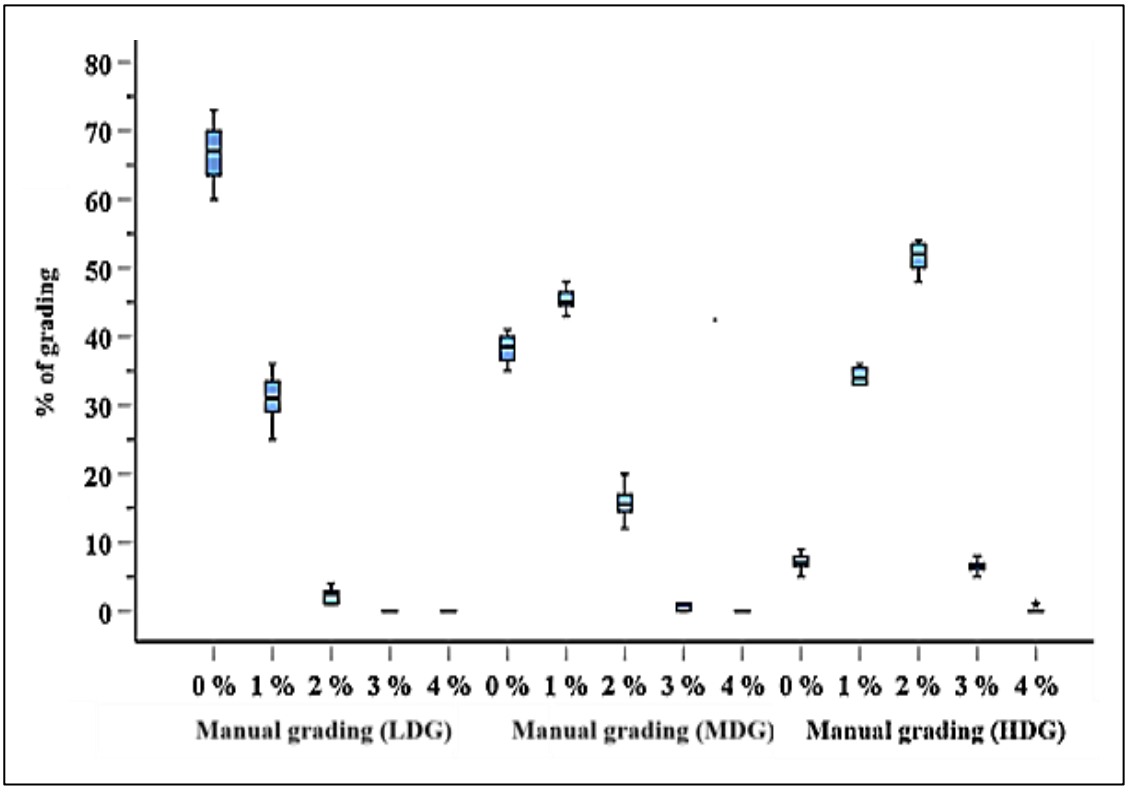


Figure 3.11 The manual grading of GDI categories at study baseline
LDG: low DNA damage; MDG: medium DNA damage and HDG: high DNA damage

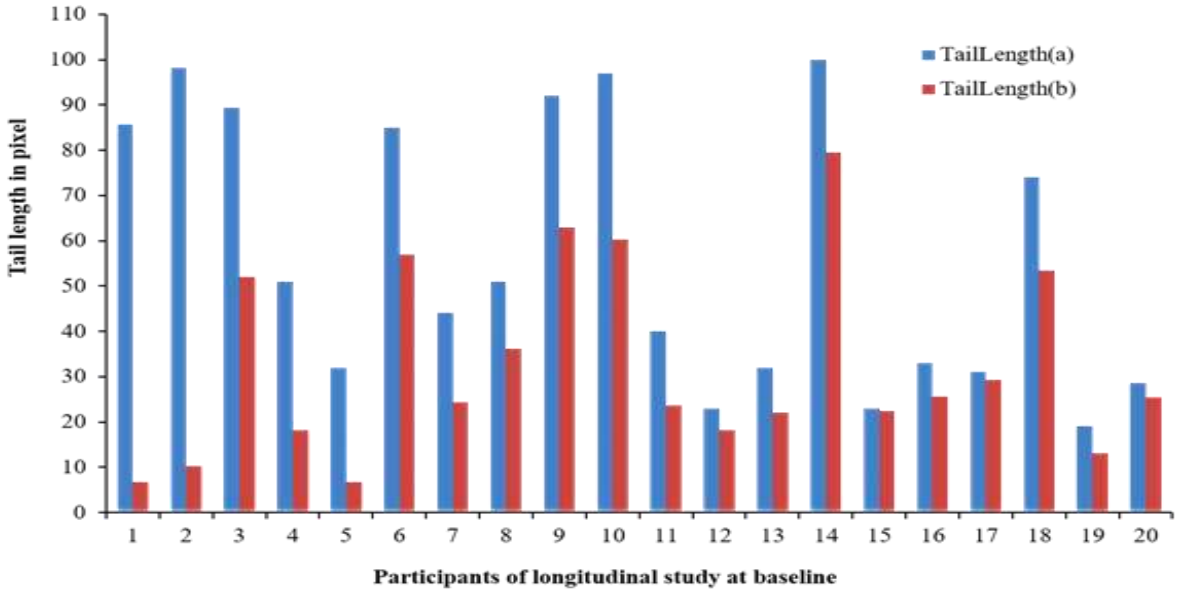


Figure 3.12 Tail length variability for all participants at study baseline
a: Image J software and b: Tritek software

Figure 3.13 below shows the results of the GDI analyses for the longitudinal study samples (excluding participant number 3 for which only one sample was available). Most GDI values

did not vary by more than 20-30% throughout the study period. Of the participants, in 5, 4 and 10, the GDI levels were classed in the HDG, MDG and LDG groups, respectively. The MDG and LDG groups were clearly and consistently very distinct from the HDG group and 3 of the MDG group were consistently quite distinct from the LDG group.

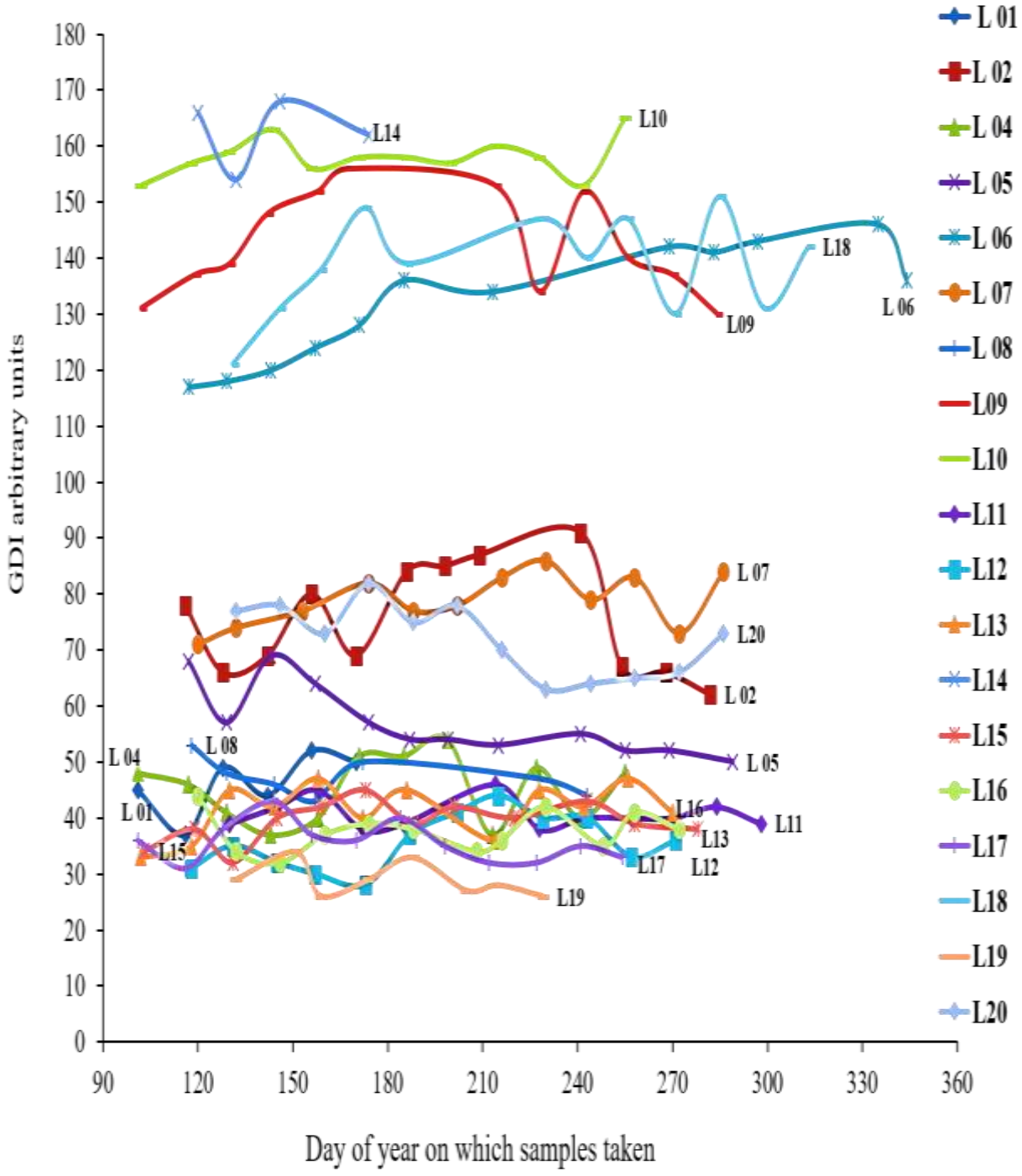


Figure 3.13 Variation of GDI throughout the longitudinal study
L = participant

The longitudinal study means values for Comet tail length and GDI together with the corresponding CV, for each participant are presented in Table 3.4.

Table 3.4: The variabilities in sperm DNA damage indicators in the longitudinal study

Participant ID (n)	Sperm DNA damage indicator			
	Tail Length		GDI	
	mean ± SD	CV%	mean ± SD	CV%
P01 (6)	77.8±9.7	12	46.2±5.4	12
P02 (12)	82.6± 8.2	10	75.3±10	13.2
P04 (12)	45.6± 9.7	21	45±6	13
P05 (12)	42.3± 6	14	57±6.4	11.2
P06 (12)	100± 8.4	8	132±10.4	7.8
P07 (12)	42.6± 4.2	10	79±4.8	6
P08 (7)	50± 1.4	3	47.3±3.5	7
P09 (12)	94.2± 8	9	142.4±9.3	6.5
P10 (12)	93.5± 7	7	158±3.5	2.2
P11 (12)	42± 5	12	40.7±2.6	6.4
P12 (12)	31.8± 5	16	35.6±5	14
P13 (12)	38.5± 4.4	11	41.5±4.7	11.4
P14 (4)	103± 2.2	2	162.5±6.2	4
P15 (12)	27± 3.5	13	39.3±3.7	9.3
P16 (12)	30.3±2.7	9	37.5±3.6	9.5
P17 (12)	30.2± 2	6	35.7±3.6	9.5
P18 (12)	67± 3.5	5	138.8±9	6.6
P19 (8)	20.4±4. 5	22	29±3	10
P20 (12)	30.6± 2	6	72±6.3	8.8

P1 to P20: participants from 1 to 20 (P3 excluded – see text). CV: coefficient of variation. SD: standard deviation, n: number of times the Comet assay was performed for each participant through the study.

3.3.3.1 The correlations between semen quality and sperm DNA damage indicators at study baseline

Table 3.5 shows that there was no significant correlation between the sperm parameters and GDI at baseline, except there was a highly significant negative correlation between % vital sperm and GDI. There was also a significant negative correlation between both % vital sperm and total sperm count with tail length determined using ImageJ software.

Table 3.5: Correlations between sperm DNA damage indicators and semen parameters at study baseline

Sperm parameters	Sperm DNA damage indicator	
	GDI	Tail length
	Spearman's rho correlation (R) (n=20)	
Volume (ml)	0.14	0.34
% of progressively motile sperm	-0.32	-0.41
% of non-motile sperm	0.38	0.43
Sperm concentration $X10^6$/ml	0.43	0.44
Total sperm count $X10^6$	0.28	**0.45***
% vital sperm	**-0.57****	**-0.66****
% of morphologically normal sperm	0.20	0.41

Two-tailed test indicated significant positive or negative correlations *at the 0.05 level **at the 0.01 level. n: number of participants.

3.3.3.2 The correlations between sperm DNA damage indicators and demographic parameters at study baseline

To find if there was a correlation between the GDI and tail length determined by Image J software with male age, abstinence period and BMI at the study baseline the Spearman correlation test was applied. There was no significant correlation between the GDI and tail length with age, abstinence period and BMI at baseline (Table 3.6).

Table 3.6: Correlations between sperm DNA damage indicators and abstinence, age, and BMI at study baseline

Sperm DNA damage indicator	Abstinence	Age years	BMI(Kg/m2)
	Spearman's correlation (R) (n=20)		
GDI	0.24	-0.01	-0.19
Tail Length	0.31	-0.03	-0.13

n: number of participants

There were no significant differences in sperm DNA damage indicators between ethnicity groups, BMI categories, sexual activity, LSI categories, wearing of underwear or travel (Table 3.7). There was also no significant correlation between the $PM_{2.5}$ and GDI and tail length (data not shown).

Table 3.7: Sperm DNA damage indicators and demographics at study baseline

Variables		Sperm DNA damage indicator (Mean ±SD)	
		GDI	Tail Length
Ethnicity	White British (n=11)	80.2±49.0	53.7±34.9
	Indian (n=4)	63.8±38.8	60.4±24.3
	Other White (n=4)	74.8±44.9	61.6±29.8
BMI categories	Low normal BMI (n=6)	90.7±36.3	49.4±30.4
	High normal BMI (n=6)	60.3±31.9	52.3±27.3
	Overweight (n=7)	77.7±57.6	53.7±32.2
Sexual activity	Daily (n=7)	68.6±46.3	55.7±33.4
	At least once week (n=12)	72.8±42.3	53.9±28.4
LSI categories	Active (n=3)	90.3±40.3	69.1±31.4
	Insufficiently active (n=17)	71.4±44.6	54.2±29.9
Underwear	Worn(n=18)	75.2±45.8	58.3±30.7
	Not worn(n=2)	65±17	40.0±15.6
[A] Travel	Travel (n=10)	81.2±47.8	63.5±31.8
	No travel (n=10)	67.2±40.2	49.5±27,5

[A]: Tavel in the last three months; LSI; leisure score index, n: number of participants.

There was no significant difference in sperm DNA damage indicators between those men exposed or not exposed to one or more chemical or physical agents as shown in Table 3.8.

Table 3.8: Impact of Physical and chemical exposures on sperm DNA damage indicator at study baseline

Hazardous agents	Sperm DNA damage indicator (Mean ± SD)	
	GDI	Tail Length
Zero (n=2)	44.5±0.7	59.3±37.2
One (n=5)	92.5±34.7	58.5±37.5
Two (n=5)	89.4±50.0	64.8±27.5
Three (n=4)	66.0±66.7	44.5±37.2
Four (n=4)	65.8±42.5	38,3±24.4

Zero: participants not exposed to any of these agents. One, Two, Three, four: participants exposed to any 1, 2, 3 or 4 of these agents**.** n: number of participants, SD: standard deviation.

Concerning the general health categories, there was no significant difference in sperm DNA damage indicators between general health categories, as shown in Table 3.9.

Table 3.9: Sperm DNA damage indicator differences in categories of health issues at study baseline

Health categories	Sperm DNA damage indicator (Mean ± SD)	
	GDI	Tail Length
Zero (n=2)	46.5±2.1	68.3±24.5
One (n=4)	101±60	77.3±39.0
Two (n=5)	75.8±53.2	58.3±32.1
Three (n=5)	75.8±42.1	51.4±27.0
Four (n=4)	57.3±20.1	33.8±12.1

Zero: participants with no health issues; One: men with one health issues; Two: men with two or three health problem; Three: men with four health issues and Four: men with five or more health issues; n: number of participants; SD: standard deviation.

Additionally, there were no significant differences in sperm DNA damage between groups categorized by exposure to cigarette smoking, alcohol, and drug consumption at baseline as shown in Table 3.10.

Table 3.10: Sperm DNA damage indicator and exposure to cigarette smoking, alcohol and drugs at baseline

Exposure categories	Sperm DNA damage indicator not exposed/exposed (Mean ± SD)	
	GDI	Tail Length
Exposed to cigarette smoking (11/9)	72.0±42.3/ 76.9±47.5	26.0±29.3/ 56.4±33.4
Regular alcohol consumption (7/13)	67.4±46.4/ 77.8±43.5	44.0±25.8/ 63.2±30.6
Drug consumption (15/5)	74.5±41.3/ 73.4±55.3	57.1±28.8/ 54.5±36.1

Table 3.11 shows there was a highly significant negative correlation between tail length and alcohol consumption categories. Further, there was a significant difference between tail length in Image J software in Non- or light-drinkers group with moderate and heavy drinkers' groups (Table 3.11).

Table 3.11: Sperm DNA damage indicator and alcohol consumption categories at study baseline

Exposure categories	Sperm DNA damage indicator (Mean ± SD)	
	GDI	Tail Length
Non- or light-drinkers (n=7)	86.7±50.7	**76.5±30.2***
Moderate drinkers (n=10)	71.6±43.4	49.1±26.2
Heavy drinkers (n=3)	53.3±23.0	34.3±14.7
Spearman correlation R (n=20)	-0.22	**-0.57****

Non- or light-drinkers consumed <1 unit of alcohol per week; moderate drinkers consumed 1-20 units per week; Heavy drinkers consumed >20 units per week. Two-tailed test indicated significant positive or negative correlations *at the 0.05 level **at the 0.01 level. n: number of participants; SD: standard deviation.

There were no significant differences in sperm DNA damage between groups categorized by smoking, alcohol and drug consumption at baseline as shown in Table 3.12.

Table 3.12: Sperm DNA damage indicators in four exposure categories at study baseline

Exposure categories	Sperm DNA damage indicator (Mean ± SD)	
	GDI	Tail Length
(i) (n=3)	41.7±7.8	38.7±10.7
(ii) (n=9)	82.3±46.5	65.2±32.9
(iii) (n=4)	101.3±44.5	80.1±22.3
(iv) (n=4)	53.5±37.8	43.9±31.4

(i): refers to those men who were not exposed to any of the three items (smoking cigarette, alcohol, or drugs); (ii): refers to men who were exposed to any of the three; (iii): refers to men who were exposed to any two of the three; (iv): refers to men who were exposed to all three. n: number of participants; SD: standard deviation

3.3.3.3 The correlations between sperm DNA damage and diet and dietary supplements at study baseline

There were no significant differences in GDI and tail length between dietary habits (Table 3.13). Additionally, there was no significant difference in sperm DNA damage indicators between the participants and any other food kinds such as fruits, other vegetables, and nuts & seeds (data not shown).

Table 3.13: Sperm DNA damage indicator differences in two diet categories at study baseline

Diet	Sperm DNA damage indicator (Mean ± SD)	
	GDI	Tail Length
Meat and fish eater (n=4)	76.5±63.4	42.8±38.4
Fish eater (n=15)	75.6±40.6	61.7±27.7

n: number of participants; SD: standard deviation

Furthermore, there was no significant correlation between the consumption of different foods items such as different meat types, Crisps /chips, pulses, spicy cereals and pizza with sperm DNA damage indicator at baseline (data not are shown).

3.4 Discussion

There is growing evidence that the semen quality of adult males can be affected by a variety of lifestyle, environmental and/or occupational factors. However, until relatively recently, most studies investigating these relationships have considered only how environment and lifestyle might affect the traditional semen variables (e.g. sperm concentration and motility), (Jurewicz et al., 2009; Sharpe 2010). More recent studies have begun to include an assessment of sperm DNA damage and consequently, insights into how environment and lifestyle can influence sperm DNA integrity are beginning to emerge (Cherry et al., 2008; Aitken et al., 2009).

In the present studies the neutral Comet assay was initially performed on HepG2 cells to establish and optimize the assay and the cell harvesting by trypsinization and scraping were also compared. H_2O_2 was used as a positive control because it is known to produce SSB and DSB (Petersen et al., 2000; Banáth & Olive 2003; Driessens et al., 2009;). Previously reported findings using a rat thyroid cell line (Driessens, et al.,2009), showed that non-lethal concentrations of H_2O_2 (0.1–0.5 mM) as well as irradiation (1–10 Gy) induced SSB at ~2–3 times control DNA damage levels and DSB at 1.2–2.3 times control DNA damage levels. Takahashi & Ohnishi (2005) demonstrated the formation of DSB and histone H2AX phosphorylation by immunocytochemistry in normal human fibroblasts exposed to H_2O_2 and showed that H_2O_2 can form DSB at various doses (Olive & Johnston 1997). These results support the present findings in terms of the H_2O_2 dose response as seen in Figure 3.3.

Alkylating agents, such as bleomycin, daunomycin, and TMZ, are also able to induce DNA damage and in the present work TMZ was used as an additional positive in the neutral Comet assay. As described in Chapter 1, when DNA MMR enzymes attempt to process O^6 alkylG-induced mispairs in DNA, SSB and DSB can be generated leading to activation of apoptotic pathways involving massive degradation of DNA, as was observed in Figure 3.5 and Figure 3.6 which showed increasing DNA damage. Consistent with these results, earlier studies used the neutral Comet assay and _H2AX focus formation to detect DSB following treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) an alkylating agent similar to TMZ (Takahashi & Ohnishi 2005; Yu et al., 2006).

Because there is no clear agreement about the best software to analyze Comet images to estimate DNA damage, I compared two software packages (Image J and Tritek) and manual scoring (Collins 2004) to classify DNA damage. There was a highly significant correlation between the manual scoring results and tail profiles from both software packages. However, the software packages were unable to recognise heavily damaged cells in which the tails were completely separated from the heads. GDI was therefore considered the better indicator of DNA damage.

In total two hundred and six longitudinal and 20 off-study sperm samples were processed by the neutral Comet assay. Each image was analyzed manually and then the GDI was calculated. In addition, the images were analyzed by both Image J and Tritek software to determine the specific tail profiles which are generally reported as an indicator for estimating DNA fragmentation (Lovell & Omori 2008; Lovell 2016).

The participants were categorized depending upon their GDI level as either HDG (n=5), MDG (n=4) or LDG (n=10), and the conclusion was that there were 5 participants in this study that had remarkably and consistently high levels of DNA damage in their sperm DNA. There were no significant correlations for the HDG group with any exposures, dietary or health issues, although this might be due to the low number of men displaying this characteristic. Therefore, the reason for these participants having such high levels of DNA damage remains to be established. There were significant highly negative correlations between tail parameters in Image J software and alcohol consumption categories, and there was a significant difference between tail parameters in Image J software in non- or light-drinkers and those in moderate and heavy drinkers' groups.

Unfortunately, the number of participants in this study was not high enough to confirm any impact of lifestyle or environmental exposure on sperm DNA damage, but the studies do confirm that quantifying DNA damage using the Comet assay is feasible, rapid and inexpensive and should be included routinely in semen quality analyses.

3.5 Conclusion

The reproducibility and reliability of the Comet assay were very good and there is a strong positive correlation between % of vital sperm and DNA damage indicator, GDI, indicating that this technique could be used clinically to support semen fluid analysis in infertility diagnostics.

Chapter 4: Determination of O^6-alkylguanine adducts by ASB in sperm and leucocyte DNA in longitudinal samples

4.1 Introduction

In spite of a possible effect on fertility of alkyl-DNA adducts that can be formed by exposure to alkylating agents such as Cyclophosphamide and Cisplatin, and despite the ability of these agents to induce DNA damage in testicular tissue *in vivo* (Garcia et al., 2015; Hosseini et al., 2018), just one study has investigated the level of the methylated base, N7-MedG in human sperm DNA (Stocks et al., 2010). In this study, a significant association was reported between N7-MedG levels and male infertility, the assisted reproduction outcomes of 67 IVF/ICSI couples and the percentage of oocytes successfully fertilized.

Although N7-MedG is relatively innocuous biologically (De Bont & Larebeke. 2004), its presence in DNA indicates that other nucleophilic sites within the DNA bases have also been alkylated such as the O^6 of guanine and N3 of adenine, (Montesano 1981). As described in Chapter 1, O^6-alkylG lesions in DNA are among the most toxic, mutagenic and recombinogenic, and therefore an assessment of their levels in sperm DNA might be a possible marker of male fertility.

In this chapter, the very high affinity of the *Schizosaccharomyces pombe* Atl1 protein for O^6-alkylG-DNA adducts (Pearson et al., 2005; Pearson et al., 2006) has been exploited in developing a convenient, sensitive and inexpensive Atl1-slot-blot (ASB) method, which does not require expensive instrumentation and requires only small amounts of DNA.

Importantly, the binding of Atl1 is to all O^6-alkylG adducts assessed so far and is therefore not specific for any one alkyl lesion type, potentially improving the sensitivity of the assay in comparison with other alkyl group specific methods. Hence, this is the first study to investigate the presence of O^6-alkylG adducts in sperm and buffy coat DNA in the same participant and enabling comparisons with semen parameters and sperm DNA damage measured by the neutral Comet assay.

For brevity, "the levels of O^6-alkylG adducts" is usually referred to as "O^6-alkylG levels"

4.2 Aims and objectives

The overall aims of the work were to:

1) Optimise the ASB methodology

2) Quantify O^6-alkylG levels in human DNA from sperm and buffy coat and

3) Correlate semen quality and DNA damage as detected by the neutral Comet assay with O^6-alkylG levels DNA in a longitudinal study.

4.3 Materials and methods

4.3.1 Materials

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Blood and cell culture DNA Midi Kits were purchased from Qiagen and used according to the included protocols unless otherwise stated. Isopropanol (laboratory reagent grade), Ethanol (analytical reagent grade) Whatman 3MM Chromatography paper (46×57cm) and Amersham Hybond-N+ membranes (Portran, BA79, 300mm×3m, pore size 0.1µm) were from Fisher Scientific. Amersham Hyperfilm ECL was from Scientific Laboratory Supplies. ECL select was purchased from GE Healthcare, PicoGreen dye was from Invitrogen and DTT was from Thermo Scientific. Propidium iodide (PI) was from (Thermo Scientific USA).

4.3.2 Methods

4.3.2.1 Density gradient centrifugation of human sperm samples

Semen samples stored at -80^{o}C were thawed at RT. To remove debris and round cells, up to 1 ml of the thawed semen sample was layered on the top of a layer of 80% SupraSperm™ media (1 ml; Origio, Denmark) and centrifuged at 300g for 20 min. The top layer was removed and stored at -80^{o}C and the sperm pellet was washed by mixing twice with 1 ml of PBS and then centrifuging at 300g for 5 min. The sperm cells were then resuspended in 1 ml PBS, counted as described in Section 2.2.2.4.2 and stored at -80^{o}C until used.

4.3.2.2. DNA extraction from sperm and buffy coat samples

For both sperm and buffy coat samples, the blood and cell culture DNA Midi Kits were used. As previously described (Stocks et al., 2010), frozen sperm pellets were thawed at RT for 10 min. They were resuspended in 1 ml of PBS, added to a falcon tube containing 9.5 ml of G2 buffer with 19 µl RNase (10 mg/ml) and 250 µl proteinase K (1 mg/ml) and incubated at 4°C overnight on a rotator. The next day, 0.5 ml of DTT (5 mM) was added and the mixture incubated again at 4°C on a rotator for 1 hour, then transferred to a 37°C incubator for one hour.

The frozen buffy coats were thawed at RT and each sample counted manually using a Haemocytometer as described in Section 2.2.2.5.1. Five ml of PBS was added to every 1-1.2 $x10^7$cells in a 50 ml Corning tube and the sample mixed with one volume of ice-cold Buffer C1 and 3 volumes of ice-cold dH_2O. The tubes were inverted several times until the suspension became white, and then the tube was incubated for 10 min on ice and centrifuged at RT for 25 min at 1750 rpm. The supernatant was then discarded and 1 ml of ice-cold Buffer C1 and 3 ml of ice-cold dH_2O was added, the pelleted nuclei re-suspended by vortexing and the sample centrifuged again at RT for 10 min, at 1000 rpm. The supernatant was discarded and if the pellet was not white, the wash was repeated until it was, then 5 ml of buffer G2 was added and the nuclei completely resuspended by vortexing for 30 seconds. Qiagen Protease (95uL) was added and the samples incubated at 50°C for 60 min.

The following steps then were applied to both sperm and buffy coat DNA extractions. Filter columns were equilibrated with 4ml of QBT buffer. Each falcon tube was vortexed for 20 sec and the entire sample was poured onto the Qiagen column and the sample passed through by gravity flow until the cross frit (a mark at the bottom of the column) was visible.

The columns were washed twice with 7.5 ml QC buffer and the DNA was eluted with 4 ml of QF buffer. DNA was precipitated by adding 2.8 ml RT isopropanol to each collection tube and inverting several times. Samples were then centrifuged 12,000g for 15 min at 4°C. The isopropanol has removed the pellet resuspended in 1 ml of 70% ethanol and the sample centrifuged at 12,000g for 5 min at 4°C. The ethanol was removed and the pellet was spun again for 2 min. All the remaining ethanol was removed and the pellets were allowed to air

dry for approximately 2 min before the pellet was completely dissolved in 500-1000 µl of TE buffer (pH 7.5). The DNA solution was stored at -80˚C.

4.3.2.2 DNA quantification by PicoGreen and Nanodrop assays

PicoGreen and Nanodrop (ND-8000 spectrophotometer) assays were used to determine the concentration of DNA in the samples. DNA purity was evaluated by Nanodrop values for 260nm/230nm and 260nm/280nm ratios for which values of approximately 2 and 1.8 respectively are considered as pure DNA (Luebbehusen 2006; Ghatak et al., 2013).

These DNA concentrations were then used to guide the preparation of dilutions for the PicoGreen assay. If the DNA concentration was high enough (more than 90 µg/µl), the PicoGreen assay was conducted to obtain a more accurate value of the DNA concentration (Blotta et al., 2005). DNA dilutions were prepared in TE buffer (pH7.2) and 10 µL of each unknown DNA and a series of CT-DNA standards were added to duplicate wells of a 96 well black microplate (Greiner, Scientific Laboratory Supplies).

To each well was then added 200 µL of PicoGreen reagent (diluted 1:1000 in TE buffer). The plate was protected from the light and the fluorescence was measured using a TECAN Genios plate reader (excitation wavelength 485 nm, emission wavelength 535 nm). The standard curve was accepted when R^2>0.99, and the DNA concentrations were extrapolated from this.

4.3.2.3 Atl1 slot blot assay (ASB)

The positive controls for this assay were aliquots of Calf thymus DNA (CT- DNA) that had been methylated in vitro using TMZ at 3 different concentrations (0.2, 2, and 20 µg/ml) labelled as TMZ (0.2 CT- DNA), TMZ (2 CT- DNA) and TMZ (20 CT- DNA) respectively. Unmodified CT-DNA was labelled as DMSO (CT- DNA) and was the negative control, or background.

The levels of O^6-meG in these standards were determined by an MGMT competition assay to be 0.13, 0.25,2.5 and 27.8 fmole O^6-meG/µg DNA. Stock solutions of affinity purified Atl1 protein conjugated with horseradish peroxidase (HRP) were firstly diluted 1:10 in PBS and this then diluted 1:5000 in 5 %, Marvel milk powder in PBS (final dilution 1:50000). A

medium salt wash (2mM EDTA, 20mM HEPES, 325mM NaCl, 0.1% (w/v) SDS and 1% (v/v) Triton X-100) and a high salt wash (2mM EDTA, 20mM HEPES, 500mM NaCl, 0.1% (w/v) SDS and 1% (v/v) Triton X-10) and three concentrations of guanidine hydrochloride (1, 0.5, 0.1 and 0.05 M) were all prepared in dH_2O.

An immunoslot-blot protocol that was the basis for the ASB protocol has already been described (Harrison et al., 2001), briefly, DNA was diluted in TE buffer in Eppendorf tubes to obtain a final volume of at least 400 µl at a concentration of 1µg/100µl. Each tube was sonicated in ice for 10 seconds at 50% power using a sonicator (Sonicator, Bandeline electronic sonopuls, UW 2070, Probe: SH70G) with a 4mm probe, denatured by heating at 96^{o}C for 5min and cooled in ice for 10 min. Meanwhile, two pieces of 3MM paper and one Hybond-N+ membrane were cut to fit into the slot-blot apparatus (72-well Schleicher & Schuell Minifold II Slot-Blotter). The 3MM papers were soaked in TE buffer and placed in the apparatus and then the Hybond-N+ membrane was soaked in TE buffer and placed on the top of the 3MM layer.

The apparatus was assembled then connected to a vacuum-electric pump and turned on. Into the wells, 100 µl of each CT-DNA standard or "unknown" DNA sample was loaded in triplicate. Once the wells had run dry, 100 µl of TE buffer was added to each well. After all the wells had run dry again, the apparatus was dismantled and the Hybond-N+ membrane was placed in an oven for 30 min at 80˚C. Subsequent steps were at RT. Next, the membrane was blocked by covering it with 20 ml of 5% (w/v) Marvel (milk powder) in PBS-Tween (0.02 %) in a dish placed on an orbital shaker for 30 min. In the basic ASB, the membrane was then incubated in a sealed plastic bag with 20 ml of Atl1-HRP conjugate (called simply Atl1 hereafter) dilution (1:50000) in 0.5% Marvel on a platform shaker for 1 hour. Subsequently, the membrane was washed with 1M guanidine hydrochloride once for 5 min, then washed with medium salt wash buffer three times for 5 min each on an orbital shaker.

Eight ml of enhanced chemiluminescent (ECL) reagents (a mix of 4 ml solution A and 4 ml solution B) was poured all over the membrane and left for 3 min. The membrane was then held vertical for 10 sec to remove ECL reagent and wrapped in plastic film. In a darkroom, the wrapped membrane was placed in a cassette (Amersham Biosciences) and an X-ray film was placed over it, and the cassette was closed. After approximately one hour the X-ray film was developed using a JP-33 Automatic X-ray Film Processor machine (JPI Healthcare

Solution). Finally, the developed film was scanned (HP Scanjet 4850) and the intensity of the bands for the samples and the standards was quantified by using ImageJ software (version of Fiji using Java 6). A standard curve was generated from the methylated CT-DNA standards and the levels of O^6-alkylG in the samples were extrapolated.

4.3.2.4 Propidium iodide staining of membrane-bound DNA

The consistency of DNA binding to Hybond N+ in each slot was assessed by Propidium iodide (PI) staining of the membrane after the ASB procedure. The membrane was washed in PBS for 30 min and then stained with 5 µg/ml PI in PBS for 2h at RT protected from light.

The membrane was then washed twice in PBS for 30 min, left to dry for 20 min and finally scanned using a Typhoon scanner (Typhoon 9200 Variable Mode Image, Amersham Biosciences, UK) using 300nm absorption and 560nm emission wavelengths. The intensity of DNA bands in the obtained image was quantified by using the ImageJ program.

4.3.2.5 ASB image and statistical analyses

O^6-alkylG adduct levels were estimated in 28 sperm and 40 buffy coat DNA samples for which the DNA concentration obtained by Nanodrop (Section 4.4.3) was adequate. From the Image J analyses, the mean and standard deviation was calculated for the triplicate samples. Additionally, the signal to noise ratio was evaluated in each of the series of optimization experiments to determine the most sensitive assay condition. For this, the band intensity signal of each of the methylated CT-DNA standards were divided by the intensity signal of the blank (DMSO CT DNA). The highest sensitivity was taken as the highest signal/noise ratio recorded for the highest methylated CT-DNA standards (27.8 fmole O^6-MeG/µg DNA). One-way Anova was used to compare the levels of O^6-MeG in the methylated CT-DNA standards among the groups. The Spearman test was used to find the correlations between O^6- alkylG adduct levels in DNA of sperm and buffy coat samples and sperm parameters and sperm DNA damage indicators.

4.3.2.6 ASB assay and treatment of DNA with MGMT

To make sure that the signals on the film were generated by O6-alkylG and not by any cross-reacting DNA lesion, methylated CT-DNA standards and DNAs from sperm and buffy coat

samples (with sufficient DNA) were incubated in Eppendorf tubes with 10 µl of Affinity purified MGMT that had been diluted 1:100 IBSA (50mM Tris pH8.3, 1mM EDTA, 3mM DTT and 0.1% (w/v) bovine serum albumin (BSA) for two hours at 37oC.

The control samples were incubated only with 10 µl of IBSA. After treatment, all tubes were sonicated for 10 seconds at 50% power, heated for 5 min and cooled in ice for 10 min and the samples assayed using the above ASB protocol.

4.4 Results

Of the forty-one sperm samples prepared by density gradient centrifugation and DNA extracted, only 28 samples had sufficient DNA for ASB analysis. Of the 43 buffy coat DNA preparations only 3 did not have sufficient DNA.

4.4.1 Optimization of ASB assay

To optimize the sensitivity of the ASB assay by reducing non-specific binding and hence increasing the signal to noise ratio, several approaches were examined that modified specific steps of the assay of Atl1-binding to methylated CT-DNA standards as described below.

4.4.1.1 The effect of temperature (RT and 4ºC) on Atl1 binding

To determine the impact of temperature on the binding of Atl1 to methylated DNA standards, two different conditions were used i.e. RT (RT) and 4ºC, shown in Figure 4.1A as I and II, respectively. The signal for all the standards was higher using RT than 4ºC but this increment was not statistically significantly different (Figure 4.1B).

The signal: noise ratio (RLU for the highest standard/RLU for blanks) for Atl1 binding at 4ºC (1.9) was closely similar to that for RT (2.0). Temperature therefore resulted in no obvious difference in Atl1 binding and was not a parameter that could be usefully exploited in subsequent experiments. There was no noticeable difference in DNA binding on both membranes as shown by PI staining and quantifying the band intensities using image J as shown in Figure 4.1C, indicating that the amount of DNA bound to the membranes was almost identical.

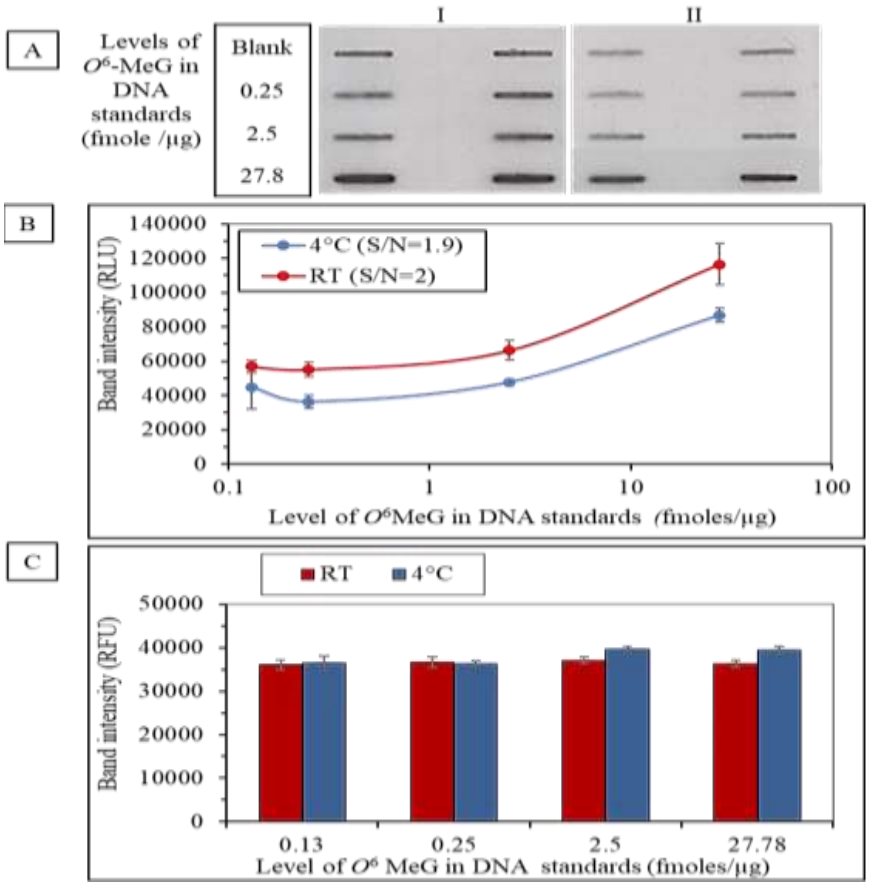


Figure 4.1 The impact of two incubation temperatures on Atl1 binding

A: ASB image, B: ECL band intensities of scanned images analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank, I: The membrane was blocked in 5% marvel in PBS only and the Atl1 binding step was for 1 hour at RT (I) or 4ºC (II).

4.4.1.2 The effect of various blocking protocols on Atl1 binding

Milk powder (in this thesis, Marvel), BSA, Tween 20 and NaCl at different concentrations are common constituents of membrane blocking buffers, and the effect of these on Atl1-binding and signal/noise ratios were assessed as described below.

4.4.1.2.1 Comparison of blocking with milk or BSA on Atl1 binding

To determine the impact of the hybond N+ membrane blocking protocol on the binding of Atl1 to methylated DNA standards, two different conditions were used i.e. 5% milk and 3% BSA, shown in Figure 4.2A as I and II, respectively. In Figure 4.2A II the membrane was completely black so that there was no possibility to quantify the bands. It seems likely that the membrane was very poorly, or not at all, blocked by 3% BSA and hence the Atl1 bound

everywhere and generated this signal. However, 5% marvel resulted in a good dose response and allowed the intensity of the bands to be quantified, giving a signal: noise ratio of 2.1. (Figure 4.2B). Figure 4.2C showed there is no clear difference in PI staining band intensity between both membranes.

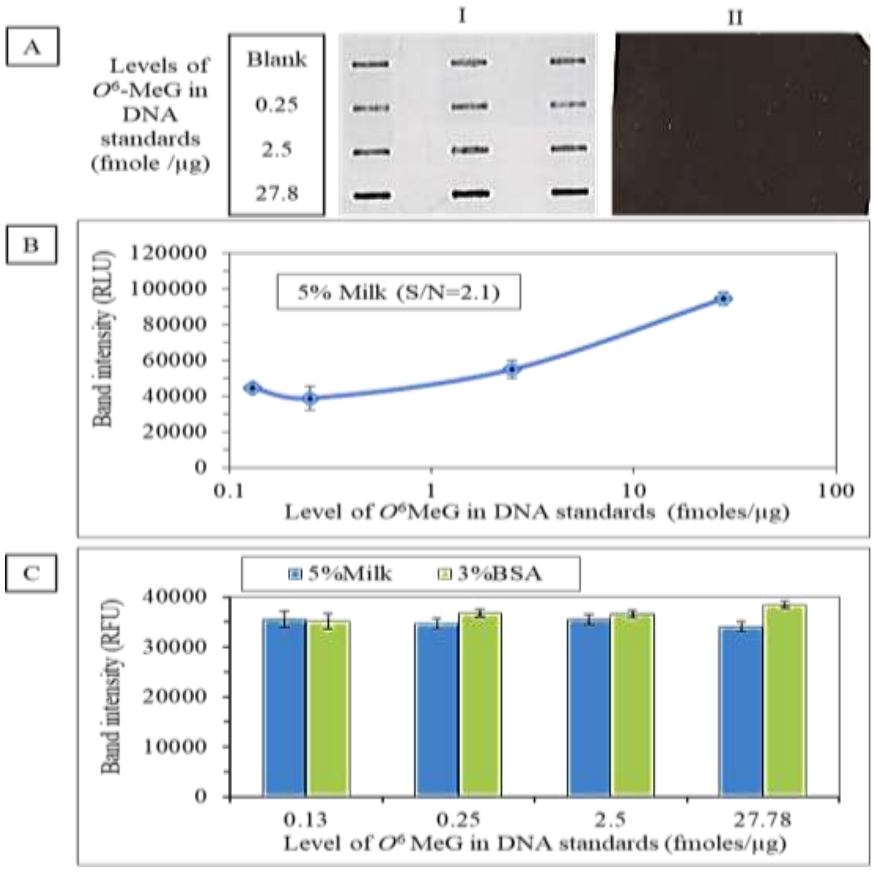


Figure 4.2 Comparison between the blocking the membrane with 3% of BSA and 5% of marvel milk

A: ASB image, B: ECL band intensities of scanned images analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. I: The membrane was blocked in (I) 5% marvel milk only or (II) 3% BSA only and the Atl1 binding step was for 1 hour at RT.

4.4.1.2.2 Comparison of blocking with milk, BSA and combinations on Atl1 binding

To investigate further the use of BSA at the membrane blocking and Atl1 binding steps, five different conditions were used as shown in Figure 4.3. Again, BSA alone was not an effective blocking agent, producing a very high membrane signal (Figure 4.3A III) and a very flat response curve (Figure 4.3B). The best signal/noise ratio (4.7) was recorded for the membrane blocked with 5% marvel as shown in Figure 4.3B. There were no consistent

differences between PI staining band intensities among the membranes when quantified by image J (Figure 4.3C).

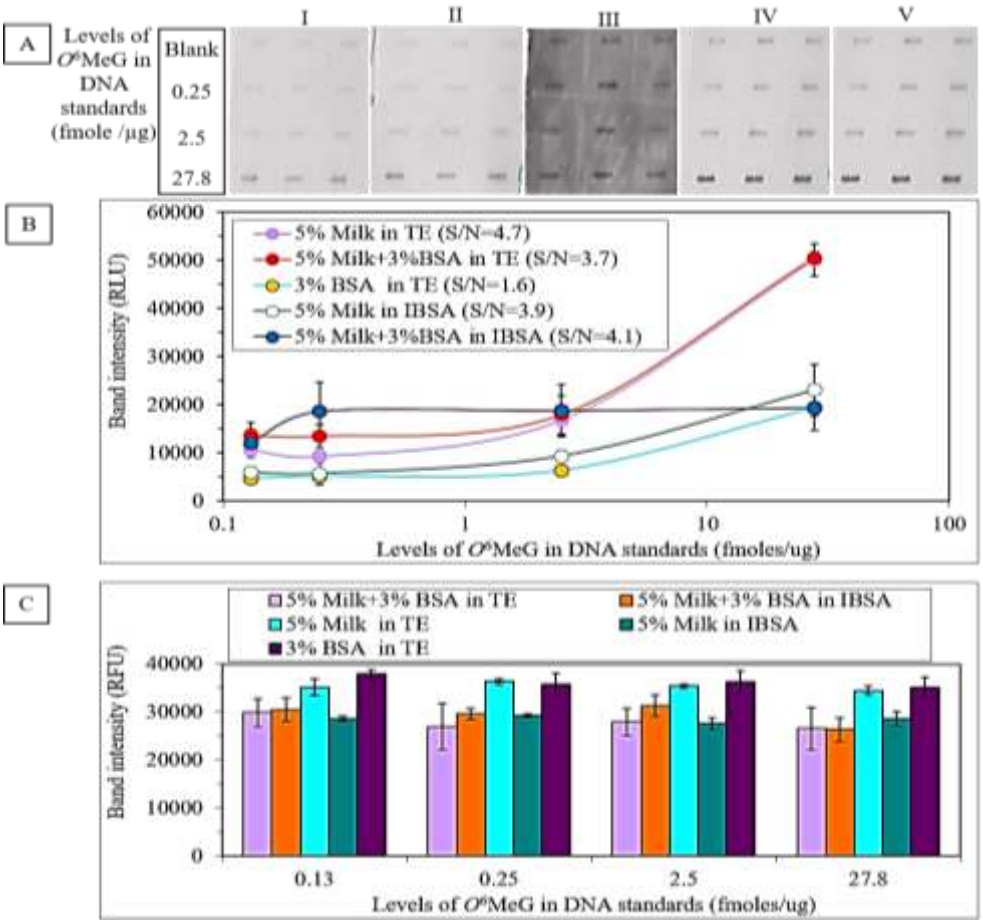


Figure 4.3 The effect of blocking buffer composition on Atl1 binding

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. Buffer compositions: I: 5% milk in TE; II: 5% milk+3%BSA in TE; III: 3% BSA in TE, IV: 5% milk in IBSA; V: 5% milk+3%BSA in IBSA. The Atl1 binding step was for 1 hour at RT.

4.4.1.2.3 The effect of Tween in the blocking and Atl1 binding steps

To examine the possible impact of different Tween concentrations (0, 0.02 %and 0.1 %), on both blocking and Atl1 binding steps, three hybond-N^+ membranes were used as shown in Figure 4.4. Initially, the visual appearance of the membranes showed that there was little impact of Tween on band intensity as shown in Figure 4.4A. Image J quantitation showed that 0.02% Tween (signal/noise 1.7) was slightly better than no Tween (signal/noise 1.6) while 0.1% Tween increased the background signal and significantly affected the standard

curve. There were no consistent differences in PI staining band intensity on the three membranes after quantification by image J (Figure 4.4C).

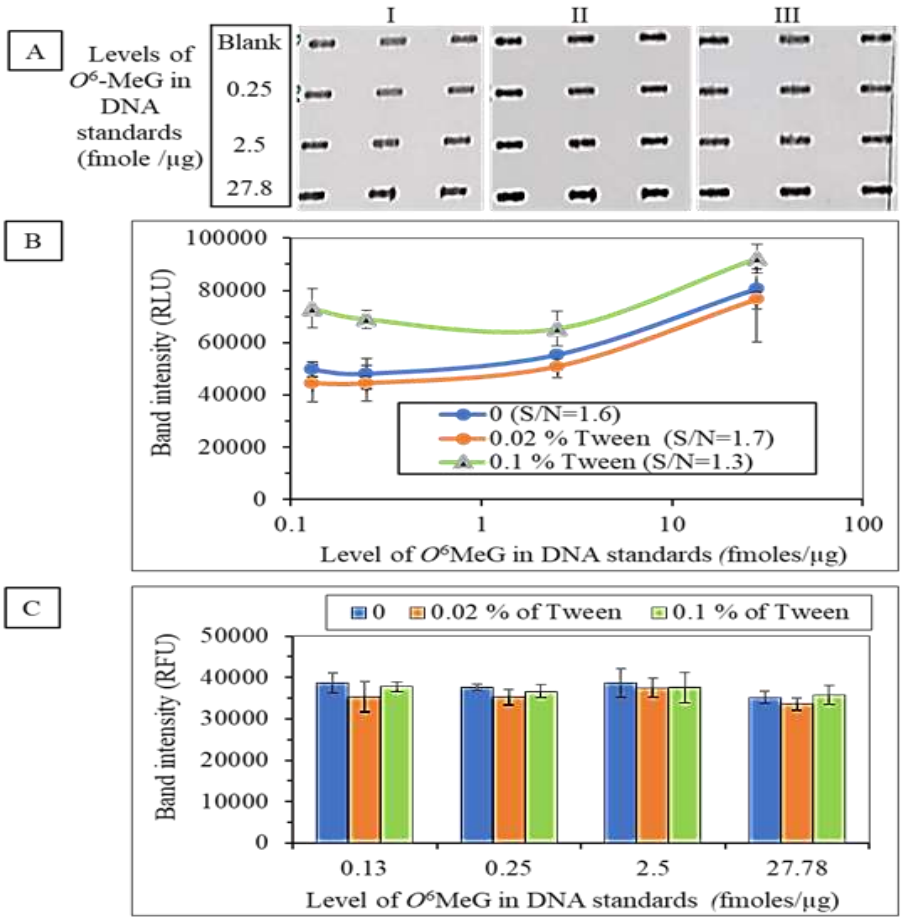


Figure 4.4 The effect of Tween on both blocking and Atl1 binding steps
A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. In all cases, the membrane was blocked in 5% marvel and Atl1 binding was for 1 hour at RT: I, without Tween; II: with 0.02% Tween and III: with 0.1% Tween. The Atl1 binding step was for 1 hour at RT.

4.4.1.3 Effect of membrane baking time on Atl1 binding

A marked increase in ECL band intensity in all CT-DNA standards was seen with 120 minutes of baking when compared with 30, 60, and 90 minutes, which were all closely similar (Figure 4.5 A and B). The highest signal /noise ratio (6.1) was seen using 30 min of baking. (Figure 4.5B). There was no consistent difference in PI staining band intensities following quantification by Image J as shown in Figure 4.5C, so the basis of the increased ECL signals was not related to the amount of DNA bound.

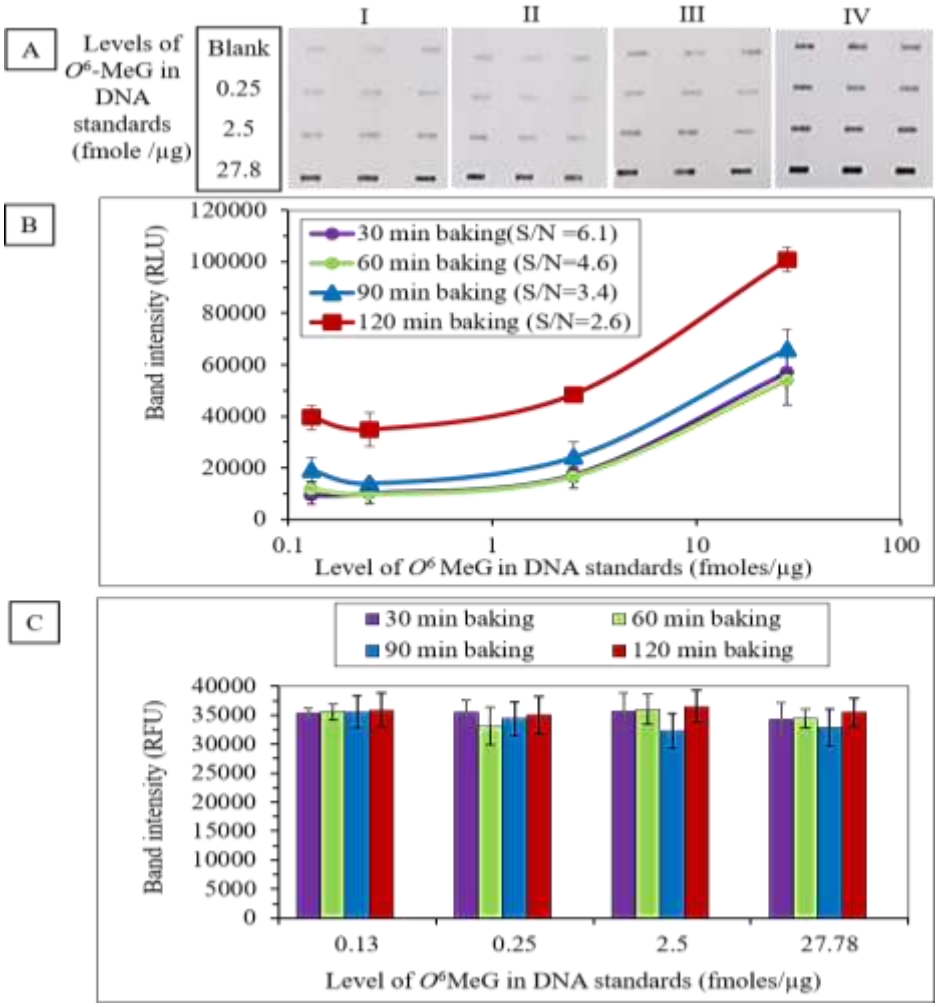


Figure 4.5 Different baking times effect on bands intensity signals

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signalnoise ratio for 27.8 fmole/ug vs Blank. Membrane baking was for I: 30 min; II: 60 min; III: 90 minutes; IV: 120 min. The Atl1 binding step was for 1 hour at RT.

4.4.1.4 Effect of washing temperature and wash buffer composition on Atl1 binding

To determine the impact of washing temperature membranes were washed three times at RT for 5 min each time with; medium salt (350mM NaCl) at RT, medium salt at 37^{o}C medium salt at 4^{o}C and high salt (500mm NaCl) at RT and the results are shown in Figure 4.6. A marked increase in ECL band intensity in all CT-DNA standards was seen with medium salt at 4^{o}C washing when compared with the other conditions which were all closely similar except for the highest standard (Figure 4.6 A and B).

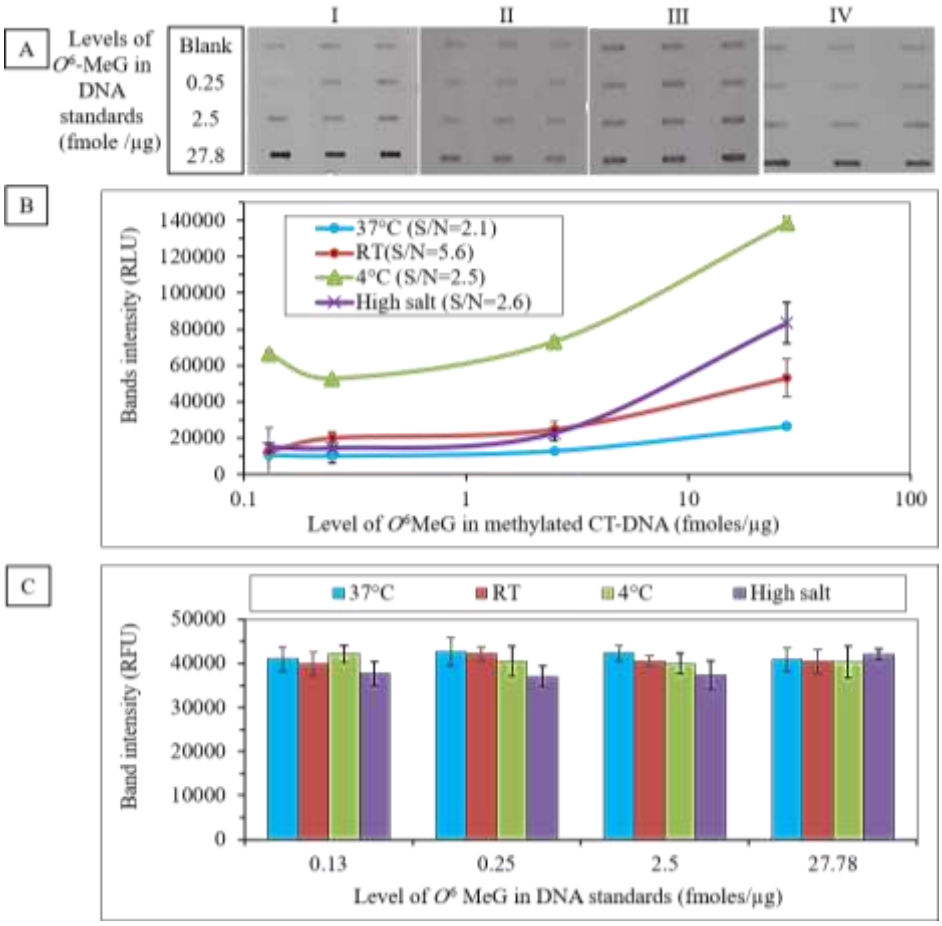


Figure 4.6 The effect of washing temperature and wash buffer composition on Atl1 binding

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. The Atl1 binding step was for 1 hour at RT and membranes were washed three times at RT for 5 min each time with; I: medium salt at RT, II: medium salt at 37^{o}C III: medium salt at 4^{o}C and IV: high salt at RT.

The membrane washed with medium salt at RT showed the highest signal/noise ratio (5.6) as shown in Figure 4.6 B. There was no consistent difference in PI staining band intensities following quantification by Image J as shown in Figure 4.6 C.

4.4.1.5 The effect of washing membranes with guanidine hydrochloride after Atl1 binding

In attempts to improve the signal/noise ratio by reducing the background signal, hybond-N^{+} membranes were washed, after Atl1 binding, with guanidine hydrochloride multiple times, for different lengths of time, and using different molarities as described below.

4.4.1.5.1 The effect of multiple washes with guanidine hydrochloride

After Atl1 binding individual membranes were either not washed with 1 M guanidine hydrochloride or washed once, twice or three times for 5 min each at RT and the results are shown in Figure 4.7A (I, II, III and IV).

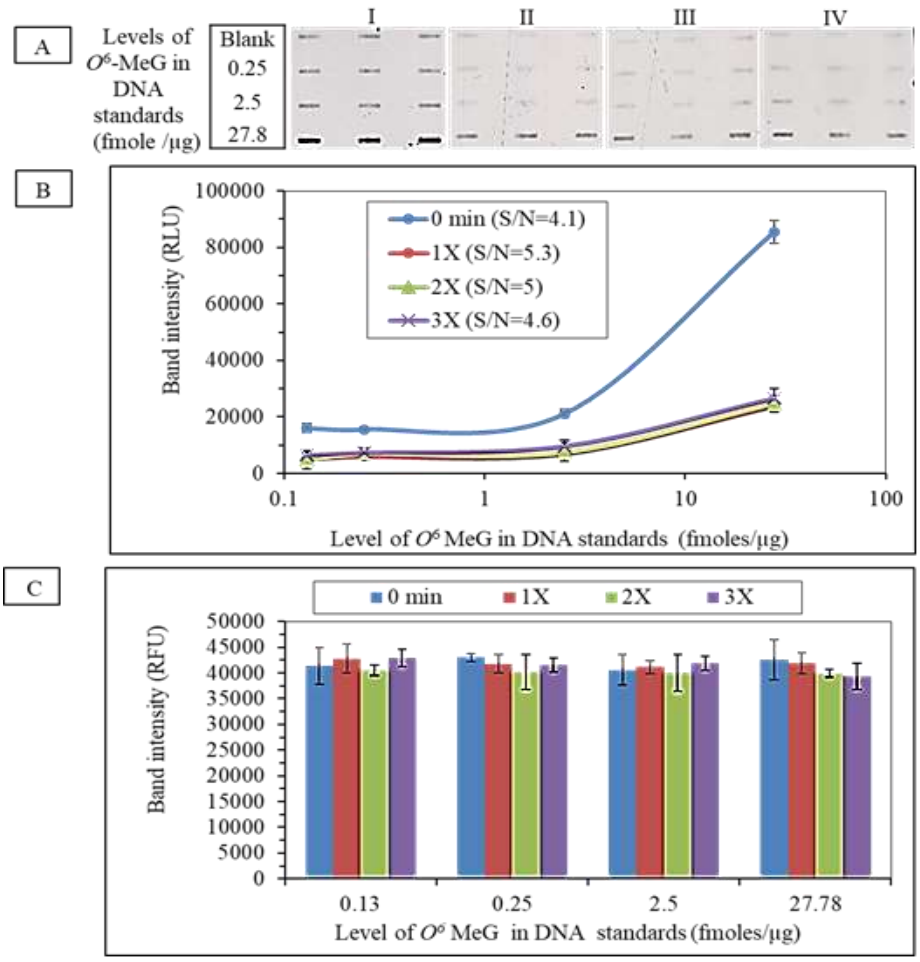


Figure 4.7 The impact of multiple washes with 1 M guanidine hydrochloride on Atl1 binding

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. The Atl1 binding step was for 1 hour at RT and the membrane was not washed (I) or washed with 1 M guanidine hydrochloride once (II), twice (III) or three times (IV) each time at RT for 5 min.

A marked lower ECL band intensity in all CT-DNA standards was seen for all three membranes washed with guanidine hydrochloride in comparison with not washed (Figure 4.7 B). However, all washings increased the signal/noise ratios, the highest (5.3) being after a single wash. There were no significant differences in PI staining band intensities following quantitation by image J (Figure 4.7C).

4.4.1.5.2 The effect of guanidine hydrochloride washing times

The results for different washing times (0, 1, 3, 5 and 7 minutes) with 1 M guanidine hydrochloride after Atl1 binding are shown in Figure 4.8.

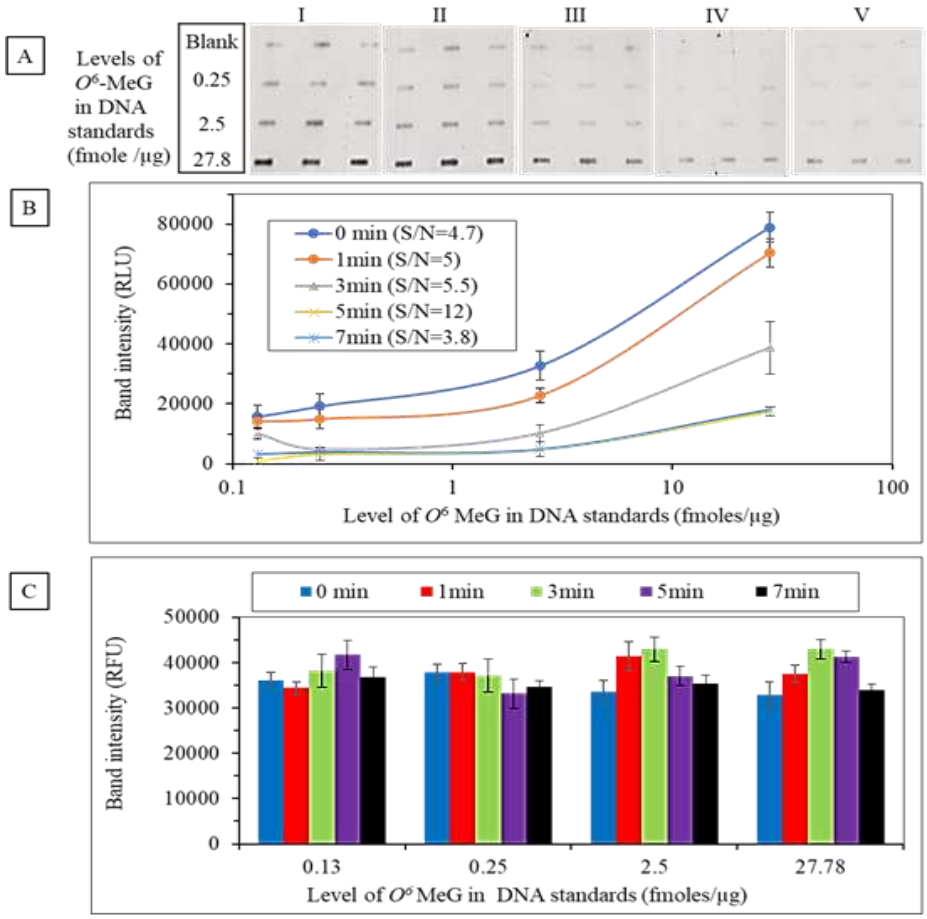


Figure 4.8 The effect of washing times with 1 M guanidine hydrochloride after Atl1 binding

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. The Atl1 binding step was for 1 hour at RT and membrane was not washed with 1 M guanidine hydrochloride (I) or washed at RT for 1min (II), 3 min (III), 5min (IV) or 7 min (V).

There was a consistent trend for the ECL band intensity in all CT-DNA standards to decrease with increased washing times in comparison with a membrane that was not washed (Figure 4.8 A and B). The highest signal/noise ratio (12) was obtained for the membrane washed for 5 min (Figure 4.8B) although this was likely to be due to the much lower blank CT DNA signal as the remainder of the values on the curve were almost identical to those obtained with a 7-min wash. There were no significant differences in PI staining band intensities following quantitation by image J (Figure 4.8C).

4.4.1.5.3 The effect of guanidine hydrochloride concentration

The results of washing membranes, after Atl1 binding, with different guanidine hydrochloride concentrations (0.05, 0.1, 0.5 and 1M) are shown in Figure 4.9. A marked lower ECL band intensity in all CT-DNA standards was seen for the membrane washed with 1M guanidine hydrochloride in comparison with other concentrations (Figure 4.9 B).

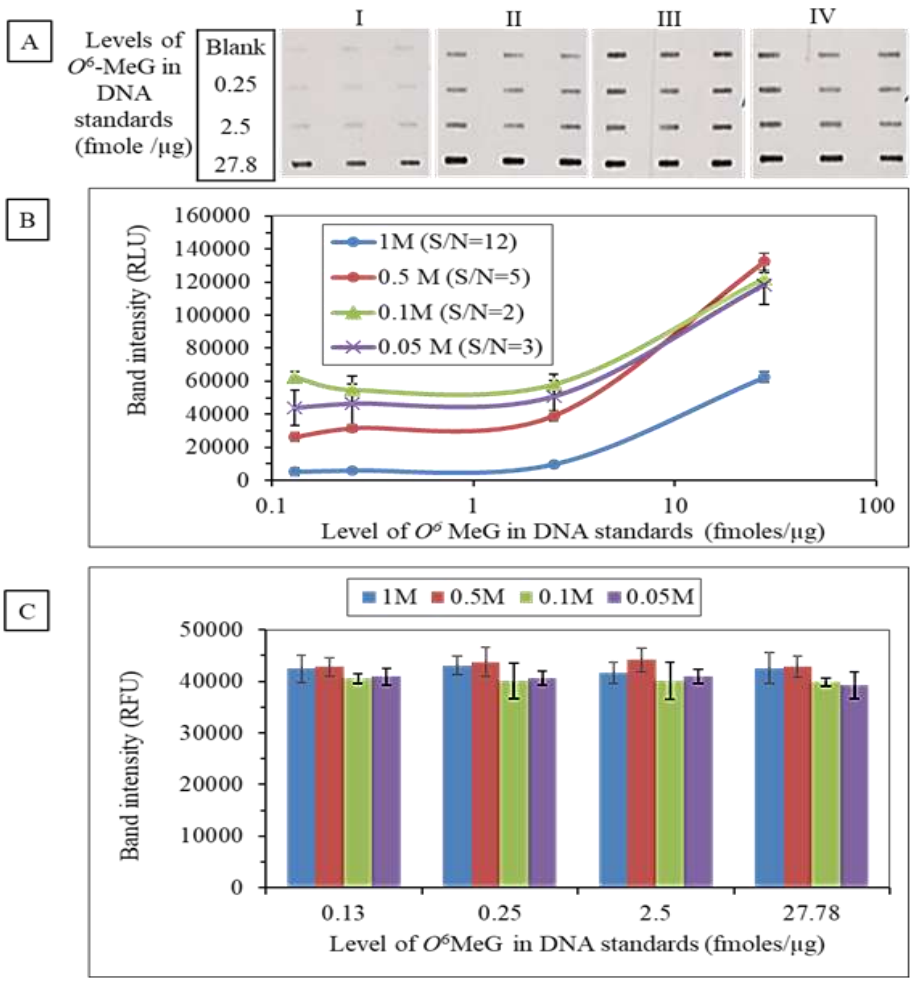


Figure 4.9 The effect of washing with different molarities of guanidine hydrochloride on Atl1 binding

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank. The Atl1 binding step was for 1 hour at RT and the membranes were washed with 1 M (I) 0.5 M (II) 0.1 M (III) or 0.05 M (IV) guanidine hydrochloride.

After quantification of the band intensity signals by image J the membrane washed with 1M guanidine hydrochloride recorded by far the highest signal: noise ratio (12) as shown in Figure 4.9B. There were no statistically significant differences in PI staining band intensities after quantification by image J (Figure 4.9C).

4.4.1.6 The effect of re-purification of CT-DNA on Atl1 binding

To determine if the background signal with Atl1 was due to a non-DNA contaminant, the blank CT-DNA standard was repurified by passing through a Qiagen midi column without any treatment or after addition of G2 buffer or G2 buffer +Proteinase K+ RNAase. The control was blank CT-DNA that was not Qiagen purified. Two hybond-N+ membranes were used and one was not washed (I) and the other, washed once with 1M guanidine hydrochloride for five minutes at RT (II) and the results are shown in Figure 4.10.

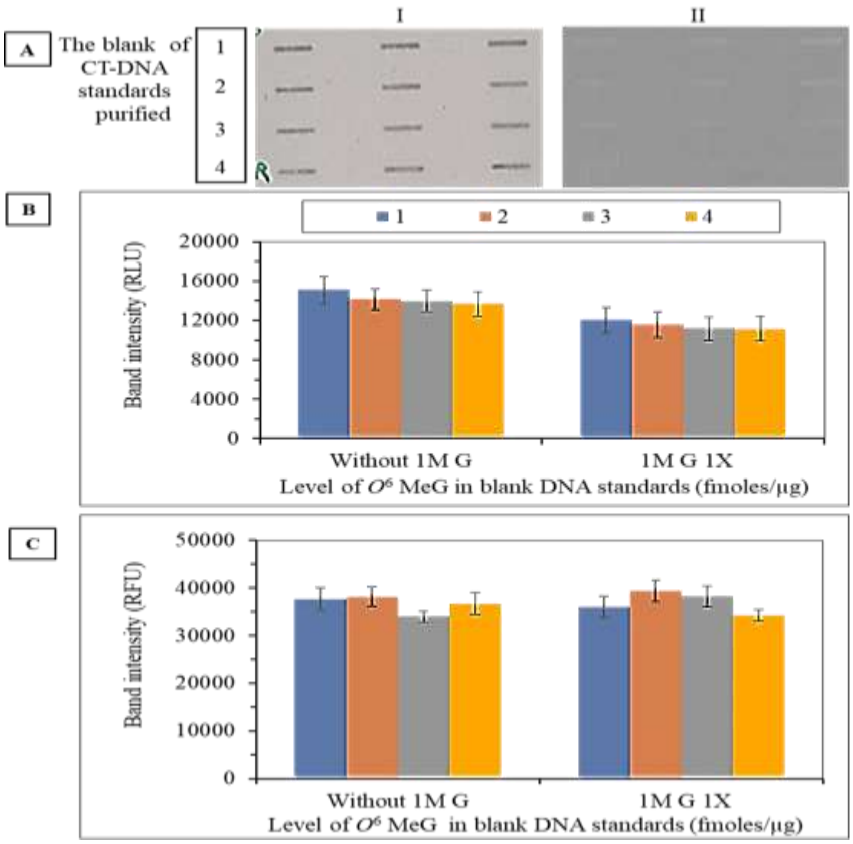


Figure 4.10 The effect of repurifying CT-DNA on non-specific binding
A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. The blank CT-DNA standard was (1) not purified by Qiagen DNA extraction kit column or purified (2) using only the kit column, (3) after addition of the kit G2 solution (4) after addition of G2, Proteinase K and RNAse and the membrane was not washed (I) or washed (II) with 1M guanidine hydrochloride once for 5 min at RT. The Atl1 binding step was for 1 hour at RT.

The 1M guanidine hydrochloride wash again had a clear impact on the ECL band intensity as shown in Figure 4.10A. After the band intensity signals were quantified by Image J, there

was a decrease but not a significant one, in band intensity of the blank CT-DNA after re-purification (Figure 4.10B). Repurification of the blank CT DNA therefore had no significant effect on Atl1 binding. There was no noticeable difference in PI staining band intensity after quantitation by image J (Figure 4.10C).

4.4.1.7 The effect of treatment of methylated CT-DNA with MGMT on Atl1 binding

To confirm that the Atl1 detected bands originated from O^6-MeG, two hybond-N+ membranes were used to analyse methylated CT-DNA standards that were sonicated and denatured and subsequently either incubated with MGMT for 2 hours at 37^{o}C (Figure 4.11, I) or left untreated (Figure 4.11, II) as shown below in Figure 4.11A and B.

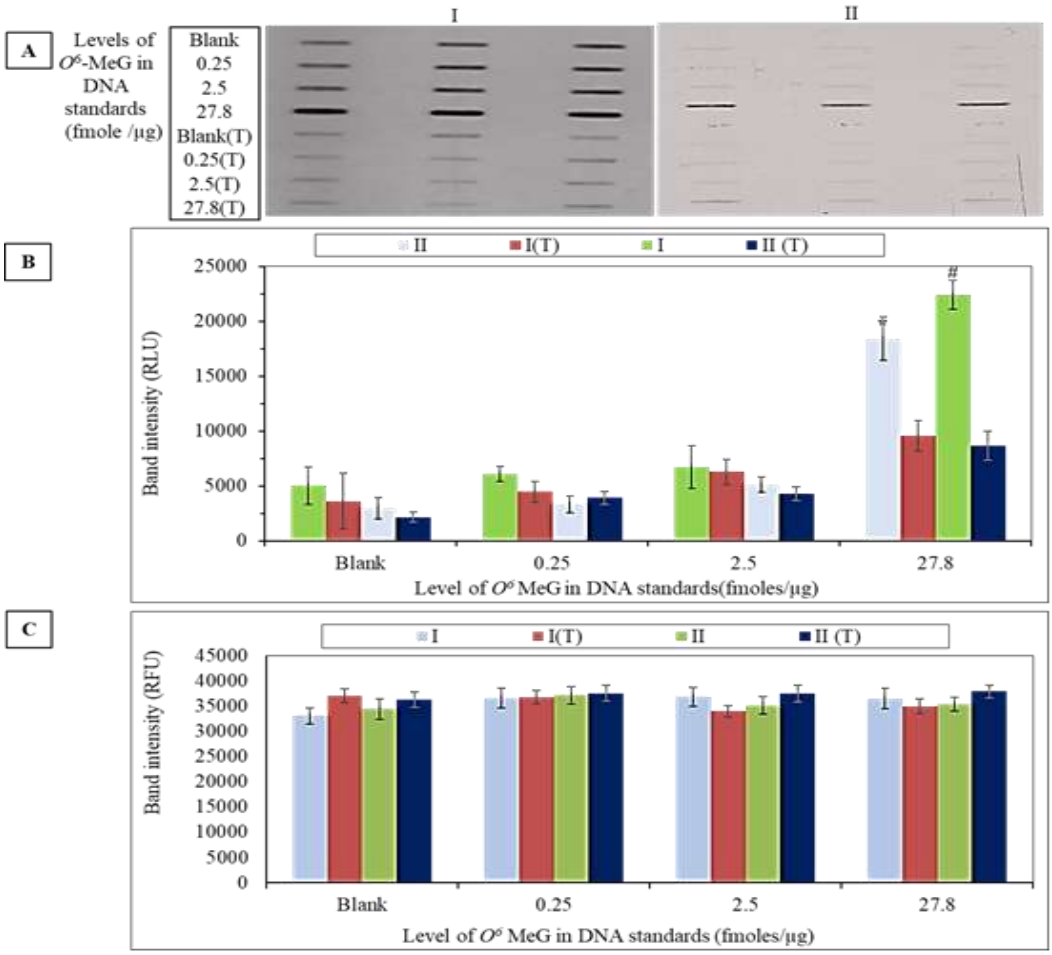


Figure 4.11 Atl1 binding to methylated CT-DNA standards with and without treatment with MGMT

A: ASB image, B: band intensities of scanned image analysed by Image J, C: PI staining band intensities analysed by Image J. Error bars represent SD. T: CT -DNA standards treated with MGMT for 2 hours at 37^{o}C, and neither sonicated nor denatured (I) or both sonicated and denatured (II). The Atl1 binding step was for 1 hour at RT and washing was for three times at RT for 5 minutes each with medium salt, *Band intensity of 27.8 standard not treated

with MGMT (I) was significantly higher than for treated with MGMT (IT), * $P \leq 0.05$; # Band intensity of 27.8 standard not treated with MGMT (II) was significantly higher than that for the treated standard (IIT) * $P \leq 0.05$.

The ECL band intensities were reduced by treatment with MGMT but these decreases were not statistically significant, except at the highest level of O^6-MeG (27.8 fmole/µg) as shown in Figure 4.11B. There was no clear difference in PI staining band intensities after quantitation by Image J as shown in Figure 4.11C.

These results suggest that both Atl1 and MGMT have the ability to bind and/or repair the O^6 MeG in both single and double-strand DNA. The ability of Atl1 to bind to O^6 MeG appears higher in double than the single-strand DNA, while the ability of MGMT to repair the O^6 MeG appears higher for single than for double-stranded DNA. By contrast PI appears to have the same ability to bind to single and double-strand DNA.

4.4.2 O^6-alkylG levels in sperm and buffy coat DNA

There were 28 sperm and 40 buffy coat DNA samples that had sufficient DNA for the ASB analysis. They were analysed using the optimised ASB protocol which involved baking at 80 °C for 30 min, then at RT: blocking with 5% milk/0.02% Tween, Atl1 (1:50,000 dilution) binding in the same buffer for 1 hour and washing once with medium salt buffer containing 1M guanidine hydrochloride. The O^6-alkylG level in the DNA samples was extrapolated from the methylated CT-DNA standard curve as shown in Figure 4.12 and Figure 4.14.

4.4.2.1 O^6- alkylG levels in sperm DNA

The ECL signals for the standards (Figure 4.12 A) and the standard curve (Figure 4.12 B) were consistent with previous results and gave a signal/noise ratio of 8.2. Note that the standards labelled with 0.13, 0.25, 2.5 and 27.8 were Qiagen purified as described above. The ECL signals for the sperm DNA samples showed wide variations both between participants and, where samples were available, between samples taken at different times from the same participant (Figure 4.12 C). This was reflected in the extrapolated O^6-alkylG levels which ranged from undetectable in two samples to 33.5 fmole/µg and the mean was 17.0±8.6 fmole/µg. For the three samples producing ECL bands that were denser than the highest standard, it was assumed that the standard curve proceeded linearly, but it is accepted

that the levels may be less accurate than the other samples. Participant 09 was an extreme case where the 1, 6 and 12 week samples had O^6-alkylG levels of 19.6, 2.2 and 19.3 fmole/µg, respectively. In contrast, for Participant 16 the 1, 6 and 12 week samples had O^6-alkylG levels of 22.8, 23.5 and 29.7 fmole/µg, respectively and for Participant 20 the 1, 6 and 12 week samples had O^6-alkylG levels of n/d, n/d and 4.0 fmole/µg, respectively.

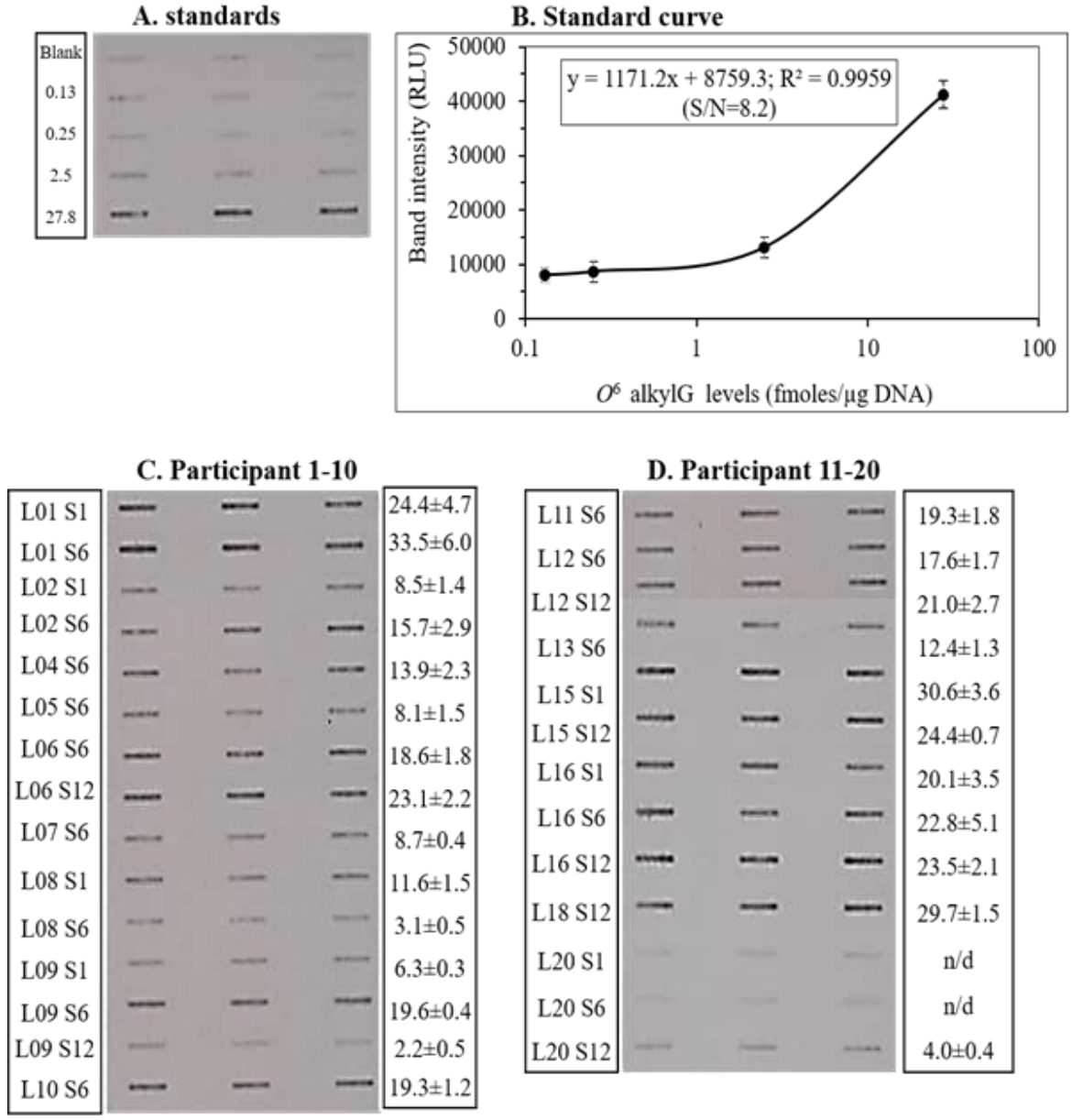


Figure 4.12 O^6-alkylG levels in sperm DNA

A: ASB image, B: band intensities of scanned image in A analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank; C and D: ASB image of sperm DNA samples from participants 1-10 and 11-20, respectively. Calculation of O^6-alkylG levels in C and D are based on standard curve (B). n/d: signal not detectably higher than blank.

There was no clear difference in PI staining band intensities (Figure 4.13 A and B) after quantitation by Image J as shown in Figure 4.13 C, so none of the ECL signal differences could be attributed to different levels of DNA binding to the membranes.

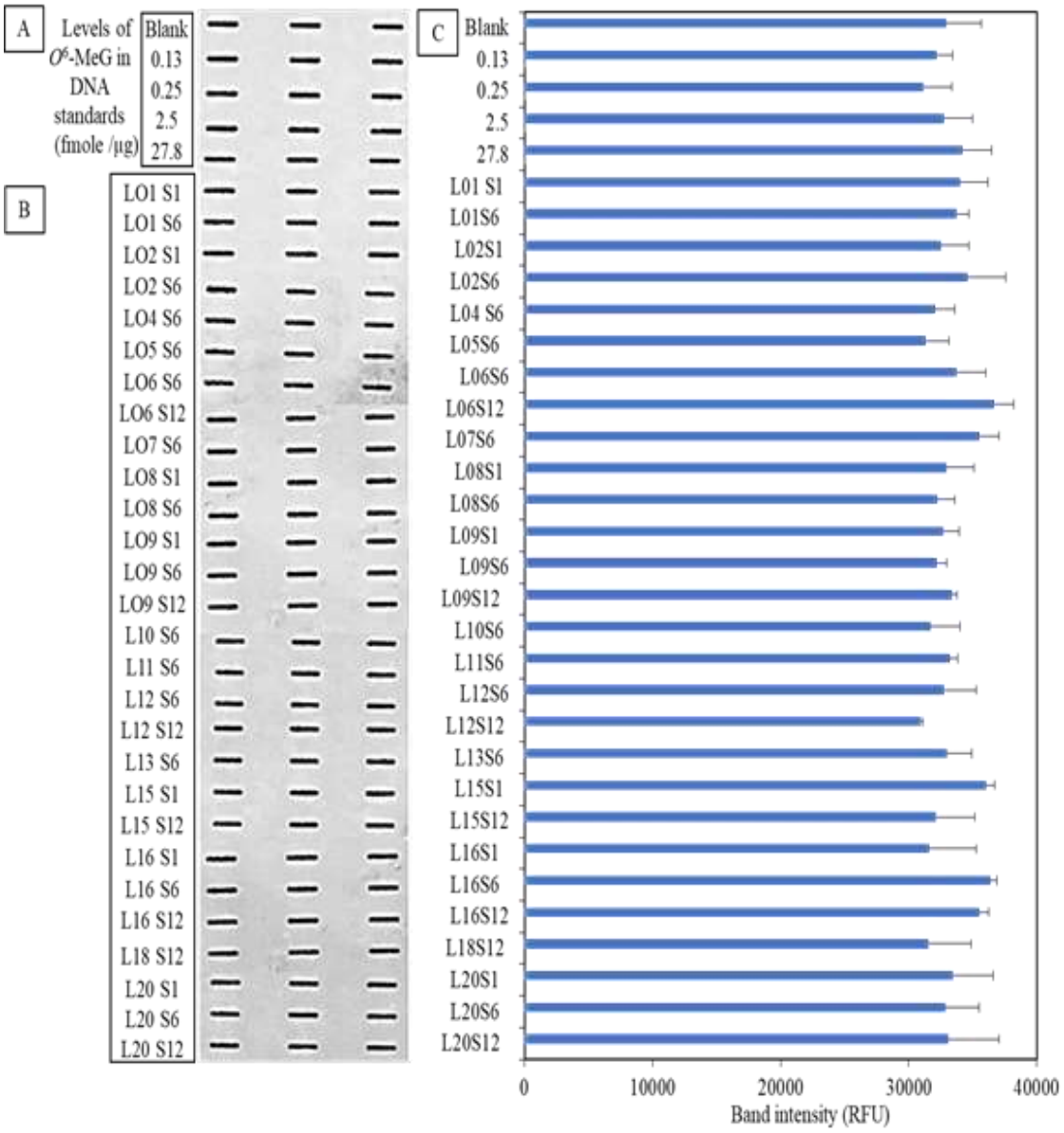


Figure 4.13 PI staining of standards and sperm DNA

A: PI staining of methylated CT-DNA standards and B, sperm DNA samples, C: Quantification of band intensity by Image J for membranes shown in A. Error bars represent SD.

4.4.2.2 *O*^{6}-alkylG levels in buffy coat DNA

The ECL signals for the standards (Figure 4.14 A) and the standard curve (Figure 4.14 B) were consistent with previous results and gave a signal/noise ratio of 8. The ECL signals for

the buffy coat DNA samples showed wide variations both between participants and, where samples were available, between samples taken at different times from the same participant (Figure 4.14 C). This was reflected in the extrapolated O^6-alkylG levels shown in Figure 4.14 D, which ranged from undetectable in 5 samples to 15.7 fmole/µg and the mean was 5.9 ±4.2 fmole/µg. Five DNA samples had detectable signals but these were not quantifiable.

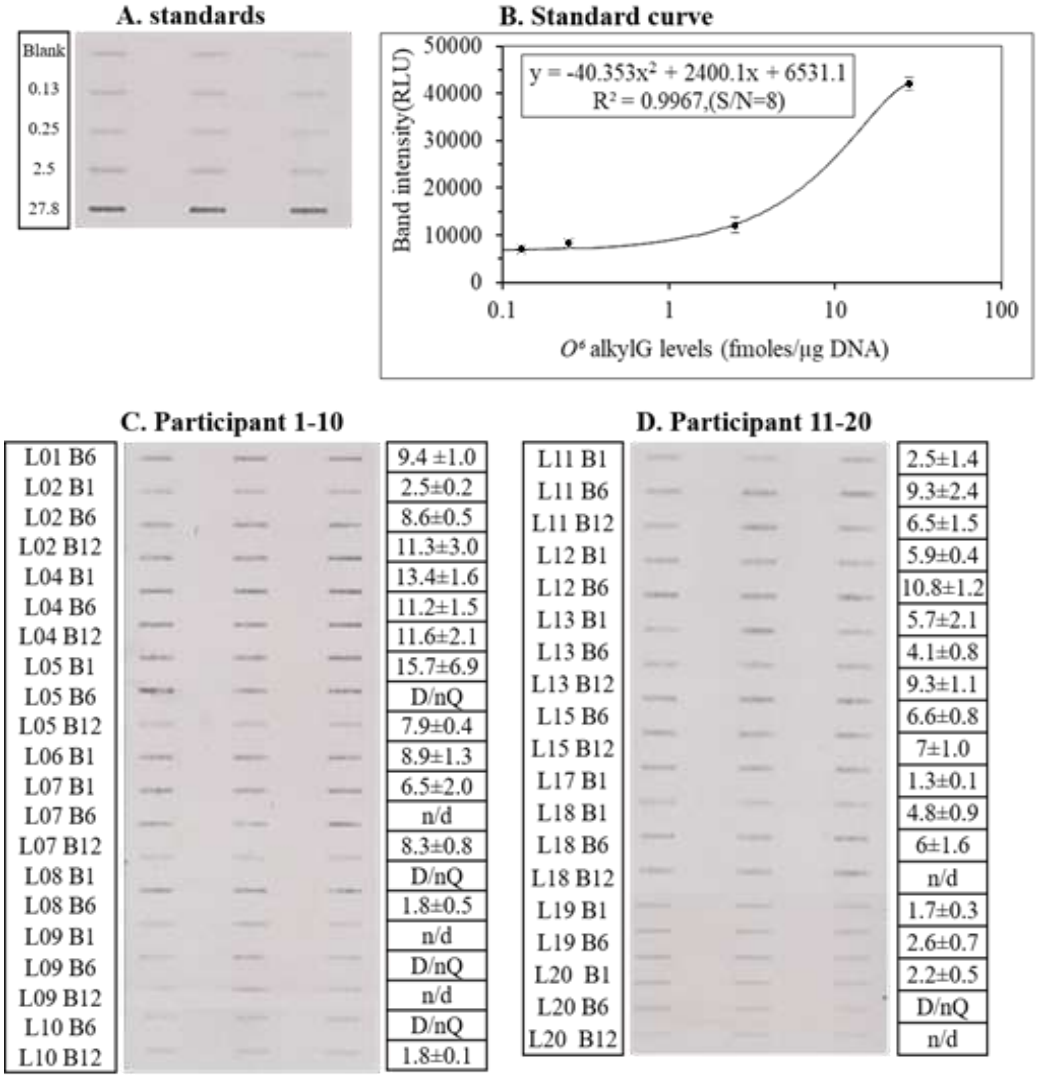


Figure 4.14 O^6-alkylG levels in buffy coat DNA

A: ASB image, B: band intensities of scanned image in A analysed by Image J. Error bars represent SD. S/N = signal/noise ratio for 27.8 fmole/ug vs Blank; C and D: ASB image of buffy coat DNA samples from participants 1-10 and 11-20, respectively. Calculation of O^6-alkylG levels in C and D are based on standard curve. n/d: signal not detectably higher than blank; D/nQ signal visibly above blank but not quantifiable.

The visual appearance of PI band intensity on the hybond-N+ membranes revealed no significant differences in the standards and buffy coat DNA binding as shown below in Figure 4.15 A and this was confirmed by quantification by Image J as shown in Figure 4.15

B. Hence it is reasonable to conclude that none of the ECL signal differences could be attributed to different levels of DNA binding to the membranes.

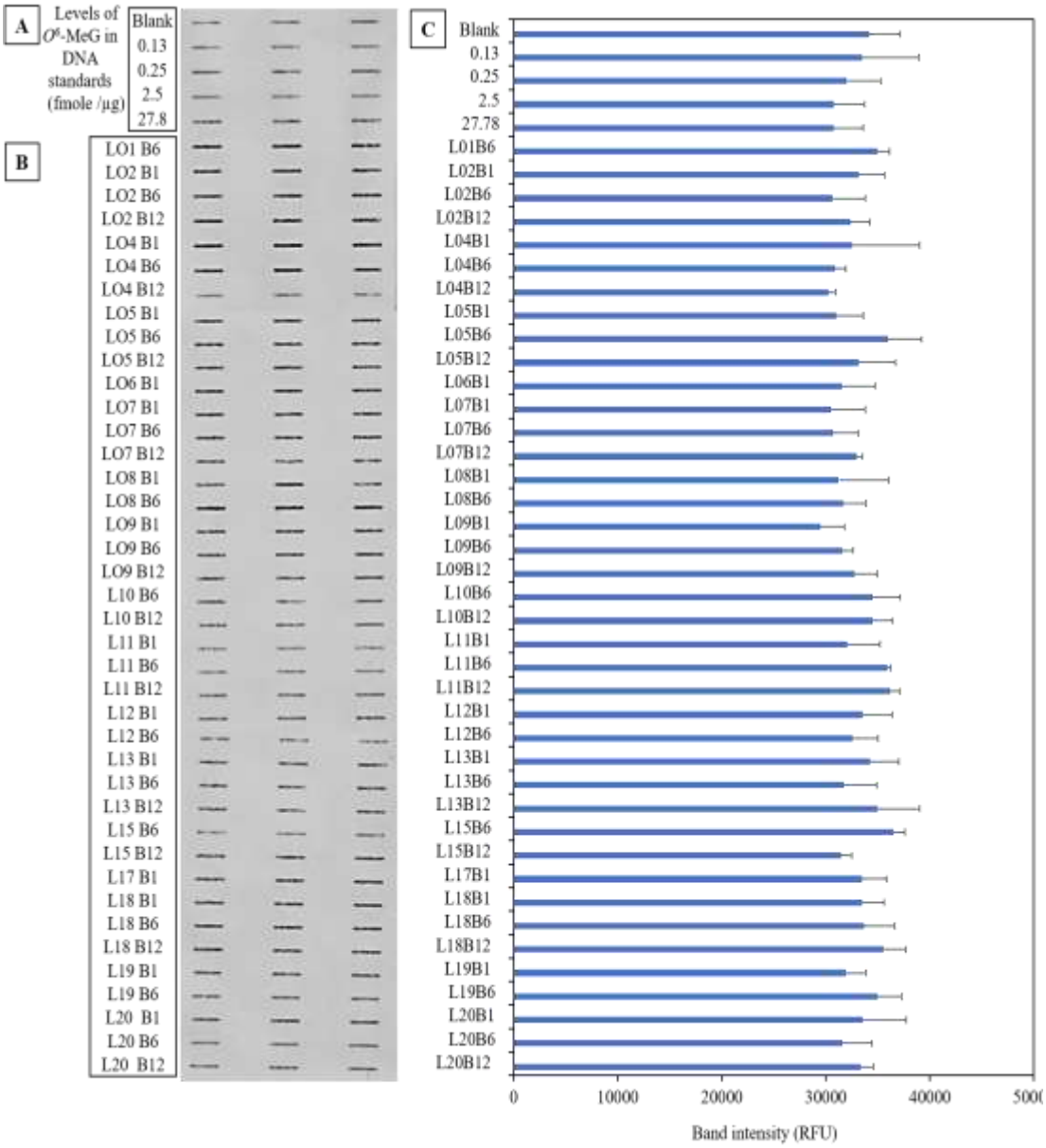


Figure 4.15 PI staining of standards and buffy coat

A: PI staining of methylated CT-DNA standards, B: longitudinal of buffy coat DNA samples; and C: Quantitation of band intensity by Image J for membranes shown in A and B. Error bars represent SD.

4.4.2.3 Correlation between O^6-alkylG levels in sperm and buffy coat DNA

As shown in Figure 4.16, where DNA samples were available from the same participant at the same cycle number, the correlation between the O^6-alkylG levels in sperm and buffy coat, were not significant (Spearman's rho 0.4). For the buffy coat DNA samples that had 2

fmoles/ug or more, the corresponding levels in the sperm DNA were 1.2 to 3.5 times higher than buffy coat DNA. However, where the values for buffy coat were measurable but lower than 2 fmoles/ug, there may have been up to >30 times more O^6-alkylG in the corresponding sperm DNA.

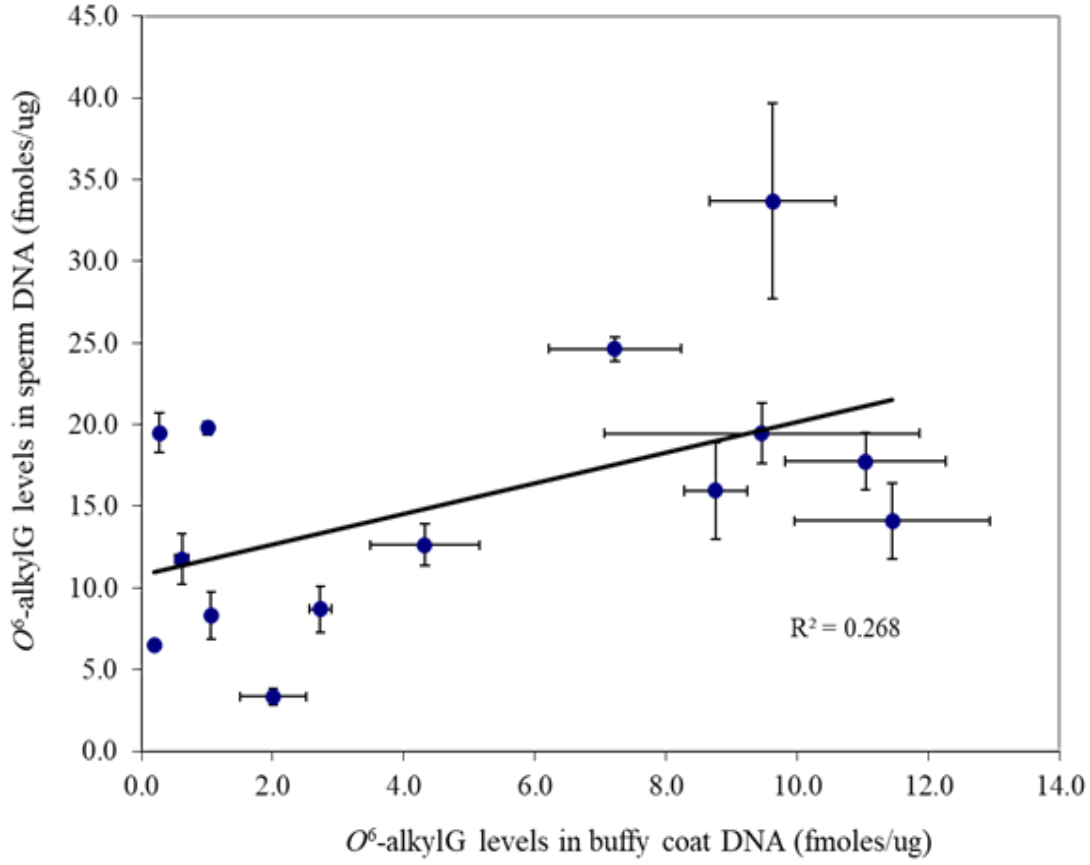


Figure 4.16 O^6-alkylG levels in sperm and buffy coat DNA
Values that are shown as n/d or Q/nd in Figures 4.12 and 4.14 are not plotted. Error bars indicate SD. Excel generated the regression line.

4.4.2.4 Effect of treatment of sperm and buffy coat DNA with MGMT on Atl1 binding

Six DNA samples that had a sufficient DNA were incubated with MGMT as described previously and analysed by ASB. Figure 4.17 A and B shows that there was a decrease in the ECL signals obtained from methylated CT-DNA standards after treatment with MGMT, but only for the 2.5 and 27.8 fmole/µg standards.

However, there was a significant reduction in the band intensity of all sperm and buffy coat DNA samples after treatment with MGMT as shown in Figure 4.17B. In addition, the visual

appearance of PI band intensity on the hybond-N+ membrane revealed no significant differences in the treated and non-treated as shown in Figure 4.18 A, and there was no significant difference in PI band intensities between the treated and non-treated as shown in Figure 4.18B.

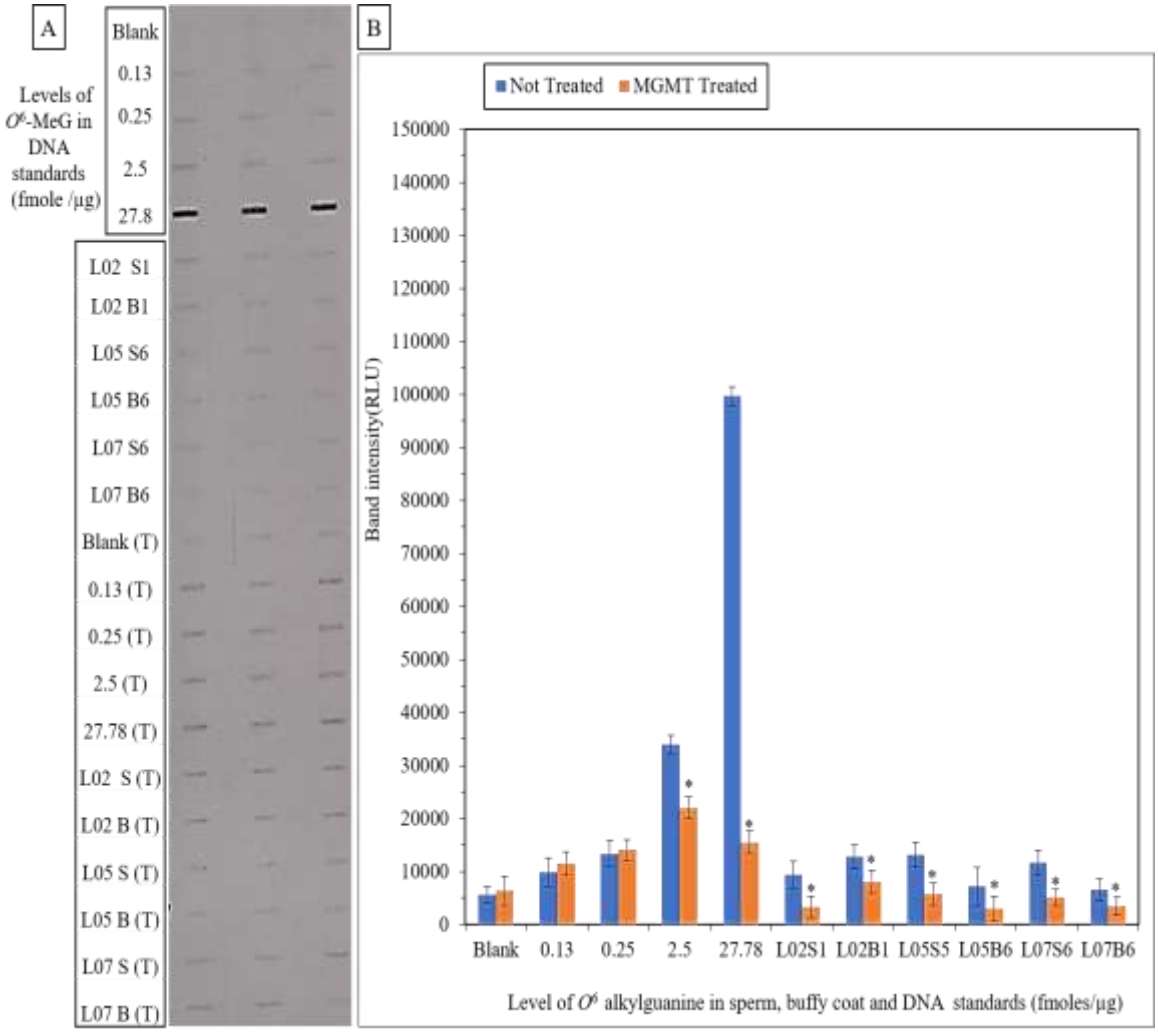


Figure 4.17 Effect of treatment of sperm and buffy coat DNA with MGMT on Atl1 binding

A: ASB image, B: band intensities of scanned image in A analysed by Image J. Error bars represent SD.; L: longitudinal, S: sperm DNA; B: buffy coat DNA; T: DNA treated with MGMT for 2 hours at 37^{o}C *Band intensity signals significantly decreased after treated with MGMT, $P \leq 0.05$.

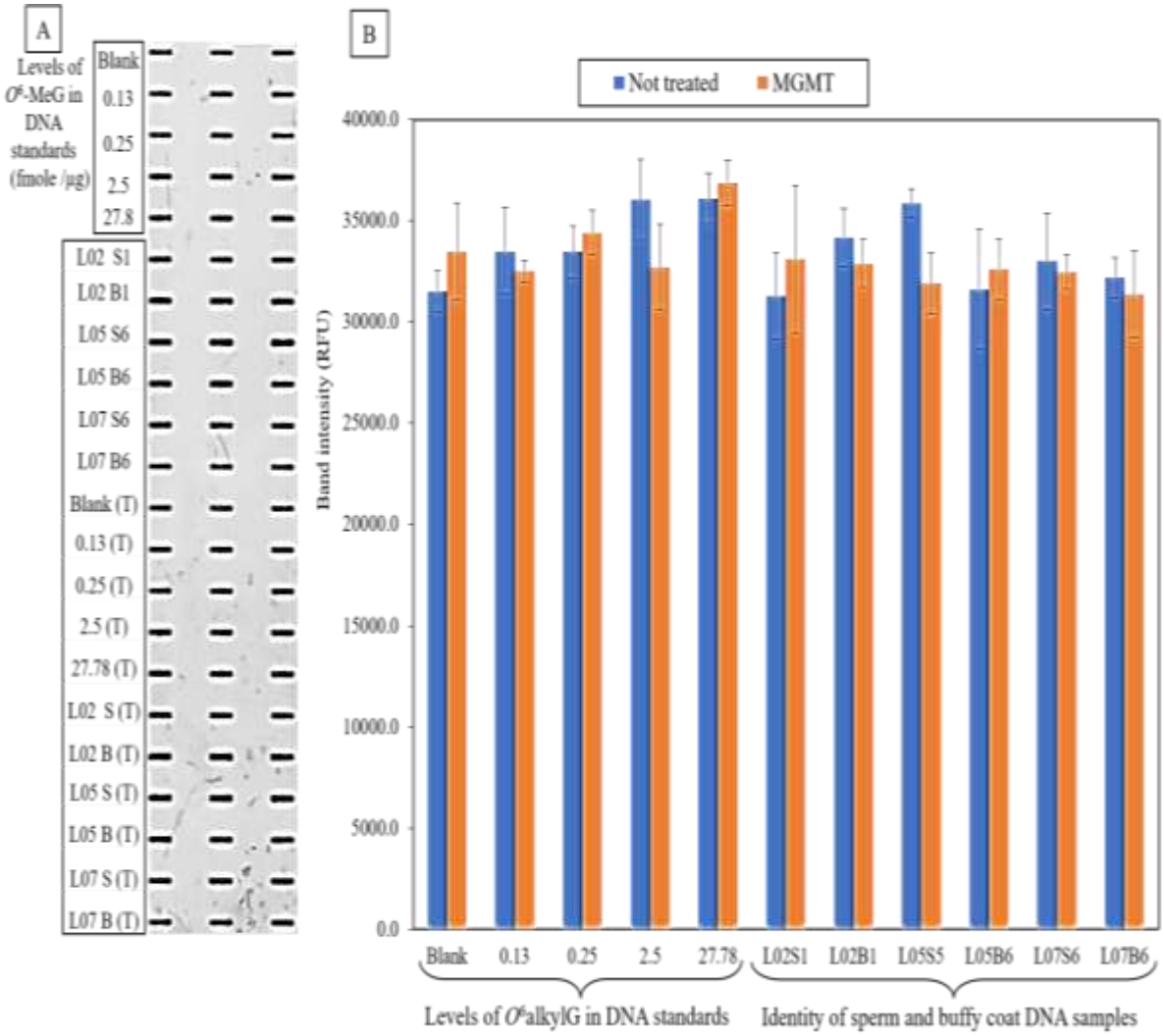


Figure 4.18 PI staining of methylated CT-DNA standards, sperm and buffy coat DNA with and without MGMT treatment

A: PI staining of methylated CT-DNA standards and buffy coat DNA samples, B: quantitation of band intensity by Image J. Error bars represent SD. L: longitudinal, S: sperm DNA; B: buffy coat DNA; T: DNA treated with MGMT for 2 hours at 37^{o}C.

4.4.2.5 Correlations between O^6-alkylG levels in sperm and buffy coat DNA and sperm DNA damage measured by neutral Comet assay

Figure 4.19 shows the variation in GDI and O^6-alkylG levels in sperm and buffy coat DNA for each participant at each time the samples were taken. There were no significant correlations between O^6-alkylG levels in sperm and buffy coat DNA samples with sperm DNA damage at baseline, six and twelve weeks during the study period. However, based on 7 participants' data, there was a significant negative correlation ($R = -0.94$, $P<0.01$) between GDI and the level of O^6-alkylG adducts in sperm DNA at baseline.

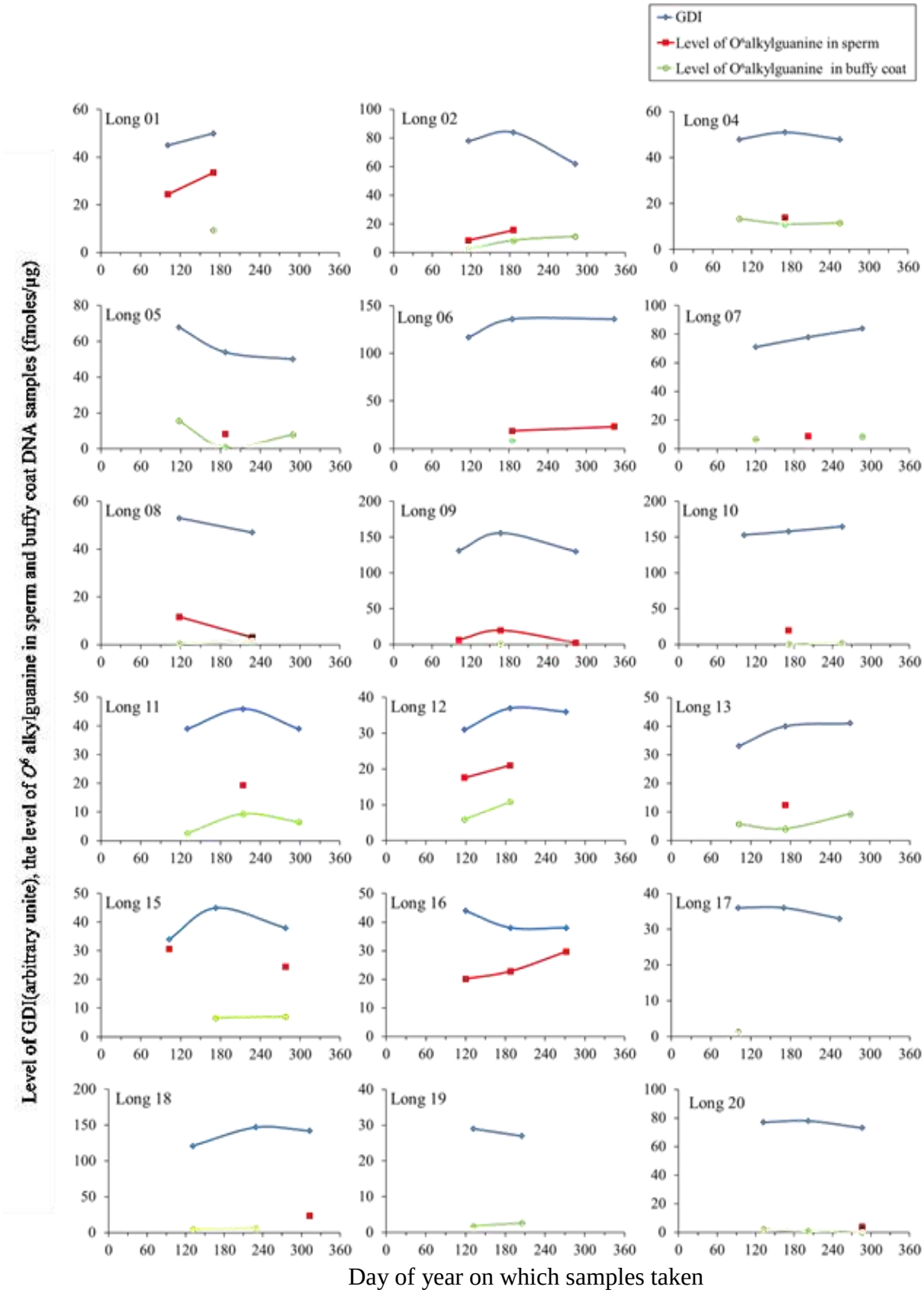


Figure 4.19 Sperm GDI and O^6-alkylG levels in sperm and buffy coat DNA

4.4.2.6 Correlations between semen parameters and O^6-alkylG levels in sperm and buffy coat DNA

There were no significant correlations between any of the semen quality parameters and the O^6-alkylG levels in sperm DNA at baseline, six and twelve weeks during the study period (data not shown). However, there was a significant negative correlation ($R = -0.86$, $P < 0.05$) between the O^6-alkylG levels in buffy coat DNA and % progressive sperm motility at the sixth week of the study.

4.4.2.7 Correlations between O^6-alkylG levels in sperm and buffy coat DNA with age, abstinence period and BMI

There were no significant correlations between the levels of O^6-alkylG levels in buffy coat DNA and sperm with age, BMI and abstinence at baseline, six and twelve weeks during the study period. However, there were significant positive correlations between O^6-alkylG levels in sperm DNA and BMI at baseline ($R = 0.83$, $P<0.05$) and between O^6-alkylG levels in buffy coat DNA and age at baseline ($R = 0.69$, $P< 0.05$). In addition, there was a significant negative correlation between O^6-alkylG levels in sperm DNA and age in the sixth week of the study period ($R = -0.57$, $P< 0.05$).

4.5 Discussion

As described in the introduction, O^6-alkylG in DNA can have several deleterious cellular effects as this is one of the principal cytotoxic, mutagenic and carcinogenic lesions induced by exposure to alkylating agents (Povey et al., 2000).

In this chapter a sensitive and reproducible ASB assay was developed and optimised for the quantification of O^6-alkylG levels in human sperm and buffy coat DNA samples in order to determine if this might be related to any of the DNA damage, semen or lifestyle, etc, factors described in Chapters 2 and 3.

Optimisation involved assessing a number of parameters in the assay in order to decrease Atl1 binding to non-methylated DNA, which was considered to be due to non-specific binding, without decreasing the signal from methylated DNA which was considered to be

due to specific binding (see below). The effectiveness of the condition was indicated by the signal/noise ratio. Standard curves were fitted by excel to a 2^{nd} order polynomial function which determined correlation coefficients that were usually greater than 0.96 indicating that all assay results were generally highly reliable. It should be noted that none the conditions used affected the signals obtained with PI staining and therefore did not affect the amount of DNA bound to the membrane.

The effect of temperature on the binding of Atl1 was investigated and while Atl1 bound more extensively at RT, the signal/noise ratio was not different between this and 37^{o}C, so RT was used in subsequent assays. When BSA and Marvel were compared for the membrane blocking step the former resulted in a completely black film, so Marvel was used in subsequent assays.

The effect of different percentages of Tween 20 was then compared in the blocking and Atl1 binding steps. The results suggested that 0.02% was the most appropriate concentration to use in subsequent experiments because it produced the largest signal/noise ratio, although at 1.7, this was only marginally higher than obtained without Tween (S/N, 1.6).

Different times for membrane baking at 80^{o}C were also studied. There was a significant increase in ECL band intensity with 120 min baking for all CT-DNA standards when compared with 30, 60 or 90 min. but the highest signal/noise ratio was seen at 30 min (6.1). It was concluded that 30 min baking was optimal for routine ASB assays. PI staining was almost identical for each standard and each baking time, so this was not the basis of the increased sensitivity.

The membrane washing steps post Atl1 binding were then modified by varying the temperature and the salt concentration of the washing solution. These experiments showed that the optimal washing conditions were medium salt at RT which recorded the highest signal/noise ratio (5.6).

Another attempt to decrease nonspecific binding involved washing the hybond-N^{+} membrane after the RT Atl1 binding step, with guanidine hydrochloride solution using conditions that varied the number of wash times, length of time, and the molarity. The highest signal/noise ratio was observed after 1 wash with 1M guanidine hydrochloride which is a strong chaotropic agent and one of the strongest denaturants used in physiochemical

studies of protein folding and it increases the solubility of hydrophobic molecules (Monera et al., 1994).

The final protocol was as follows: membrane baking was for 30 min at 80°C, then, all at RT: for the blocking and Atl1 binding steps the buffer contained 5% Marvel and 0.02% Tween and were for 1 hour each; the membrane was then washed with medium salt buffer three times for 5min each, then once with 1M guanidine hydrochloride for five minutes. The optimised ASB requires only small quantities of DNA is rapid and sensitive and can now be applied to large-scale epidemiological studies.

To investigate the possibility that the blank signal was caused by a non-DNA contaminant of the CT-DNA, it was re-purified using different conditions in combination with the Qiagen midi DNA extraction kit and columns. Repurification did not affect the band intensity, hence it was unlikely that the background was due to a non-DNA contaminant.

To confirm that the Atl1-detected bands originate from O^6-MeG in the methylated CT-DNA standards, they were treated with MGMT. In general, the band intensities decreased indicating that the bands originated from O^6-MeG.

There have been no previous reports of the detection of O^6-alkylG in sperm and buffy coat DNA so this is the first study to investigate this, and to study the correlation between O^6-alkylG levels in sperm and buffy coat DNA with semen quality and sperm DNA damage measured by neutral Comet assay in the same individual.

Forty-one and forty-three longitudinal sperm and buffy coat DNA samples respectively were extracted, although only 28 sperm and 40 buffy coat samples contained sufficient DNA for ASB analysis. All but two sperm DNA samples and 5 buffy coat DNA samples had quantifiable O^6-alkylG levels and another five buffy coat DNA samples had trace but not quantifiable levels. The O^6-alkylG levels were universally higher in sperm than the buffy coat. In two cases, sperm DNA levels were probably more than 30 times higher than buffy coat levels. This might be an indication that the repair of these lesions in white blood cells by MGMT, or other repair pathways, is more active than in sperm cells, which are generally accepted to have no, or deficient, DNA repair systems. The origin of the Atl1 generated signal in both sperm and buffy coat DNA was assessed by pretreating the purified DNA with the MGMT protein, but even after MGMT treatment, signals were still detectable. This may

be due to" true" non-specific binding of Atl1 to DNA or binding to DNA O^6-alkylG lesions that were not substrates for, or repaired by, MGMT.

There were some significant positive or negative correlations between O^6-alkylG levels in sperm and/or buffy coat DNA samples with GDI, BMI and sperm parameters. However, the number of participants and number of DNA samples that were in sufficient quantity for ASB analysis were insufficient to arrive at any firm conclusion.

4.6 Conclusion

The present studies demonstrated the feasibility of the application of a novel ASB methodology that had sufficient sensitivity to quantify O^6-alkylG levels in small amounts of human DNA. This novel method could therefore potentially contribute to the characterization of the overall load of O^6-alkylG in sperm or any other human DNA samples.

The numbers of samples involved precluded any meaningful conclusions to be reached about the relationships of O^6-alkylG levels in either sperm or buffy coat DNA with any other parameter measured in these studies.

Chapter 5 Overall discussion

This study has attempted to accumulate data on semen quality parameters and the levels of sperm DNA damage in terms of DSB and O^6-alkylG in order to establish if there are any correlations between these and various participant exposure factors. While the participants were not attempting to become parents, it was considered that any clear correlations might, in future studies, help to elucidate the basis of decreases in male fertility. In particular, O^6-alkylG levels in sperm DNA have not previously been investigated in this way.

Chapter 2 determined the semen parameters for all the samples obtained. As expected, there was a positive statistically significant correlation between CASA and manual haemocytometer counting. All semen parameters were in the range of WHO 2010 reference values. The total sperm count displayed the highest variations through six months of the longitudinal study, while the % vital sperm had the lowest variations. No clear impact of lifestyle, occupational or environmental exposures was seen.

Chapter 3 assessed DNA DSB by the neutral comet assay and compared various scoring methods. While two software packages and manual scoring were closely correlated, GDI was required for the study as neither software recognised the Comets resulting from highly damaged sperm. The main findings were that each participants' GDI was remarkably consistent throughout the study period, with the CV ranging between 2 and 14 % but that there was a substantial difference between the highest and lowest GDI figures. This might suggest that different individuals were consistently exposed to different levels of DNA damaging agents although the possible role of individual differences in the effectiveness of DNA repair pathways might be speculated. There was a significant negative correlation between GDI and % vital sperm at baseline of the study. However, there was no clear impact of lifestyle, occupational or environmental exposures and no correlation with any semen parameter, so the basis of these differences remain to be established.

Chapter 4 described the development, optimisation and application of a novel assay for the levels of O^6-alkylG in DNA which was shown to be both sensitive and reproducible. Interestingly, the levels of O^6-alkylG in DNA of sperm was higher than the buffy coat and

that might be again due to DNA repair mechanisms being more active in leukocytes than in sperm, although this remains to be verified experimentally. Pretreating the DNA samples with the MGMT protein did not completely eliminate the signal obtained by Atl1 binding suggesting that some of the lesions recognised by the latter are not repaired by the former. This requires verification with much larger numbers of samples. Again, there was no clear impact of lifestyle, occupational or environmental exposures and no correlation with any semen parameter, or GDI, so the basis of these differences remain to be established.

Future work

The present studies have established that it is practical to apply all of the described methodologies to participants and their semen samples. In order to establish if significant correlations exist between the semen and the other parameters, the major future objective would be to increase the numbers of participants so that any null hypotheses can be confirmed or refuted. Calculations as described by Jones et al (2003) and in http://statulator.com/SampleSize/ss2PM.html could be employed to determine the number of participants required to address specific questions. For example, using the baseline data for Comet tail length of 56±30 (n=20), to detect a 25% difference in tail length with a power of 80% and a level of significance of 5% (two sided), then a study would require a total sample size of 146, assuming equal group sizes.

Another example is: the mean difference in tail length between S1 (baseline sample) and S6 (6th sample collected) samples for 18 men in this feasibility study was 2.1 with a SD of 9.1; 2 men were excluded as they didn't provide the S6 sample. The mean tail length at baseline was 52.2±28 (n=18). Therefore, a future study would require a sample size of 161 men (providing both samples) to achieve a power of 80% and a level of significance of 5% (two sided), for detecting a mean of the differences of 2 between pairs, assuming the standard deviation of the differences to be 9.

However, for the DNA adduct work there are some sensitivity limitations and it is narrow in scope, so other adducts should be included where possible. DNA strand break damage assessed by the neutral Comet assay might also be compared with the alkaline Comet assay. Relevant serum hormones and seminal plasma components might also be investigated in these studies.

Suggestions are therefore to:

- Establish a routine applicable methodology for DNA adducts such as 8oxoguanine, N7-alkylguanine and N3-alkyladenine.
- Increase the sensitivity of the O^6-alkylG assay so that much less DNA is required.
- Obtain funding for a large-scale study involving at least 200 participants in order to establish if any significant correlations exist that might help couples to conceive.
- Carry out a pilot study of DNA damage measured by neutral and alkaline Comet assay in both sperm and leucocytes in the same participants.
- Determine the semen and DNA damage parameters and all lifestyle etc parameters in the large-scale study, part of which could be to.
- Study the effect of a specific dietary intervention, such as honey, which contains antioxidant activity on the variability of semen quality and DNA damage.
- Study the correlation between the blood concentrations of hormone such as testosterone, LH, FSH, Inhibin, TSH, T3, and T4 with semen parameters and sperm DNA damage.
- Study the correlation between the seminal plasma concentrations of fructose, zinc and 8-oxoG with semen parameters and sperm DNA damage.

Reference List

Adamopoulos, D.A., Lawrence, D.M., Vassilopoulos, P., Contoyiannis, P.A. and Swyer, G.I.M., 1978. Pituitary-testicular interrelationships in mumps orchitis and other viral infections. Br Med J, 1(6121), pp.1177-1180.

Adamopoulos, D.A., Pappa, A., Nicopoulou, S., Andreou, E., Karamertzanis, M., Michopoulos, J., Deligianni, V. and Simou, M., 1996. Andrology: Seminal volume and total sperm number trends in men attending subfertllity clinics in the greater Athens area during the period 1977–1993. Human Reproduction, 11(9), pp.1936-1941.

Adler, M., Mitchell, H., Richens, J., Cowan, F. and French, P., 2004. ABC of sexually transmitted infections. BMJ Publishing Group.

Afeiche, M., Williams, P.L., Mendiola, J., Gaskins, A.J., Jørgensen, N., Swan, S.H. and Chavarro, J.E., 2013. Dairy food intake in relation to semen quality and reproductive hormone levels among physically active young men. Human Reproduction, 28(8), pp.2265-2275.

Afeiche, M.C., Gaskins, A.J., Williams, P.L., Toth, T.L., Wright, D.L., Tanrikut, C., Hauser, R. and Chavarro, J.E., 2014. Processed meat intake is unfavorably and fish intake favorably associated with semen quality indicators among men attending a fertility clinic. The Journal of Nutrition, 144(7), pp.1091-1098.

Agarwal, A., Mulgund, A., Hamada, A. and Chyatte, M.R., 2015. A unique view on male infertility around the globe. Reproductive Biology and Endocrinology, 13(1), p.37.

Aitken, R.J. and De Iuliis, G.N., 2010. On the possible origins of DNA damage in human spermatozoa. Molecular Human Reproduction, 16(1), pp.3-13.

Aitken, R.J., De Iuliis, G.N. and mclachlan, R.I., 2009. Biological and clinical significance of DNA damage in the male germ line. International Journal of Andrology, 32(1), pp.46-56.

Altakroni, B., 2015. Occupational and environmental exposures, sperm DNA damage and infertility (Doctoral dissertation, The University of Manchester UK).

Alvarez, C., Castilla, J.A., Martinez, L., Ramirez, J.P., Vergara, F. and Gaforio, J.J., 2003. Biological variation of seminal parameters in healthy subjects. Human Reproduction, 18(10), pp.2082-2088.

Amann, R.P. and Waberski, D., 2014. Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology, 81(1), pp.5-17.

Amireault, S. and Godin, G., 2015. The Godin-Shephard leisure-time physical activity questionnaire: validity evidence supporting its use for classifying healthy adults into active and insufficiently active categories. Perceptual and motor skills, 120(2), pp.604-622.

Anderson, D., Dobrzyńska, M.M. and Basaran, N., 1997. Effect of various genotoxins and reproductive toxins in human lymphocytes and sperm in the Comet assay. Teratogenesis, carcinogenesis, and mutagenesis, 17(1), pp.29-43.

Auger, J., Eustache, F., Andersen, A.G., Irvine, D.S., Jørgensen, N., Skakkebaek, N.E., Suominen, J., Toppari, J., Vierula, M. and Jouannet, P., 2001. Sperm morphological defects related to environment, lifestyle and medical history of 1001 male partners of pregnant women from four European cities. Human Reproduction, 16(12), pp.2710-2717.

Auger, J., Kunstmann, J.M., Czyglik, F., Jouannet, P., 1995. Decline in semen quality among fertile men in Paris during the past 20 years. N. Engl. J. Med. 332, 281–285.

Auger, J., Mesbah, M., Huber, C. and Dadoune, J.P., 1990. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. International Journal of Andrology, 13(6), pp.452-462.

Badouard, C., Ménézo, Y., Panteix, G., Ravanat, J.L., Douki, T., Cadet, J. and Favier, A., 2008. Determination of new types of DNA lesions in human sperm. Zygote, 16(01), pp.9-13.

Balbo, S., Turesky, R.J. and Villalta, P.W., 2014. DNA adductomics. Chemical Research in Toxicology, 27(3), pp.356-366.

Baldi, E. and Muratori, M. eds., 2014. Genetic damage in human spermatozoa. Springer.

Banáth, J.P. and Olive, P.L., 2003. Expression of phosphorylated histone H2AX as a surrogate of cell killing by drugs that create DNA double-strand breaks. Cancer Research, 63(15), pp.4347-4350.

Bansal, S.K., Singh, V., Singh, K. and Singh, R., 2017. Sex Chromosomal Genes in Male Infertility. In Male Infertility: Understanding, Causes and Treatment (pp. 253-270). Springer, Singapore.

Barlow, N.J. and Foster, P.M., 2003. Pathogenesis of male reproductive tract lesions from gestation through adulthood following in utero exposure to di (n-butyl) phthalate. Toxicologic Pathology, 31(4), pp.397-410.

Barrett, K.E., Barman, S.M. and Boitano, S., 2010. Ganong's review of medical physiology. New Delhi: mcgraw Hill, 2010.

Bartsch, H. and Nair, J., 2004. Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis. Cancer Detection and Prevention, 28(6), pp.385-391.

Bartsch, H. and Spiegelhalder, B., 1996. Environmental exposure to N-nitroso compounds (NNOC) and precursors: an overview. European journal of cancer prevention, 5, pp.11-17.

Başaran, N., Duydu, Y., Üstündağ, A., Taner, G., Aydin, S., Anlar, H.G., Yalçin, C.Ö., Bacanli, M., Aydos, K., Atabekoğlu, C.S. and Golka, K., 2019. Evaluation of the DNA damage in lymphocytes, sperm and buccal cells of workers under environmental and occupational boron exposure conditions. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 843, pp.33-39.

Baumgartner, A., Cemeli, E. and Anderson, D., 2009. The comet assay in male reproductive toxicology. Cell Biology and Toxicology, 25(1), pp.81-98.

Baumgart, P.M., Kliem, H.C., Gottfried-Anacker, J., Wiessler, M. and Schmeiser, H.H., 1993. Site-specific mutagenesis induced by single O^6-alkylguanines (O^6-n-propyl, O^6-n-butyl, O^6-n-octyl) *in vivo*. Nucleic acids research, 21(16), pp.3755-3760.

Bennetts, L.E., De Iuliis, G.N., Nixon, B., Kime, M., Zelski, K., McVicar, C.M., Lewis, S.E. and Aitken, R.J., 2008. Impact of estrogenic compounds on DNA integrity in human spermatozoa: evidence for cross-linking and redox cycling activities. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 641(1-2), pp.1-11.

Beranek, D.T., 1990. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 231(1), pp.11-30.

Bergamo, P., Volpe, M.G., Lorenzetti, S., Mantovani, A., Notari, T., Cocca, E., Cerullo, S., Di Stasio, M., Cerino, P. and Montano, L., 2016. Human semen as an early, sensitive biomarker of highly polluted living environment in healthy men: A pilot biomonitoring study on trace elements in blood and semen and their relationship with sperm quality and RedOx status. Reproductive Toxicology, 66, pp.1-9.

Beydola, T., Sharma, R.K., Lee, W. and Agarwal, A., 2013. Sperm preparation and selection techniques. Male Infertility Practice. New Delhi: Jaypee Brothers Medical Publishers, pp.244-51.

Blackmore, P.F., Beebe, S.J., Danforth, D.R. and Alexander, N., 1990. Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. Journal of Biological Chemistry, 265(3), pp.1376-1380.

Blotta, I., Prestinaci, F., Mirante, S. and Cantafora, A., 2005. Quantitative assay of total dsDNA with PicoGreen reagent and real-time fluorescent detection. Annali-Istituto Superiore Di Sanita, 41(1), p.119.

Boué, F., Bérubé, B., De Lamirande, E., Gagnon, C. and Sullivan, R., 1994. Human sperm-zona pellucida interaction is inhibited by an antiserum against a hamster sperm protein. Biology of Reproduction, 51(4), pp.577-587.

Bowden, R.D., Buckwalter, M.R., McBride, J.F., Johnson, D.A., Murray, B.K. and O'Neill, K.L., 2003. Tail profile: a more accurate system for analyzing DNA damage using the Comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 537(1), pp.1-9.

Bravo, Z. and Valdivia, M., 2018. Follicular fluid stimulates capacitation and acrosome reaction in alpaca sperm (Vicugna pacos). Reproduction in domestic animals, 53(3), pp.629-635.

Bujan, L; A. Mansat; F. Pontonnier; R. Mieusset. 1996 Time series analysis of sperm concentration in fertile men in Toulouse, France between 1977 and 1992. BMJ, 312, 471-472.

Bukowska, D., Kempisty, B., Piotrowska, H., Sosinska, P., Wozna, M., Ciesiolka, S., Antosik, P., Jaskowski, J.M., Brüssow, K.P. and Nowicki, M., 2013. The structure and role of mammalian sperm RNA: a review. Veterinarni Medicina, 58(2), pp.57-64.

Bungum, M., 2012. Role of sperm DNA Integrity in Fertility. Embryology: Updates and Highlights on Classic Topics, p.23.

Bungum, M., Bungum, L. and Giwercman, A., 2011. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. Asian Journal of Andrology, 13(1), p.69.

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J. And Giwercman, A., 2007. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Human Reproduction, 22(1), pp.174-179.

Bussche, J.V., Moore, S.A., Pasmans, F., Kuhnle, G.G.C. and Vanhaecke, L., 2012. An approach based on ultra-high-pressure liquid chromatography–tandem mass spectrometry to quantify O^6-methyl and O^6-carboxymethylguanine DNA adducts in intestinal cell lines. Journal of Chromatography A, 1257, pp.25-33.

Carlsen, E., Giwercman, A., Keiding, N., Skakkebaek, N.E., 1992. Evidence for decreasing quality of semen during past 50 years. BMJ 305, 609–613. doi:10.1136/bmj.305.6854.609.

Carlsen, E., Swan, S.H., Petersen, J.H. and Skakkebæk, N.E., 2005. Longitudinal changes in semen parameters in young Danish men from the Copenhagen area. Human Reproduction, 20(4), pp.942-949.

Carrell, D.T. The genetics of male infertility.2007. Humana Press Inc999 Riverview Drive, Suite 208 Totowa, New Jersey 07512. pp.77-99.

Carrell, D.T., Emery, B.R. and Hammoud, S., 2007. Altered protamine expression and diminished spermatogenesis: what is the link? Human Reproduction Update, 13(3), pp.313-327.

Carrera, A., Gerton, G.L. and Moss, S.B., 1994. The major fibrous sheath polypeptide of mouse sperm: structural and functional similarities to the A-kinase anchoring proteins. Developmental Biology, 165(1), pp.272-284.

Cederroth, C.R., Zimmermann, C., Beny, J.L., Schaad, O., Combepine, C., Descombes, P., Doerge, D.R., Pralong, F.P., Vassalli, J.D. and Nef, S., 2010. Potential detrimental effects of a phytoestrogen-rich diet on male fertility in mice. Molecular and Cellular Endocrinology, 321(2), pp.152-160.

Centola, G.M., Blanchard, A., Demick, J., Li, S. and Eisenberg, M.L., 2016. Decline in sperm count and motility in young adult men from 2003 to 2013: observations from a US sperm bank. Andrology, 4(2), pp.270-276.

Chavarro, J.E., Toth, T.L., Wright, D.L., Meeker, J.D. and Hauser, R., 2010. Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic. Fertility and Sterility, 93(7), pp.2222-2231.

Cherry, N., Moore, H., McNamee, R., Pacey, A., Burgess, G., Clyma, J.A., Dippnall, M., Baillie, H. and Povey, A., 2008. Occupation and male infertility: glycol ethers and other exposures. Occupational and Environmental Medicine, 65(10), pp.708-714.

Cherry, N., Povey, A.C., McNamee, R., Moore, H., Baillie, H., Clyma, J.A., Dippnall, M. and Pacey, A.A., 2014. Occupation exposures and sperm morphology: a case-referent analysis of a multi-centre study. Occup Environ Med, 71(9), pp.598-604.

Chi, H.J., Chung, D.Y., Choi, S.Y., Kim, J.H., Kim, G.Y., Lee, J.S., Lee, H.S., Kim, M.H. and Roh, S.I., 2011. Integrity of human sperm DNA assessed by the neutral comet assay and its relationship to semen parameters and clinical outcomes for the IVF-ET program. Clinical and Experimental Reproductive Medicine, 38(1), pp.10-17.

Chia, S.E., Lim, S.T.A., Ho, L.M. and Tay, S.K., 2001. Monthly variation in human semen quality in male partners of infertile women in the tropics. Human Reproduction, 16(2), pp.277-281.

Chiu, Y.H., Afeiche, M.C., Gaskins, A.J., Williams, P.L., Petrozza, J.C., Tanrikut, C., Hauser, R. and Chavarro, J.E., 2015. Fruit and vegetable intake and their pesticide residues in relation to semen quality among men from a fertility clinic. Human Reproduction, 30(6), pp.1342-1351.

Claassens, O.E., Menkveld, R., Franken, D.R., Pretorius, E., Swart, Y., Lombard, C.J. and Kruger, T.F., 1992. The acridine orange test: determining the relationship between sperm morphology and fertilization *in vitro*. Human Reproduction, 7(2), pp.242-247.

Cohen-Dayag, A., Tur-Kaspa, I., Dor, J., Mashiach, S. and Eisenbach, M., 1995. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proceedings of the National Academy of Sciences, 92(24), pp.11039-11043.

Collins, A.R., 2004. The comet assay for DNA damage and repair. Molecular Biotechnology, 26(3), p.249.

Collins, A.R., Dobson, V.L., Dušinská, M., Kennedy, G. and Štětina, R., 1997. The comet assay: what can it really tell us?. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 375(2), pp.183-193.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C. and Štětina, R., 2008. The comet assay: topical issues. Mutagenesis, 23(3), pp.143-151.

Collodel, G., Capitani, S., Pammolli, A., Giannerini, V., Geminiani, M. and Moretti, E.,2010. Semen quality of male idiopathic infertile smokers and nonsmokers: an ultrastructural study. Journal of Andrology, 31(2), pp.108-113.

Cortés-Gutiérrez, E.I., Dávila-Rodriguez, M.I., Fernández, J.L., Gosálvez, J., Johnston, S.D. and López-Fernández, C., 2009. Mapping alkali-labile sites in mammalian spermatozoa.

Corzett, M., Mazrimas, J. and Balhorn, R., 2002. Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Molecular reproduction and development, 61(4), pp.519-527.

Cupid, B.C., Zeng, Z., Singh, R. and Shuker, D.E., 2004. Detection of O^6-Carboxymethyl-2 ‘-deoxyguanosine in DNA Following Reaction of Nitric Oxide with Glycine and in Human Blood DNA Using a Quantitative Immunoslot Blot Assay. Chemical Research in Toxicology, 17(3), pp.294-300.

Da Pieve, C., Sahgal, N., Moore, S.A. and Velasco‐Garcia, M.N., 2013. Development of a liquid chromatography/tandem mass spectrometry method to investigate the presence of biomarkers of DNA damage in urine related to red meat consumption and risk of colorectal cancer. Rapid Communications in Mass Spectrometry, 27(21), pp.2493-2503.

De Bont, R. and Van Larebeke, N., 2004. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis, 19(3), pp.169-185.

De Fleurian, G., Perrin, J., Ecochard, R., Dantony, E., Lanteaume, A., Achard, V., Grillo, J.M., Guichaoua, M.R., Botta, A. and Sari‐Minodier, I., 2009. Occupational exposures obtained by questionnaire in clinical practice and their association with semen quality. Journal of Andrology, 30(5), pp.566-579.

De Jonge, C.J. and Barratt, C. eds., 2006. The sperm cell: production, maturation, fertilization, regeneration. Cambridge University Press.p.49-50.

De Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H., 1999. Molecular mechanism of nucleotide excision repair. *Genes & Development*, *13*(7), pp.768-785.

DEFRA, 2011. What are the causes of air Pollution. Available at: https://ukair.defra.gov.uk/assets/documents/What_are_the_causes_of_Air

DEFRA, 2019. What are the causes of air Pollution. Available at: https://ukair.defra.gov.uk/assets/documents/What_are_the_causes_of_Air

Denissenko, M.F., Pao, A., Tang, M.S. and Pfeifer, G.P., 1996. Preferential formation of benzo (a) pyrene adducts at lung cancer mutational hotspots in p53. Science, 274(5286), p.430.

Diemer, T., Ludwig, M., Huwe, P., Hales, D.B. and Weidner, W., 2000. Influence of urogenital infection on sperm function. Current Opinion in Urology, 10(1), pp.39-44.

Doerge, D.R., Churchwell, M.I., Fang, J.L. and Beland, F.A., 2000. Quantification of etheno-DNA adducts using liquid chromatography, on-line sample processing, and electrospray tandem mass spectrometry. Chemical research in Toxicology, 13(12), pp.1259-1264.

Donnelly, E.T., Lewis, S.E., McNally, J.A. and Thompson, W., 1998. *In vitro* fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. Fertility and Sterility, 70(2), pp.305-314.

Drabløs, F., Feyzi, E., Aas, P.A., Vaagbø, C.B., Kavli, B., Bratlie, M.S., Pena-Diaz, J., Otterlei, M., Slupphaug, G. And Krokan, H.E., 2004. Alkylation damage in DNA and RNA—repair mechanisms and medical significance. DNA repair, 3(11), pp.1389-1407.

Driessens, N., Versteyhe, S., Ghaddhab, C., Burniat, A., De Deken, X., Van Sande, J., Dumont, J.E., Miot, F. and Corvilain, B., 2009. Hydrogen peroxide induces DNA single-and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. Endocrine-related cancer, 16(3), pp.845-856.

Drinkaware. (2016). Unit Calculator. Retrieved January 1, 2016, from Drinkaware: https://www.drinkaware.co.uk/understand-yourdrinking/unit-calculator.

Duty, S.M., Silva, M.J., Barr, D.B., Brock, J.W., Ryan, L., Chen, Z., Herrick, R.F., Christiani, D.C. and Hauser, R., 2003. Phthalate exposure and human semen parameters. Epidemiology, 14(3), pp.269-277.

Duty, S.M., Singh, N.P., Ryan, L., Chen, Z., Lewis, C., Huang, T. and Hauser, R., 2002. Reliability of the comet assay in cryopreserved human sperm. Human Reproduction, 17(5), pp.1274-1280.

Duty, S.M., Singh, N.P., Silva, M.J., Barr, D.B., Brock, J.W., Ryan, L., Herrick, R.F., Christiani, D.C. and Hauser, R., 2003(a). The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. Environmental health perspectives, 111(9), pp.1164-1169.

Duydu, Y., Başaran, N., Üstündağ, A., Aydın, S., Ündeğer, Ü., Ataman, O.Y., Aydos, K., Düker, Y., Ickstadt, K., Waltrup, B.S. and Golka, K., 2012. Assessment of DNA integrity (COMET assay) in sperm cells of boron-exposed workers. Archives of toxicology, 86(1), pp.27-35.

Eastman, A. and Barry, M.A., 1992. The Origins of DNA Breaks: A Consequence of DNA Damage, DNA Repair, or Apoptosis? New Drugs. Cancer investigation, 10(3), pp.229-240.

Eddy, E.M., Toshimori, K. And O'Brien, D.A., 2003. Fibrous sheath of mammalian spermatozoa. Microscopy Research and Technique, 61(1), pp.103-115.

Ellison, K.S., Dogliotti, E., Connors, T.D., Basu, A.K. and Essigmann, J.M., 1989. Site-specific mutagenesis by O6-alkylguanines located in the chromosomes of mammalian cells: influence of the mammalian O6-alkylguanine-DNA alkyltransferase. *Proceedings of the National Academy of Sciences*, *86*(22), pp.8620-8624.

Eoff, R.L., Irimia, A., Egli, M. and Guengerich, F.P., 2007. Sulfolobus solfataricus DNA polymerase Dpo4 is partially inhibited by “wobble” pairing between O^6-methylguanine and cytosine, but accurate bypass is preferred. Journal of Biological Chemistry, 282(2), pp.1456-1467.

Erenpreisa, J., Erenpreiss, J., Freivalds, T., Slaidina, M., Krampe, R., Butikova, J., Ivanov, A. and Pjanova, D., 2003. Toluidine blue test for sperm DNA integrity and elaboration of image cytometry algorithm. Cytometry Part A: The Journal of the International Society for Analytical Cytology, 52(1), pp.19-27.

Erenpreiss, J.U.R.I.S., Hlevicka, S.O.L.V.E.I.G.A., Zalkalns, J.A.N.I.S. and Erenpreisa, J.E.K.A.T.E.R.I.N.A., 2002. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. Journal of Andrology, 23(5), pp.717-723.

Evenson, D.P. and Wixon, R., 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology, 65(5), pp.979-991.

Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R., 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. Science, 210(4474), pp.1131-1133.

Evenson, D.P., Larson, K.L. and Jost, L.K., 2002. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. Journal of Andrology, 23(1), pp.25-43.

Farmer, P.B. and Singh, R., 2008. Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: the increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens. Mutation Research/Reviews in Mutation Research, 659(1-2), pp.68-76.

Ferlin, A., Raicu, F., Gatta, V., Zuccarello, D., Palka, G. and Foresta, C., 2007. Male infertility: role of genetic background. Reproductive Biomedicine Online, 14(6), pp.734-745.

Fernández, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., LaFromboise, M. and De Jonge, C., 2005. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertility and sterility, 84(4), pp.833-842.

Fernández, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R. and Alvarez, J.G., 2003. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. Journal of andrology, 24(1), pp.59-66.

Fischer, M.A., Willis, J. and Zini, A., 2003. Human sperm DNA integrity: correlation with sperm cytoplasmic droplets. Urology, 61(1), pp.207-211.

Forti, G. and Serio, M., 1993. Male infertility: is its rising incidence due to better methods of detection or an increasing frequency? Hum. Reprod. 8, 1153e1154.

Fried, M.G., Kanugula, S., Bromberg, J.L. and Pegg, A.E., 1996. DNA binding mechanism of O 6-alkylguanine-DNA alkyltransferase: stoichiometry and effects of DNA base composition and secondary structure on complex stability. Biochemistry, 35(48), pp.15295-15301.

Friedberg, E.C., 2003. DNA damage and repair. Nature, 421(6921), p.436.

Gabrielsen, J.S. and Tanrikut, C., 2016. Chronic exposures and male fertility: the impacts of environment, diet, and drug use on spermatogenesis. Andrology, 4(4), pp.648-661.

Gaffney, B.L., Marky, L.A. and Jones, R.A., 1984. Synthesis and characterization of a set of four dodecadeoxyribonucleoside undecaphosphates containing O^6-methylguanine opposite adenine, cytosine, guanine, and thymine. *Biochemistry*, *23*(24), pp.5686-5691.

Garcia, J.M., Chen, J.A., Guillory, B., Donehower, L.A., Smith, R.G. and Lamb, D.J., 2015. Ghrelin prevents cisplatin-induced testicular damage by facilitating repair of DNA double strand breaks through activation of p53 in mice. Biology of reproduction, 93(1), pp.24-1.

Garrett, C., Liu, D.Y., Clarke, G.N., Rushford, D.D. and Baker, H.W.G., 2003. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight‐line velocity are related to pregnancy rate in subfertile couples. Human Reproduction, 18(8), pp.1643-1649.

Gaskins, A.J., Colaci, D.S., Mendiola, J., Swan, S.H. and Chavarro, J.E., 2012. Dietary patterns and semen quality in young men. Human reproduction, 27(10), pp.2899-2907.

Gaskins, A.J., Mendiola, J., Afeiche, M., Jørgensen, N., Swan, S.H. and Chavarro, J.E., 2015. Physical activity and television watching in relation to semen quality in young men. Br J Sports Med, 49(4), pp.265-270.

Gaspari, L., Chang, S.S., Santella, R.M., Garte, S., Pedotti, P. and Taioli, E., 2003. Polycyclic aromatic hydrocarbon-DNA adducts in human sperm as a marker of DNA damage and infertility. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 535(2), pp.155-160.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S.A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. The Journal of cell biology, 119(3), pp.493-501.

Ghatak, S., Muthukumaran, R.B. and Nachimuthu, S.K., 2013. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. Journal of biomolecular techniques: JBT, 24(4), p.224.

Giojalas, L.C., 1998. Correlation between response to progesterone and other functional parameters in human spermatozoa. Fertility and sterility, 69(1), pp.107-111.

Gomathi, C., Balasubramanian, K., Bhanu, N.V., Srikanth, V. And Govindarajulu, P., 1993. Effect of chronic alcoholism on semen—studies on lipid profiles. International Journal of Andrology, 16(3), pp.175-181.

Gomez, E., Buckingham, D.W., Brindle, J., Lanzafame, F., Irvine, D.S. and Aitken, R.J., 1996. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. Journal of andrology, 17(3), pp.276-287.

Gore, S.M. and Altman, D.G., 2001. How large a sample. Statistics in practice. London: BMJ Publishing, 6(8).

Greco, E., Romano, S., Iacobelli, M., Ferrero, S., Baroni, E., Minasi, M.G., Ubaldi, F., Rienzi, L. and Tesarik, J., 2005. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. Human Reproduction, 20(9), pp.2590-2594.

Guthauser, B., Boitrelle, F., Plat, A., Thiercelin, N. And Vialard, F., 2014. Chronic Excessive Alcohol Consumption and Male Fertility: A Case Report on Reversible Azoospermia and a Literature Review. Alcohol and Alcoholism, 49(1), pp.42-44.

Guttikunda, S., 2008. What is Particulate matter ?. Composition & Science, SIM-air Working Paper Series, pp.10-2008.

Guyton, A. and Hall, J., 2006. Textbook of medical physiology, 11th.

Guzick, D.S., Overstreet, J.W., Factor-Litvak, P., Brazil, C.K., Nakajima, S.T., Coutifaris, C., Carson, S.A., Cisneros, P., Steinkampf, M.P., Hill, J.A. and Xu, D., 2001. Sperm morphology, motility, and concentration in fertile and infertile men. New England Journal of Medicine, 345(19), pp.1388-1393.

Gyori, B.M., Venkatachalam, G., Thiagarajan, P.S., Hsu, D. and Clement, M.V., 2014. OpenComet: an automated tool for comet assay image analysis. Redox biology, 2, pp.457-465.

Hanmayyagari, B. and Guntaka, M., 2015. A rare case of male infertility: Sertoli only syndrome. CHRISMED Journal of Health and Research, 2(1), p.64.

Hargreave, T.B., 2000. Genetic basis of male fertility. British Medical Bulletin, 56(3), pp.650-671.

Harris, C.C., Vahakangas, K., Newman, M.J., Trivers, G.E., Shamsuddin, A., Sinopoli, N., Mann, D.L. and Wright, W.E., 1985. Detection of benzo [a] pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. Proceedings of the National Academy of Sciences, 82(19), pp.6672-6676.

Harrison, K.L., Wood, M., Lees, N.P., Hall, C.N., Margison, G.P. and Povey, A.C., 2001. Development and application of a sensitive and rapid immunoassay for the quantitation of N7-methyldeoxyguanosine in DNA samples. Chemical research in toxicology, 14(3), pp.295-301.

Hefferin, M.L., and Tomkinson, A.E., 2005. Mechanism of DNA double-strand break repair by non-homologous end joining. DNA repair, 4(6), pp.639-648.

Homa, S.T., Vessey, W., Perez-Miranda, A., Riyait, T. and Agarwal, A., 2015. Reactive Oxygen Species (ROS) in human semen: determination of a reference range. Journal of assisted reproduction and genetics, 32(5), pp.757-764.

Hosseini, A., Zare, S., Borzouei, Z. And Pakdel, F.G., 2018. Cyclophosphamide-induced testicular toxicity ameliorates by American ginseng treatment: An experimental study. International Journal of Reproductive Biomedicine, 16(11), p.711.

Hovatta, O., Venäläinen, E.R., Kuusimäki, L., Heikkilä, J., Hirvi, T. and Reima, I., 1998. Aluminium, lead and cadmium concentrations in seminal plasma and spermatozoa, and semen quality in Finnish men. Human reproduction (Oxford, England), 13(1), pp.115-119.

Howell, S.J. and Shalet, S.M., 2005. Spermatogenesis after cancer treatment: damage and recovery. *JNCi Monographs*, *2005*(34), pp.12-17.

Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J. and Thompson, W., 1996. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. MHR: Basic science of reproductive medicine, 2(8), pp.613-619.

Ibadin, O.K. and Ibeh, I.N., 2008. Bacteriospermia and sperm quality in infertile male patient at University of Benin Teaching Hospital, Benin City, Nigeria. Mala Journal Microbiol, 4(2), pp.65-67.

Ibañez-Perez, J., Santos-Zorrozua, B., Lopez-Lopez, E., Irazusta, J., Prieto, B., Aparicio, V., Corcostegui, B., Gracia-Orad, Á. and Matorras, R., 2019. Impact of physical activity on semen quality among men from infertile couples. European Journal of Obstetrics & Gynecology and Reproductive Biology, 237, pp.170-174.

Irvine, D.S., 1998. Epidemiology and aetiology of male infertility. Human Reproduction, 13(suppl 1), pp.33-44.

Irvine, D.S., Macleod, I.C., Templeton, A.A., Masterton, A. and Taylor, A., 1994. Andrology: A prospective clinical study of the relationship between the computer-assisted assessment of human semen quality and the achievement of pregnancy *in vivo*. Human Reproduction, 9(12), pp.2324-2334.

Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A. and Aitken, R.J., 2000. DNA integrity in human spermatozoa: relationships with semen quality. Journal of andrology, 21(1), pp.33-44.

Jackson, P.E., Hall, C.N., Badawi, A.F., O'Connor, P.J., Cooper, D.P. and Povey, A.C., 1996. Frequency of Ki‐ras mutations and DNA alkylation in colorectal tissue from individuals living in Manchester. Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center, 16(1), pp.12-19.

Jayachandra, N. and Srinivasa, J., 2011. Impact of occupational heat exposure on male reproductive health. Pakistan Journal of Physiology, 7(2), pp.53-55.

Jeng, H.A. and Yu, L., 2008. Alteration of sperm quality and hormone levels by polycyclic aromatic hydrocarbons on airborne particulate particles. Journal of Environmental Science and Health, Part A, 43(7), pp.675-681.

Jensen, T.K., Bonde, J.P. and Joffe, M., 2006. The influence of occupational exposure on male reproductive function. Occupational Medicine, 56(8), pp.544-553.

Jensen, T.K., Carlsen, E., Jørgensen, N., Berthelsen, J.G., Keiding, N., Christensen, K., Petersen, J.H., Knudsen, L.B. and Skakkebaek, N.E., 2002. Poor semen quality may contribute to recent decline in fertility rates. Human Reproduction, 17(6), pp.1437-1440.

Jensen, T.K., Swan, S.H., Skakkebæk, N.E., Rasmussen, S. and Jørgensen, N., 2010. Caffeine intake and semen quality in a population of 2,554 young Danish men. American journal of epidemiology, 171(8), pp.883-891.

Jeulin, C., Feneux, D., Serres, C., Jouannet, P., Guillet-Rosso, F., Belaisch-Allart, J., Frydman, R. and Testart, J., 1986. Sperm factors related to failure of human in-vitro fertilization. Reproduction, 76(2), pp.735-744.

Jiang, Z.L., Li, Q.W., Li, W.Y., Hu, J.H., Zhao, H.W. and Zhang, S.S., 2006. Effect of low-density lipoprotein on DNA integrity of freezing–thawing boar sperm by neutral comet assay. Animal reproduction science, 99(3-4), pp.401-407.

Joffe, M., 2003. Infertility and environmental pollutants. British medical bulletin, 68(1), pp.47-70.

Johnson, S.L., Dunleavy, J., Gemmell, N.J. and Nakagawa, S., 2015. Consistent age dependent declines in human semen quality: a systematic review and meta-analysis. Ageing research reviews, 19, pp.22-33.

Jones, S., Carley, S. and Harrison, M., 2003. An introduction to power and sample size estimation. Emergency medicine journal: EMJ, 20(5), p.453.

Jones, R.E. and Lopez, K.H., 2013. Human reproductive biology. Academic Press.

Jungwirth, A., Diemer, T. and Dohle, G.R., Guidelines on male infertility. 2014., European Association of Urology, p.11-12.

Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., Krausz, C. And EAU Working Group on Male Infertility, 2012. European Association of Urology guidelines on Male Infertility: the 2012 update. European Urology, 62(2), pp.324-332.

Jurewicz, J., Hanke, W., Radwan, M. and Bonde, J., 2009. Environmental factors and semen quality. International journal of occupational medicine and environmental health, 22(4), pp.305-329.

Jurewicz, J., Radwan, M., Sobala, W., Radwan, P., Bochenek, M. and Hanke, W., 2018. Dietary patterns and their relationship with semen quality. American journal of men's health, 12(3), pp.575-583.

Kacsoh, B., 2000. Endocrine physiology. McGraw Hill Professional.

Kado, N.Y., Okamoto, R.A., Kuzmicky, P.A., Kobayashi, R., Ayala, A., Gebel, M.E., Rieger, P.L., Maddox, C. and Zafonte, L., 2005. Emissions of toxic pollutants from compressed natural gas and low sulfur diesel-fueled heavy-duty transit buses tested

over multiple driving cycles. Environmental science & technology, 39(19), pp.7638-7649.

Kaina, B., 2003. DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation, and signaling. Biochemical pharmacology, 66(8), pp.1547-1554.

Kaina, B., Christmann, M., Naumann, S. and Roos, W.P., 2007. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. DNA repair, 6(8), pp.1079-1099.

Kandeel, F.R. and Swerdloff, R.S., 1988. Role of temperature in regulation of spermatogenesis and the use of heating as a method for contraception. International Journal of Gynecology & Obstetrics, 27(3), pp.477-478.

Kantartzi, P.D., Goulis, C.D., Goulis, G.D. and Papadimas, I., 2007. Male infertility and varicocele: myths and reality. Hippokratia, 11(3), p.99.

Keel, B.A., 2004. How reliable are results from the semen analysis? Fertility and sterility, 82(1), pp.41-44.

Keel, B.A., 2006. Within-and between-subject variation in semen parameters in infertile men and normal semen donors. Fertility and sterility, 85(1), pp.128-134.

Keel, B.A., Stembridge, T.W., Pineda, G. and Serafy Sr, N.T., 2002. Lack of standardization in performance of the semen analysis among laboratories in the United States. Fertility and sterility, 78(3), pp.603-608.

Kidd, S.A., Eskenazi, B. and Wyrobek, A.J., 2001. Effects of male age on semen quality and fertility: a review of the literature. Fertility and sterility, 75(2), pp.237-248.

Klaene, J.J., Sharma, V.K., Glick, J. and Vouros, P., 2013. The analysis of DNA adducts: The transition from ^{32}P-postlabeling to mass spectrometry. Cancer letters, 334(1), pp.10-19.

Koeppen, B.M. and Stanton, B.A., 2009. Berne & Levy Physiology. Elsevier Health Sciences.

Köhn, F.M., Erdmann, I., Oeda, T., El Mulla, K.F., Schiefer, H.G. and Schill, W.B., 1998. Influence of urogenital infections on sperm functions. Andrologia, 30(S1), pp.73-80.

Kooy, A., de Greef, W.J., Vreeburg, J.T., Hackeng, W.H., Ooms, M.P., Lamberts, S.W. and Weber, R.F., 1990. Evidence for the involvement of corticotropin-releasing factor in the inhibition of gonadotropin release induced by hyperprolactinemia. Neuroendocrinology, 51(3), pp.261-266.

Kopp-Kuhlman, C., 2011. Impaired spermatogenesis in Finnish boars and bulls.

Kotandeniya, D., Murphy, D., Seneviratne, U., Guza, R., Pegg, A., Kanugula, S. and Tretyakova, N., 2011. Mass spectrometry-based approach to study the kinetics of O^6-alkylguanine DNA alkyltransferase-mediated repair of O^6-pyridyloxobutyl-2′-deoxyguanosine adducts in DNA. Chemical research in toxicology, 24(11), pp.1966-1975.

Kothari, S., Thompson, A., Agarwal, A. and du Plessis, S.S., 2010. Free radicals: their beneficial and detrimental effects on sperm function.

Krause, W., 1995. Computer-assisted semen analysis systems: comparison with routine evaluation and prognostic value in male fertility and assisted reproduction. Human Reproduction, 10(suppl_1), pp.60-66.

Kriek, E., Rojas, M., Alexandrov, K. and Bartsch, H., 1998. Polycyclic aromatic hydrocarbon-DNA adducts in humans: relevance as biomarkers for exposure and cancer risk. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 400(1), pp.215-231.

Kucheria, K., Saxena, R. and Mohan, D., 1985. Semen analysis in alcohol dependence syndrome. Andrologia, 17(6), pp.558-563.

Kumar, S., Thaker, R. and Verma, V., 2018 a. Occupational. Environmental exposure, and Lifestyle factors: Declining Male Reproductive Health J Gynecol Infertility, 1(1).

Kumaravel, T.S., Vilhar, B., Faux, S.P. and Jha, A.N., 2009. Comet assay measurements: a perspective. Cell biology and toxicology, 25(1), pp.53-64.

Künzle, R., Mueller, M.D., Hänggi, W., Birkhäuser, M.H., Drescher, H. and Bersinger, N.A., 2003. Semen quality of male smokers and nonsmokers in infertile couples. Fertility and Sterility, 79(2), pp.287-291.

Kupka, M.S., Ferraretti, A.P., De Mouzon, J., Erb, K., D'Hooghe, T., Castilla, J.A., Calhaz-Jorge, C., De Geyter, C., Goossens, V., Strohmer, H. and Kreuz-Kinderwunschzentrum, S.P.G., 2014. Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHRE. Human reproduction, 29(10), pp.2099-2113.

La Vignera, S., Condorelli, R.A., Balercia, G., Vicari, E. And Calogero, A.E., 2013. Does alcohol have any effect on male reproductive function? A review of literature. Asian Journal Androl, 15(2), pp.221-5.

Larsen, L., Scheike, T., Jensen, T.K., Bonde, J.P., Ernst, E., Hjollund, N.H., Zhou, Y., Skakkebæk, N.E., Giwercman, A. and Danish First Pregnancy Planner Study Team, 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. Human Reproduction, 15(7), pp.1562-1567.

Latini, G., Del Vecchio, A., Massaro, M., Verrotti, A. and De Felice, C., 2006. Phthalate exposure and male infertility. Toxicology, 226(2), pp.90-98.

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R. and Swan, S.H., 2017. Temporal trends in sperm count: a systematic review and meta-regression analysis. Human reproduction update, 23(6), pp.646-659.

Lewin, M.H., Bailey, N., Bandaletova, T., Bowman, R., Cross, A.J., Pollock, J., Shuker, D.E. and Bingham, S.A., 2006. Red meat enhances the colonic formation of the DNA adduct O^6-carboxymethyl guanine: implications for colorectal cancer risk. Cancer research, 66(3), pp.1859-1865.

Lewis, S.E. and Simon, L., 2010. Clinical implications of sperm DNA damage. Human Fertility, 13(4), pp.201-207.

Lewis, S.E.M., O'Connell, M., Stevenson, M., Thompson‐Cree, L. and McClure, N., 2004. An algorithm to predict pregnancy in assisted reproduction. Human Reproduction, 19(6), pp.1385-1394.

Lin, M.H., Lee, R.K.K., Li, S.H., Lu, C.H., Sun, F.J. and Hwu, Y.M., 2008. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates *in vitro* fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. Fertility and Sterility, 90(2), pp.352-359.

Liu, D.Y., Clarke, G.N. and Baker, H.G., 1991. Relationship between sperm motility assessed with the Hamilton‐Thorn motility analyzer and fertilization rates *in vitro*. Journal of andrology, 12(4), pp.231-239.

Loeb, L.A. and Harris, C.C., 2008. Advances in chemical carcinogenesis: a historical review and prospective. Cancer Research, 68(17), pp.6863-6872.

Lovell, D.P. and Omori, T., 2008. Statistical issues in the use of the comet assay. Mutagenesis, 23(3), pp.171-182.

Lovell, D.P., 2016. Statistical analysis of comet assay data. In The comet assay in toxicology (pp. 551-580).

Lu, J.C., Huang, Y.F. and Lü, N.Q., 2010. [WHO Laboratory Manual for the Examination and Processing of Human Semen: its applicability to andrology laboratories in China]. Zhonghua nan ke xue= National Journal of Andrology, 16(10), pp.867-871.

Lu, J.C., Jing, J., Chen, L., Ge, Y.F., Feng, R.X., Liang, Y.J. and Yao, B., 2018. Analysis of human sperm DNA fragmentation index (DFI) related factors: a report of 1010 subfertile men in China. Reproductive Biology and Endocrinology, 16(1), p.23.

Lue, Y.H., Sinha Hikim, A.P., Swerdloff, R.S., Im, P., Taing, K.S., Bui, T., Leung, A. and Wang, C., 1999. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity 1. Endocrinology, 140(4), pp.1709-1717.

Luebbehusen, H., 2006. The significance of the 260/230 ratio in determining nucleic acid purity. Genomic and RNA Profiling Core [online]; Baylor College of Medicine: Houston, TX, USA.

Ma, J., Wu, L., Zhou, Y., Zhang, H., Xiong, C., Peng, Z., Bao, W., Meng, T. and Liu, Y., 2018. Association between BMI and semen quality: an observational study of 3966 sperm donors. Human Reproduction, 34(1), pp.155-162.

Maldonado-Cárceles, A.B., Mínguez-Alarcón, L., Mendiola, J., Vioque, J., Jørgensen, N., Árense-Gonzalo, J.J., Torres-Cantero, A.M. and Chavarro, J.E., 2019. Meat intake in relation to semen quality and reproductive hormone levels among young men in Spain. British Journal of Nutrition, 121(4), pp.451-460.

Margison, G.P., Butt, A., Pearson, S.J., Wharton, S., Watson, A.J., Marriott, A., Caetano, C.M., Hollins, J.J., Rukazenkova, N., Begum, G. and Santibáñez-Koref, M.F., 2007. Alkyltransferase-like proteins. DNA repair, 6(8), pp.1222-1228.

Margison, G.P., Povey, A.C., Kaina, B. and Santibáñez Koref, M.F., 2003. Variability and regulation of O^6-alkylguanine–DNA alkyltransferase. Carcinogenesis, 24(4), pp.625-635.

Margison, G.P., Santibáñez Koref, M.F. and Povey, A.C., 2002. Mechanisms of carcinogenicity/chemotherapy by O^6-methylguanine. Mutagenesis, 17(6), pp.483-487.

Marques, A., Rego, A., Guilherme, S., Gaivão, I., Santos, M.A. and Pacheco, M., 2016. Evidences of DNA and chromosomal damage induced by the mancozeb-based fungicide Mancozan® in fish (Anguilla anguilla L.). Pesticide biochemistry and physiology, 133, pp.52-58.

Martin, R.H., Lin, C.C., Balkan, W.A.Y.N.E. and Burns, K.A.T.H.Y., 1982. Direct chromosomal analysis of human spermatozoa: preliminary results from 18 normal men. American Journal of Human Genetics, 34(3), p.459.

Mascarenhas, M.N., Flaxman, S.R., Boerma, T., Vanderpoel, S. and Stevens, G.A., 2012. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS medicine, 9(12).

Masutomi, N., Shibutani, M., Takagi, H., Uneyama, C., Takahashi, N. and Hirose, M., 2003. Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. Toxicology, 192(2), pp.149-170.

McLachlan, R.I., O'Donnell, L., Meachem, S.J., Stanton, P.G., De Kretser, D.M., Pratis, K. and Robertson, D.M., 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Progress in Hormone Research, 57(1), pp.149-179.

Meehan, T., Schlatt, S., O'Bryan, M.K., de Kretser, D.M. and Loveland, K.L., 2000. Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. Developmental biology, 220(2), pp.225-237.

Menkveld, R., 2010. Clinical significance of the low normal sperm morphology value as proposed in the fifth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen. Asian Journal Andrology, 12(1), pp.47-58.

Mi, H.H., Lee, W.J., Tsai, P.J. and Chen, C.B., 2001. A comparison on the emission of polycyclic aromatic hydrocarbons and their corresponding carcinogenic potencies from a vehicle engine using leaded and lead-free gasoline. Environmental health perspectives, 109(12), pp.1285-1290.

Milanizadeh, S., Zuwarali, K.N.N., Aliaghaei, A. and Bigdeli, M.R., 2018. Therapeutic potential of pretreatment with allograft Sertoli cells transplantation in brain ischemia by improving oxidative defenses. Journal of Molecular Neuroscience, 64(4), pp.533-542.

Moein, M.R., Dehghani, V.O., Tabibnejad, N. and Vahidi, S., 2007. Reactive Oxygen Species (ROS) level in seminal plasma of infertile men and healthy donors. International Journal of Reproductive BioMedicine, 5(3), pp.51-0.

Mohammad, H.N.E., Mohammad, S., Shahnaz, R., Maryam, A., Shahla, R., Fariba, M. and Mohammad, M., 2005. Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. Reproductive biomedicine online, 11(2), pp.198-205.

Monera, O.D., Kay, C.M. and Hodges, R.S., 1994. Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. Protein Science, 3(11), pp.1984-1991.

Montesano, R., 1981. Alkylation of DNA and tissue specificity in nitrosamine carcinogenesis. Journal of supramolecular structure and cellular biochemistry, 17(3), pp.259-273.

Moore, S.A., Xeniou, O., Zeng, Z.T., Humphreys, E., Burr, S., Gottschalg, E., Bingham, S.A. and Shuker, D.E., 2010. Optimizing immunoslot blot assays and application to low DNA adduct levels using an amplification approach. Analytical biochemistry, 403(1-2), pp.67-73.

Morales, C.R., Oko, R. And Clermont, Y., 1994. Molecular cloning and developmental expression of an mrna encoding the 27 kd outer dense fibre protein of rat spermatozoa. Molecular Reproduction and Development, 37(2), pp.229-240.

Morley, J.E., Distiller, L.A., Sagel, J., Kok, S.H., Kay, G., Carr, P. And Katz, M., 1977. Hormonal changes associated with testicular atrophy and gynaecomastia in patients with leprosy. Clinical Endocrinology, 6(4), pp.299-303.

Morris, I.D., Ilott, S., Dixon, L. and Brison, D.R., 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Human reproduction, 17(4), pp.990-998.

Mortimer, D., Curtis, E.F., Camenzind, A.R. and Tanaka, S., 1989. The spontaneous acrosome reaction of human spermatozoa incubated *in vitro*. Human Reproduction, 4(1), pp.57-62.

Mortimer, S.T., 2005. Essentials of sperm biology. In Office Andrology (pp. 1-9). Humana Press.

Muthusami, K.R. and Chinnaswamy, P., 2005. Effect of chronic alcoholism on male fertility hormones and semen quality. Fertility and sterility, 84(4), pp.919-924.

Naughton, C.K., Nangia, A.K. and Agarwal, A., 2001. Varicocele and male infertility: Part II: Pathophysiology of varicoceles in male infertility. Human Reproduction Update, 7(5), pp.473-481.

Nicholson, H.D., Guldenaar, S.E.F., Boer, G.J. and Pickering, B.T., 1991. Testicular oxytocin: effects of intratesticular oxytocin in the rat. Journal of Endocrinology, 130(2), pp.231-NP.

Nieschlag, E. and Kamischke, A., 2010. Empirical therapies for idiopathic male infertility. In Andrology (pp. 457-467). Springer, Berlin, Heidelberg.

Nilsen, H. and Krokan, H.E., 2001. Base excision repair in a network of defence and tolerance. Carcinogenesis, 22(7), pp.987-998.

Oldereid, N.B., Rui, H. and Purvis, K., 1992. Life styles of men in barren couples and their relationship to sperm quality. International journal of fertility, 37(6), pp.343-349.

Olesen, I.A., Joensen, U.N., Petersen, J.H., Almstrup, K., Rajpert-De Meyts, E., Carlsen, E., McLachlan, R., Juul, A. and Jørgensen, N., 2018. Decrease in semen quality and Leydig cell function in infertile men: a longitudinal study. Human Reproduction, 33(11), pp.1963-1974.

Oliva, A., Spira, A. and Multigner, L., 2001. Contribution of environmental factors to the risk of male infertility. Human Reproduction, 16(8), pp.1768-1776.

Olive, P.L. and Johnston, P.J., 1997. DNA damage from oxidants: influence of lesion complexity and chromatin organization. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics, 9(6-7), pp.287-294.

Oliveira, J.B.A., Petersen, C.G., Mauri, A.L., Vagnini, L.D., Renzi, A., Petersen, B., Mattila, M., Dieamant, F., Baruffi, R.L.R. and Franco Jr, J.G., 2018. Association between body

mass index and sperm quality and sperm DNA integrity. A large population study. Andrologia, 50(3), p. e12889.

Ollero, M., Gil-Guzman, E., Lopez, M.C., Sharma, R.K., Agarwal, A., Larson, K., Evenson, D., Thomas, A.J. and Alvarez, J.G., 2001. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Human Reproduction, 16(9), pp.1912-1921.

Ombelet, W., Bosmans, E., Janssen, M., Cox, A., Vlasselaer, J., Gyselaers, W., Vandeput, H., Gielen, J., Pollet, H., Maes, M. And Steeno, O., 1997. Semen parameters in a fertile versus subfertile population: a need for change in the interpretation of semen testing. Human Reproduction, 12(5), pp.987-993.

Oostingh, E.C., Steegers-Theunissen, R.P., de Vries, J.H., Laven, J.S. and Koster, M.P., 2017. Strong adherence to a healthy dietary pattern is associated with better semen quality, especially in men with poor semen quality. Fertility and sterility, 107(4), pp.916-923.

Ostling, 6. and Johanson, K.J., 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochemical and biophysical research communications, 123(1), pp.291-298.

Osterberg, E.C., Ramasamy, R., Masson, P. and Brannigan, R.E., 2014. Current practices in fertility preservation in male cancer patients. *Urology annals*, *6*(1), p.13.

Pacey, A.A., 2010. Environmental and lifestyle factors associated with sperm DNA damage. Human Fertility, 13(4), pp.189-193.

Pacey, A.A., Povey, A.C., Clyma, J.A., McNamee, R., Moore, H.D., Baillie, H., Cherry, N.M. and Participating Centres of Chaps-UK, 2014. Modifiable and non-modifiable risk factors for poor sperm morphology. Human Reproduction, 29(8), pp.1629-1636.

Park, J.E., Seo, J.E., Lee, J.Y. and Kwon, H., 2015. Distribution of seven N-nitrosamines in food. Toxicological research, 31(3), pp.279-288.

Pasqualotto, F.F., Sharma, R.K., Nelson, D.R., Thomas, A.J. and Agarwal, A., 2000. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertility and Sterility, 73(3), pp.459-464.

Paulsen, CA; N.G. Berman; C. Wang.1996 Data from men in greater Seattle area reveals no downward trend in semen quality: further evidence that deterioration of semen quality is not geographically uniform. Fertil Steril, 65, 1015-1020.

Pearson, S.J., Ferguson, J., Santibanez-Koref, M. and Margison, G.P., 2005. Inhibition of O^6-methylguanine-DNA methyltransferase by an alkyltransferase-like protein from Escherichia coli. Nucleic acids research, 33(12), pp.3837-3844.

Pearson, S.J., Wharton, S., Watson, A.J., Begum, G., Butt, A., Glynn, N., Williams, D.M., Shibata, T., Santibanez-Koref, M.F. and Margison, G.P., 2006. A novel DNA damage recognition protein in Schizosaccharomyces pombe. Nucleic acids research, 34(8), pp.2347-2354.

Pegg, A.E., Dolan, M.E. and Moschel, R.C., 1995. Structure, function, and inhibition of O^6-alkylguanine-DNA alkyltransferase. In Progress in nucleic acid research and molecular biology (Vol. 51, pp. 167-223). Academic Press.

Perry, M.J., 2008. Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. Human reproduction update, 14(3), pp.233-242.

Petersen, A.B., Gniadecki, R., Vicanova, J., Thorn, T. and Wulf, H.C., 2000. Hydrogen peroxide is responsible for UVA-induced DNA damage measured by alkaline comet assay in HaCaT keratinocytes. Journal of Photochemistry and Photobiology B: Biology, 59(1-3), pp.123-131.

Phillips, D.H. and Arlt, V.M., 2007. The 32 P-postlabeling assay for DNA adducts. Nature protocols, 2(11), p.2772.

Povey, A.C., 2000. DNA adducts: endogenous and induced. Toxicologic pathology, 28(3), pp.405-414.

Povey, A.C., Clyma, J.A., McNamee, R., Moore, H.D., Baillie, H., Pacey, A.A., Cherry, N.M. and Participating Centres of Chaps-uk, 2012. Modifiable and non-modifiable risk factors for poor semen quality: a case-referent study. Human reproduction, 27(9), pp.2799-2806.

Povey, A.C., Hall, C.N., Badawi, A.F., Cooper, D.P., Guppy, M.J., Jackson, P.E., O'Connor, P.J. and Margison, G.P., 2001. Host determinants of DNA alkylation and DNA repair activity in human colorectal tissue: O^6-methylguanine levels are associated with GSTT1 genotype and O^6-alkylguanine-DNA alkyltransferase activity with CYP2D6 genotype. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 495(1-2), pp.103-115.

Povey, A.C., Hall, C.N., Cooper, D.P., O'Connor, P.J. and Margison, G.P., 2000. Determinants of O^6 ‐ alkylguanine ‐ DNA alkyltransferase activity in normal and tumour tissue from human colon and rectum. International journal of cancer, 85(1), pp.68-72.

Rajashri, M., Reddy, K.R.C., Kumari, G.A. and Kumari, N.N., Coefficient of variation assessment for seminal traits evaluated by computer assisted semen analysis (CASA). International Journal of Science, Environment, and Technology, Vol. 5, No 5, 2016, 3452 – 3456

Ribas-Maynou, J. and Benet, J., 2019. Single and double strand sperm DNA damage: different reproductive effects on male fertility. Genes, 10(2), p.105.

Ribas-Maynou, J., Fernández ‐ Encinas, A., García ‐ Peiró, A., Prada, E., Abad, C., Amengual, M.J., Navarro, J. and Benet, J., 2014. Human semen cryopreservation: a sperm DNA fragmentation study with alkaline and neutral Comet assay. Andrology, 2(1), pp.83-87.

Ribas-Maynou, J., Garcia-Peiro, A., Abad, C., Amengual, M.J., Navarro, J. and Benet, J., 2012. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. Human reproduction, 27(3), pp.652-658.

Ribas-Maynou, J., García-Peiró, A., Fernandez-Encinas, A., Amengual, M.J., Prada, E., Cortes, P., Navarro, J. and Benet, J., 2012a. Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. PloS one, 7(9), p.e44679.

Ribas-Maynou, J., García‐Peiró, A., Fernández‐Encinas, A., Abad, C., Amengual, M.J., Prada, E., Navarro, J. and Benet, J., 2013. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology, 1(5), pp.715-722.

Rolland, M., Le Moal, J., Wagner, V., Royere, D., De Mouzon, J., 2013. Decline in semen concentration and morphology in a sample of 26 609 men close to general population between 1989 and 2005 in France. Human Reproduction 28, 462–470.

Romero-Otero, J., Medina-Polo, J., García-Gómez, B., Lora-Pablos, D., Duarte-Ojeda, J.M., García-González, L., García- Cruz, E. and Rodríguez-Antolín, A., 2015. Semen quality assessment in fertile men in Madrid during the last 3 decades. Urology, 85(6), pp.1333-1338.

Safarinejad, M.R., Azma, K. and Kolahi, A.A., 2009. The effects of intensive, long-term treadmill running on reproductive hormones, hypothalamus-pituitary-testis axis, and semen quality: a randomized controlled study. Journal of Endocrinology, 200(3), p.259.

Saffhill, R., Margison, G.P., and O'Connor, P.J., 1985. Mechanisms of carcinogenesis induced by alkylating agents. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 823(2), pp.111-145.

Sakkas, D. and Alvarez, J.G., 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertility and Sterility, 93(4), pp.1027-1036.

Sakkas, D., Mariethoz, E. and John, J.C.S., 1999. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Experimental cell research, 251(2), pp.350-355.

Sakkas, D., Seli, E., Bizzaro, D., Tarozzi, N. And Manicardi, G.C., 2003. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. Reproductive Biomedicine Online, 7(4), pp.428-432.

Santi, D., Magnani, E., Michelangeli, M., Grassi, R., Vecchi, B., Pedroni, G., Roli, L., De Santis, M.C., Baraldi, E., Setti, M. and Trenti, T., 2018. Seasonal variation of semen parameters correlates with environmental temperature and air pollution: A big data analysis over 6 years. Environmental pollution, 235, pp.806-813.

Sassone-Corsi, P., 2002. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science, 296(5576), pp.2176-2178.

Schmid, T.E., Eskenazi, B., Baumgartner, A., Marchetti, F., Young, S., Weldon, R., Anderson, D. and Wyrobek, A.J., 2007. The effects of male age on sperm DNA damage in healthy non-smokers. Human Reproduction, 22(1), pp.180-187.

Schmid, T.E., Kamischke, A., Bollwein, H., Nieschlag, E. and Brinkworth, M.H., 2003. Genetic damage in oligozoospermic patients detected by fluorescence in‐situ hybridization, inverse restriction site mutation assay, sperm chromatin structure assay and the Comet assay. Human Reproduction, 18(7), pp.1474-1480.

Schulte, R.T., Ohl, D.A., Sigman, M. and Smith, G.D., 2010. Sperm DNA damage in male infertility: etiologies, assays, and outcomes. Journal of assisted reproduction and genetics, 27(1), pp.3-12.

Shamsi, M.B., Venkatesh, S., Tanwar, M., Talwar, P., Sharma, R.K., Dhawan, A., Kumar, R., Gupta, N.P., Malhotra, N., Singh, N. And Mittal, S., 2009. DNA integrity and semen quality in men with low seminal antioxidant levels. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 665(1-2), pp.29-36.

Shangula, S., Noori, M., Ahmad, I., Margison, G.P., Liu, Y., Siahmansur, T., Soran, H. and Povey, A.C., 2019. PON1 increases cellular DNA damage by lactone substrates. Archives of toxicology, pp.1-9.

Sharif, K., 2013. Sperm DNA fragmentation testing: To do or not to do?. Middle East Fertility Society Journal, 18(2), pp.78-78.

Sharpe, R.M. and Skakkebaek, N.E., 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract?. The Lancet, 341(8857), pp.1392-1396.

Sharpe, R.M., 2010. Environmental/lifestyle effects on spermatogenesis. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1546), pp.1697-1712.

Shi, X., Chan, C.P.S., Waters, T., Chi, L., Chan, D.Y.L. and Li, T.C., 2018. Lifestyle and demographic factors associated with human semen quality and sperm function. Systems biology in reproductive medicine, 64(5), pp.358-367.

Shibahara, H., Obara, H., Hirano, Y., Suzuki, T., Ohno, A., Takamizawa, S. and Suzuki, M., 2004. Prediction of pregnancy by intrauterine insemination using CASA estimates and strict criteria in patients with male factor infertility. International journal of andrology, 27(2), pp.63-68.

Shrivastav, N., Li, D. and Essigmann, J.M., 2009. Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation. Carcinogenesis, 31(1), pp.59-70.

Sifakis, S., Androutsopoulos, V.P., Tsatsakis, A.M. and Spandidos, D.A., 2017. Human exposure to endocrine disrupting chemicals: effects on the male and female reproductive systems. Environmental toxicology and pharmacology, 51, pp.56-70.

Simon, L., Brunborg, G., Stevenson, M., Lutton, D., McManus, J. and Lewis, S.E., 2010. Clinical significance of sperm DNA damage in assisted reproduction outcome. Human reproduction, 25(7), pp.1594-1608.

Simon, L., Lutton, D., McManus, J. and Lewis, S.E., 2011. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and *in vitro* fertilization success. Fertility and sterility, 95(2), pp.652-657.

Simon, L., Proutski, I., Stevenson, M., Jennings, D., McManus, J., Lutton, D. and Lewis, S.E.M., 2013. Sperm DNA damage has a negative association with live-birth rates after IVF. Reproductive biomedicine online, 26(1), pp.68-78.

Singh, N.P., Muller, C.H. and Berger, R.E., 2003. Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertility and sterility, 80(6), pp.1420-1430.

Singh, J., Su, L. and Snow, E.T., 1996. Replication across O6-Methylguanine by Human DNA Polymerase β *in vitro* insights into the futile cytotoxic repair and mutagenesis of O^6-methylguanine. journal of biological chemistry, 271(45), pp.28391-28398.

Skakkebaek, NE., Rajpert-De Meyts, E. and Main, K.M., 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects: Opinion. Human Reproduction, 16(5), pp.972-978.

Slupphaug, G., Kavli, B. and Krokan, H.E., 2003. The interacting pathways for prevention and repair of oxidative DNA damage. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 531(1), pp.231-251.

Smith, C.C., O'Donovan, M.R. and Martin, E.A., 2006. hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. Mutagenesis, 21(3), pp.185-190.

Sram, R., 1999. Impact of air pollution on reproductive health.

Stock, C.E. and Fraser, L.R., 1987. The acrosome reaction in human sperm from men of proven fertility. Human Reproduction, 2(2), pp.109-119.

Stocks, S.J., Agius, R.M., Cooley, N., Harrison, K.L., Brison, D.R., Horne, G., Gibbs, A. and Povey, A.C., 2010. Alkylation of sperm DNA is associated with male factor infertility and a reduction in the proportion of oocytes fertilised during assisted reproduction. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 698(1-2), pp.18-23.

Stojic, L., Brun, R. and Jiricny, J., 2004. Mismatch repair and DNA damage signalling. DNA repair, 3(8-9), pp.1091-1101.

Strauss, J.F. and Barbieri, R.L., 2013. Yen & Jaffe's reproductive endocrinology E-book: physiology, pathophysiology, and clinical management (Expert Consult-Online and Print). Elsevier Health Sciences.

Suarez, S.S. and Pacey, A.A., 2006. Sperm transport in the female reproductive tract. Human reproduction update, 12(1), pp.23-37.

Sun, J.G., Jurisicova, A. and Casper, R.F., 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. Biology of reproduction, 56(3), pp.602-607.

Sung, P. and Klein, H., 2006. Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nature Reviews Molecular Cell Biology, 7(10), p.739.

Swan, S.H., Kruse, R.L., Liu, F., Barr, D.B., Drobnis, E.Z., Redmon, J.B., Wang, C., Brazil, C., Overstreet, J.W., and Study for Future Families Research Group, 2003. Semen quality in relation to biomarkers of pesticide exposure. Environmental health perspectives, 111(12), pp.1478-1484.

Takahashi, A. and Ohnishi, T., 2005. Does γH2AX foci formation depend on the presence of DNA double strand breaks? Cancer letters, 229(2), pp.171-179.

Tan, S.L., Gerber, J.P., Cosgrove, L.J., Lockett, T.J., Clarke, J.M., Williams, D.B. and Head, R.J., 2011. Is the tissue persistence of O^6-methyl-2′-deoxyguanosine an indicator of tumour formation in the gastrointestinal tract? Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 721(2), pp.119-126.

Tateno, H., Iijima, S., Nakanishi, Y., Kamiguchi, Y. and Asaka, A., 1998. No induction of chromosome aberrations in human spermatozoa exposed to extremely low frequency electromagnetic fields. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 414(1), pp.31-35.

Tejada, R.I., Mitchell, J.C., Norman, A., Marik, J.J. and Friedman, S., 1984. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertility and sterility, 42(1), pp.87-91.

Tesarik, J. and Testart, J., 1989. Human sperm-egg interactions and their disorders: implications in the management of infertility. Human Reproduction, 4(7), pp.729-741.

Tesarik, J., Guido, M., Mendoza, C. and Greco, E., 1998. Human spermatogenesis *in vitro*: respective effects of follicle-stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis. The Journal of Clinical Endocrinology & Metabolism, 83(12), pp.4467-4473.

Tessmer, I. and Fried, M.G., 2014. Insight into the cooperative DNA binding of the O^6-alkylguanine DNA alkyltransferase. DNA repair, 20, pp.14-22.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F., 2000. Single-cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environmental and molecular mutagenesis, 35(3), pp.206-221.

Tiegs, A.W., Landis, J., Garrido, N., Scott Jr, R.T. and Hotaling, J.M., 2019. Total motile sperm count trend over time: evaluation of semen analyses from 119,972 men from subfertile couples. Urology, 132, pp.109-116.

Tomsu, M., Sharma, V. and Miller, D., 2002. Embryo quality and IVF treatment outcomes may correlate with different sperm Comet assay parameters. Human Reproduction, 17(7), pp.1856-1862.

Tournaye, H., Devroey, P., Camus, M., Van der Linden, M., Janssens, R. and Van Steirteghem, A., 1995. Use of pentoxifylline in assisted reproductive technology. Human Reproduction, 10(suppl_1), pp.72-79.

Tremellen, K., 2008. Oxidative stress and male infertility—a clinical perspective. Human reproduction update, 14(3), pp.243-258.

Tricker, A.R. and Preussmann, R., 1991. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutation Research/Genetic Toxicology, 259(3-4), pp.277-289.

Tricker, A.R., 1997. N-nitroso compounds and man: sources of exposure, endogenous formation and occurrence in body fluids. European journal of cancer prevention: the official journal of the European Cancer Prevention Organisation (ECP), 6(3), pp.226-268.

Trisini, A.T., Singh, N.P., Duty, S.M. and Hauser, R., 2004. Relationship between human semen parameters and deoxyribonucleic acid damage assessed by the neutral comet assay. Fertility and sterility, 82(6), pp.1623-1632.

Vaamonde, D., du Plessis, S.S. and Agarwal, A., 2016. Exercise and Human Reproduction.pp.13-16.

Valenti, D., La Vignera, S., Condorelli, R.A., Rago, R., Barone, N., Vicari, E. And Calogero, A.E., 2013. Follicle-stimulating hormone treatment in normogonadotropic infertile men. Nature Reviews Urology, 10(1), pp.55-62.

Vamvakas, S., Vock, E.H. and Lutz, W.K., 1997. On the role of DNA double-strand breaks in toxicity and carcinogenesis. Critical reviews in toxicology, 27(2), pp.155-174.

Vierula, M., Niemi, M., Keiski, A., Saaranen, M., Saarikoski, S. and Suominen, J., 1996. High and unchanged sperm counts of Finnish men. International journal of andrology, 19(1), pp.11-17.

Wallace, W.H.B., Anderson, R.A. and Irvine, D.S., 2005. Fertility preservation for young patients with cancer: who is at risk and what can be offered? The Lancet Oncology, 6(4), pp.209-218.

Watanabe, N. and Oonuki, Y., 1999. Inhalation of diesel engine exhaust affects spermatogenesis in growing male rats. Environmental health perspectives, 107(7), pp.539-544.

Weber, R.F., Dohle, G.R. and Romijn, J.C., 2005. Clinical laboratory evaluation of male subfertility. Advances in clinical chemistry, 40, pp.317-364.

Wijchman, J.G., De Wolf, B.T., Graaff, R. and ARTS, E.G., 2001. Variation in semen parameters derived from computer‐aided semen analysis, within donors and between donors. Journal of andrology, 22(5), pp.773-780.

Wolf, D.P., 1989. Acrosomal status quantitation in human sperm. American Journal of Reproductive Immunology, 20(3), pp.106-113.

Wong, V.W., Szeto, Y.T., Collins, A.R. and Benzie, I.F., 2005. The comet assay: a biomonitoring tool for nutraceutical research. Current Topics in Nutraceutical Research.

Woodside, A.M. and Guengerich, F.P., 2002. Effect of the O6 substituent on misincorporation kinetics catalyzed by DNA polymerases at O 6-methylguanine and O 6-benzylguanine. *Biochemistry*, *41*(3), pp.1027-1038.

World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus-165-Interaction, 3rd edn. Cambridge: Cambridge University, Press, 1992

World Health Organization, 1999. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction Cambridge University Press. Cambridge, UK.

World Health Organization, 2010. WHO laboratory manual for the examination and processing of human semen.

Wu, F., Tian, F.J. and Lin, Y., 2015. Oxidative stress in placenta: health and diseases. BioMed research international

Xie, M., Utzinger, K.S., Blickenstorfer, K. and Leeners, B., 2018. Diurnal and seasonal changes in semen quality of men in subfertile partnerships. Chronobiology international, 35(10), pp.1375-1384.

Xu-Welliver, M. and Pegg, A.E., 2002. Degradation of the alkylated form of the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase. Carcinogenesis, 23(5), pp.823-830.

Yu, Y., Zhu, W., Diao, H., Zhou, C., Chen, F.F. and Yang, J., 2006. A comparative study of using comet assay and γH2AX foci formation in the detection of N-methyl-N′-nitro-N-nitrosoguanidine-induced DNA damage. Toxicology *in vitro*, 20(6), pp.959-965.

Zegers-Hochschild, F., Adamson, G.D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E. and Van der Poel, S., 2009. The international committee for monitoring assisted reproductive technology (ICMART) and the world health

organization (WHO) revised glossary on ART terminology, 2009. Human reproduction, 24(11), pp.2683-2687.

Zenzes, M.T., Bielecki, R. And Reed, T.E., 1999. Detection of benzo (a) pyrene diol epoxide–DNA adducts in sperm of men exposed to cigarette smoke. Fertility and Sterility, 72(2), pp.330-335.

Zhu, J.L., Basso, O., Obel, C., Bille, C. and Olsen, J., 2006. Infertility, infertility treatment, and congenital malformations: Danish national birth cohort. Bmj, 333(7570), p.679.

Zini, A. and Agarwal, A. eds., 2018. A Clinician's Guide to Sperm DNA and Chromatin Damage. Springer.

Appendix 1. Ethical Approval for the study

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

M E M O R A N D U M

TO Michael Carroll

FROM Karen Hartley

DATE 19th December 2016

DATE OF EXPIRY: December 2017

SUBJECT Application for Ethical Approval **(SE161744)**

On the 19th December 2016 the Head of Ethics for Science & Engineering considered your application for Ethical Approval (SE161744) entitled "Investigating the effect of lifestyle and environment on human sperm – a longitudinal study". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Dr Nick Costen) and the Research Degrees Administrator. Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence. Please notify Professor Tristan McKay of any issues relating to this.

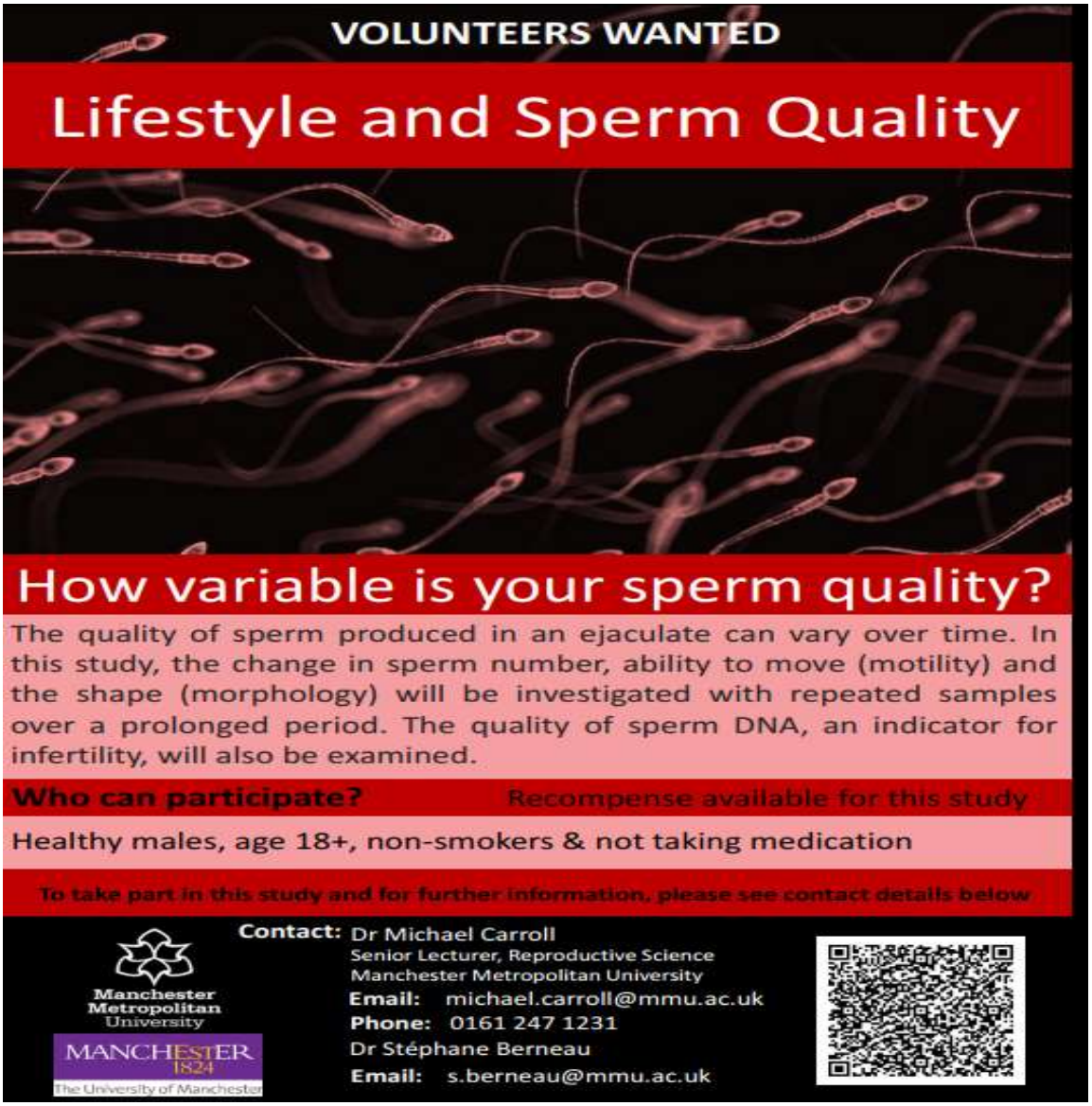
If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the MMU Request for Amendment form (found on the Graduate School website) and submit it to the Administrator.

Regards

Karen Hartley
Research Administrator
All Saints North

Page 1 of 1

Appendix 2. Advertisement for volunteers to take part in the study



Appendix 3. Study information

1. Participant information sheet (PIS)

Title of Study: *Investigating the effect of lifestyle and environment on human sperm.*

Study Background

Male factor infertility can account for 40% of infertility experienced by couples trying to conceive. As sperm are produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage.

We will investigate how lifestyle exposures can affect sperm quality. This information will offer potential therapeutic options that may improve male infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.
The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. The samples will be stored at -80°C for further analysis.
Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes.
You will also be asked to provide a blood sample and saliva (optional). The blood will be taken by an experienced phlebotomist and is a quick and painless procedure. For the saliva sample, you will just spit in a sterile container. Both blood and saliva will be prepared and stored at 80°C until required. These samples will be used for biochemical and molecular analysis.

Please note that this is not a diagnostic test and you will not be informed of the quality of your semen/sperm.

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

Participant informed consent.

ID code ……

Name: ……

Date of Birth: ……

Project title:

Investigating the effect of lifestyle and environment on human sperm.

Principal Investigator:
Dr Michael Carroll

Investigator/Collaborators:
Dr Christopher Murgatroyd
Muaamar Al-Khaiqani – PhD student, University of Manchester.
(muaamar.alkhaiqani@postgrad.manchester.ac.uk)
Dr Andy Povey (external collaborator)
Prof. Daniel Brison (external collaborator)

Ethics approval number: SE161744

I have read and understood the information sheet and all verbal explanation outlining the purpose of the study and the assessments involved.

Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without having to give a reason. I understand that my sperm will not be used, at any time, for any form of assisted reproductive technique, such as fertilising human eggs or the creation of human embryos. I understand that my semen may be also used for teaching purposes. My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of the School of Healthcare Sciences, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

I give my consent for semen collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis.

Signed ..... Date .....

Name (Print)……

Witnessed ..... Date .....

2. Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This information will be kept strictly confidential.
Please answer the following questions as accurately as possible.

Are you currently taking any prescribed medication? **YES/NO**

Are you currently attending your GP? **YES/NO**

Have you ever suffered from a cardiovascular problem? **YES/NO**
i.*e. high blood pressure, anaemia, heart attack etc*

Have you ever suffered from a neurological disorder? **YES/NO**
i.e. epilepsy, convulsions etc

Have you ever suffered from an endocrine disorder? **YES/NO**
i.e. diabètes etc.

Have you ever suffered from a chronic gastrointestinal disorder? **YES/NO**
i.e. Crohn's disease, irritable bowel syndrome etc

Have you ever suffered from a skin disorder? **YES/NO**
i.e. eczema etc

Do you suffer from any allergies? **YES/NO**
i.e. any medications, foods etc

Have you had a vasectomy or any urological surrey? **YES/NO**
i.e. testicular surgery

Have you had Mumps? **YES/NO**

Have you had any testicular injuries / torsions? **YES/NO**

Do you knowingly have, or had a Sexually Transmitted Infection? **YES/NO**

If you have answered "yes" to any of these questions, please provide details below:

3. Study Procedures

The following is a brief description of the procedures and techniques that will be employed during this study.

Semen procurement:

Semen will be produced by masturbation in to sterile containers provided. On occasions where participants cannot provide a specimen from home they will be asked to produce a sample on site in a dedicated, secured room. The specimen container will be placed in the plastic bag with the completed 'semen procurement form'.

Semen analysis:

Semen analysis is carried out within 30 minutes of specimen production. Volume, pH and other physical characteristics are noted. Sperm motility and concentration is measured and a sample of semen is smeared on to a glass slide for fixing and morphological analysis.

Blood analysis:

Blood will be taken and used for biochemical analysis. Blood hormones and biomolecules will be analysed.

DNA/RNA studies:

DNA and RNA will be isolated from both fresh and frozen sperm cells using commercial kits (QIAGEN) and examined for the expression levels of key genes responsible for sperm function. DNA methylation studies will be conducted also.
Chromatin assays such as chromatin dispersion assay and comet assays will be conducted to investigate the integrity of the sperm nucleus after exposure to various compounds.

4. MANCHESTER METROPOLITAN UNIVERSITY

Faculty of Science and Engineering
RISK ASSESSMENT COVER SHEET

FACULTY OF SCIENCE & ENGINEERING			
SCHOOL: Healthcare Science			
TITLE OF WORK: Longitudinal study of lifestyle effects on human sperm			
LOCATION OF WORK (*LLn.nn*): T1.08; T4.01			
INTENDED ACTIVITIES (attach methods sheets (e.g. standard operating practices) and work schedules to this form): Human semen procurement			
PERSONS AT RISK (including status [e.g. staff/student], for students please indicate course and level, for staff give contact email / phone number): Dr Michael Carroll & Muaamar Al-Khaiqani – PhD student visitor (research)			
HAZARDS (this should be a summary of the hazards anticipated – attach detailed assessments with appropriate risk control methods to this form): Biological: human semen and blood Low hazard from sample procurement: Slight risk of fainting due to the physical activity. Falling hazard and injury due to fainting *Are these hazards necessary in order to achieve the objectives of the activity?* Yes **Hazard Rating: low**			
HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION (appropriate COSHH data sheets / risk assessments must be attached to this form): *ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS.*			
NAME OF MATERIAL *Please provide also approximate quantity and concentration if applicable.*	**HAZARD CLASS**	**HAZARD LABEL**	**Disposal** *Hazardous materials must not be removed from laboratories. Disposal arrangements for* the materials listed below *in the location where* the work is specified to be carried out *are:*
Human semen and blood	NA	Biohazard	Samples will be stored at -80°C with participant ID number, and disposed of

			according to the HTA by incineration. Contaminated consumables in contaminated waste bins and contaminated sharps in sharps containers.

RISK CONTROL METHODS (this should be a summary of the hazards anticipated – attach detailed assessments with appropriate risk control methods to this form):

The room used for the procurement of semen (Phlebotomy room (T1.19c), IRM, Ground floor, John Dalton Building) will be maintained to minimize any clutter that may cause potential harm. The room has the capacity to be locked from the inside to ensure privacy. However, in the unlikely event of the participant fainting the door can be unlocked from the outside with a key.

Additionally, a member of the study team will be aware of the time the participant enters the room; should a stated time elapse, the study team member will knock on the door to knock to ensure the participant is OK. If there is no answer, the team member can enter the room using the key to evaluate the status of the participant.

Gloves should be worn when handling biological samples and spillage of biological samples will be treated/cleaned with 1% virkon or trigene. The workspace is to be sterilised with ethanol before and after any work.

Personal protective clothing (laboratory coat, nitrile gloves, safety spectacles) should be worn at all times and shoes must completely cover the feet.

Hazard Rating with Control Methods: Low

Will any specific **training** *be required?* No
Are there any specific **first aid** *issues?* No

PROCEDURE FOR EMERGENCY SHUT-DOWN (if applicable): NA

IF OFF-SITE INDICATE ANY OTHER ISSUES (e.g. associated with: individual's health and dietary requirements (obtain off-site health forms for all participating individuals and indicate where this information will be located); social activities, transportation, ID requirements; permissions for access and sampling). None.

	NAME	**STAFF/STUDENT No.**	**DATE**
Originator	Muaamar Al-Khaiqani	15502606	
Supervisor (*XYZ*)	Dr Michael Carroll		
Technical Manager			
Divisional Safety Representative	p.p. HoS		

DATE TO BE REVIEWED BY:

SCHOOL REFERENCE NUMBER (XYZ/ddmmyy/LLn.nn): / /

Please save the completed form as XYZ_ddmmyy_RA.doc and email to next signatory.

Appendix 4. Semen procurement form

Semen Procurement Form.

Introduction

Please follow the instructions concerning the production of a semen sample.

As a fresh sample is essential for this test, it must be brought to the laboratory within one hour of being passed at home.

NOTE: USING A PUBLIC TOILET TO PASS YOUR SAMPLE IS AGAINST THE LAW.

Instructions for collecting the semen sample

You should abstain from intercourse or masturbation for three to four days prior to providing the specimen. The sample must be obtained by masturbation (manual stimulation) and should be collected directly into the specimen container provided. A condom or artificial lubrication must not be used for semen collection, as it will kill the sperm.

The complete specimen is needed for this study, so if any is spilt you must tell us, as a repeat specimen may be required.

Label the specimen container with your full name, date of birth and the date and time the specimen was passed.

Delivery of your sample

Deliver the sample to the School of Healthcare Science within **one hour** of passing the sample.

Keep the sample at body temperature while being transported to the laboratory, for example by carrying it in an inside pocket. Excessive cold or heat can damage the sperm.

For further information contact:
Dr M. Carroll (michael.carroll@mmu.ac.uk)
Phone: 0161 247 1231
Specimen details

Please complete this form and bring it with you to the laboratory along with your sample and the request form.

Name: _____
Date of Birth: _____
Date of specimen Time passed: _____
Time specimen passed: _____
Abstinence (days): _____
I confirm that this semen specimen is mine and I consent to its use for the purpose of research and education.

Signed _____

Appendix 5. Semen and sperm parameters throughout the study

L.	Volume (ml)	Sperm concentration ($X10^6$/ml)	Total sperm count ($X10^6$)	% progressively motile sperm	% non-motile sperm	% vital sperm	% morphologically normal sperm
L01							
1	7.8	50.7	395.2	50.7	37.9	80.1	4
2	7.2	3.0	21.6	54.5	39.6	78.7	
3	8.7	16.1	140.2	61.5	31.7	84.2	
4	7.5	50.4	377.9	51.6	37.2	77.0	
5	7.0	21.7	151.7	43.6	40.5	73.3	
6	2.0	49.5	99.0	53.1	35.2	84.8	4
L02							
1	4.0	15.2	60.9	72.8	16.5	94.7	10
2	3.4	119.7	406.9	87.2	6.9	96.0	
3	4.4	150.1	660.5	82.3	10.8	88.4	
4	5.0	73.3	366.4	66.1	19.0	89.0	
5	7.0	73.3	513.0	52.3	38.2	83.6	
6	9.6	71.5	686.6	69.4	21.5	84.7	5
7	8.0	52.6	420.8	46.6	46.9	85.0	
8	3.5	102.5	358.9	73.5	17.0	88.6	
9	2.5	2.1	5.2	18.2	79.3	53.8	
10	8.3	17.3	143.4	32.5	57.3	70.3	
11	6.8	121.5	825.9	78.1	15.8	86.9	
12	6.6	35.3	233.0	48.1	43.1	74.5	3
L03							
1	3.5	26.4	92.4	72.8	22.0	95.0	4
L04							
1	2.2	7.3	16.0	64.8	27.2	91.3	7
2	1.1	5.9	6.5	44.0	47.0	82.2	
3	1.1	21.4	23.5	58.1	34.1	90.7	
4	1.1	46.0	50.5	68.5	23.5	87.4	
5	1.3	26.2	34.1	57.5	33.8	81.4	
6	1.3	35.0	45.5	57.4	34.6	77.0	4
7	0.6	42.0	25.2	60.9	32.3	81.6	
8	0.8	18.8	15.0	51.6	39.4	80.6	
9	0.8	13.2	10.5	44.4	47.1	74.1	
10	1.3	36.7	47.7	66.0	25.5	86.0	
11	2.0	24.7	49.4	58.6	36.3	84.5	
12	1.1	17.4	19.1	37.9	57.0	65.8	5
L05							
1	2.5	2.8	7.0	28.9	61.5	60.0	3
2	3.5	26.9	94.1	38.8	50.6	86.8	

3	2.0	31.2	62.4	41.2	48.8	76.2	
4	3.0	47.4	142.3	63.9	23.7	78.2	
5	2.0	47.3	94.6	57.5	30.6	86.2	
6	2.5	20.4	51.0	32.9	59.4	68.7	2
7	2.6	30.6	79.5	38.6	52.2	72.5	
8	2.7	33.1	89.4	40.2	50.0	77.2	
9	1.5	32.2	48.3	27.1	67.4	74.1	
10	2.7	59.4	160.4	57.2	16.4	85.2	
11	2.7	31.5	85.0	63.9	27.2	78.6	
12	1.8	25.8	46.5	48.1	32.8	75.8	2
L06							
1	2.0	6.0	11.9	51.5	36.5	90.3	4
2	2.0	59.1	118.2	60.5	30.0	82.4	
3	2.0	65.3	130.6	71.9	16.7	90.7	
4	2.3	101.1	232.5	69.0	21.1	86.1	
5	1.0	74.8	74.8	67.6	25.3	91.6	
6	2.3	33.4	76.8	48.7	46.0	78.0	2
7	1.6	43.2	69.1	68.2	23.6	80.0	
8	0.8	81.6	65.2	75.0	17.8	86.6	
9	1.6	43.3	69.3	61.4	29.4	82.8	
10	2.4	41.1	98.6	62.6	28.4	83.4	
11	1.5	84.2	126.3	72.6	18.6	87.4	
12	2.4	26.1	58.4	59.2	26.7	84.0	1
L07							
1	3.0	22.6	67.7	76.9	12.7	91.8	4
2	3.0	37.8	113.5	54.7	37.1	95.6	
3	3.5	62.4	218.3	57.3	31.8	88.1	
4	2.5	38.9	97.2	65.7	29.7	86.7	
5	3.0	37.4	112.1	51.4	42.3	88.6	
6	2.8	61.5	172.1	65.4	26.4	87.9	2
7	2.7	97.5	263.3	60.9	29.1	86.3	
8	2.5	38.6	96.4	47.1	34.5	79.5	
9	3.0	30.7	92.2	41.0	51.6	77.6	
10	2.3	35.0	80.5	61.2	27.9	87.8	
11	3.0	47.3	141.8	63.7	22.8	85.8	
12	2.8	50.2	140.7	70.9	17.7	88.0	2
L08							
1	2.4	6.6	15.8	44.8	47.5	91.2	5
2	3.0	88.7	266.0	66.6	24.0	90.2	
3	1.1	33.7	37.1	10.5	86.0	51.2	
4	1.3	16.2	21.0	43.4	47.5	83.4	
5	3.4	33.0	112.0	35.0	53.4	83.7	
6	1.5	20.6	30.0	38.3	54.6	83.2	1

7	1.4	24.7	34.6	3.6	93.6	66.3	
L09							
1	3.1	67.1	207.9	86.8	8.4	93.5	7
2	2.1	6.5	126.0	54.8	36.5	91.8	
3	2.3	69.0	158.7	67.8	20.6	87.9	
4	1.8	92.2	166.0	76.5	16.7	90.1	
5	2.4	101.1	242.5	71.6	17.0	91.2	
6	2.3	61.3	141.0	69.6	20.1	91.1	4
7	2.2	46.8	103.0	76.3	15.8	87.3	
8	3.8	43.4	165.1	73.8	19.7	85.2	
9	1.5	74.8	112.1	68.8	22.9	91.0	
10	3.2	60.6	193.9	66.8	22.9	90.9	
11	3.3	34.4	113.5	73.7	16.5	85.0	
12	2.5	34.5	86.2	70.3	21.0	90.1	1
L10							
1	1.0	59.7	59.7	63.7	24.9	93.4	7
2	1.0	27.0	27.0	85.8	11.5	92.4	
3	1.0	58.1	58.1	83.7	11.9	90.3	
4	1.1	80.4	88.4	80.2	11.8	93.5	
5	1.1	115.8	127.4	69.6	21.9	90.5	
6	1.2	78.3	94.0	68.3	7.8	91.5	10
7	1.0	82.2	82.2	83.8	12.2	93.0	
8	0.8	60.6	48.5	77.1	17.6	85.0	
9	1.2	119.4	143.3	89.6	7.9	94.8	
10	1.0	109.9	109.9	85.5	10.0	92.9	
11	0.7	101.1	70.7	80.1	13.2	89.9	
12	1.3	51.3	66.7	82.4	10.1	94.8	3
L11							
1	3.3	40.1	132.2	63.5	28.0	89.4	5
2	2.5	44.5	111.2	69.8	22.3	89.8	
3	2.5	87.5	218.7	68.2	21.0	91.9	
4	1.8	44.5	80.1	69.4	22.9	90.3	
5	1.8	88.4	159.0	71.6	17.7	92.6	
6	1.8	56.9	102.4	80.5	12.5	90.2	3
7	1.6	79.6	127.3	78.5	16.7	88.9	
8	1.4	69.4	97.2	63.4	27.2	89.4	
9	1.1	78.0	85.8	79.2	14.0	93.6	
10	1.8	38.7	69.7	71.0	23.3	90.5	
11	2.5	68.0	169.9	76.1	10.6	92.8	
12	1.3	36.2	47.1	56.3	34.7	76.5	1
L12							
1	1.1	2.3		32.4	57.8	77.0	2
2	3.7	8.7	32.2	34.5	58.2	70.7	

3	2.0	8.6	17.1	37.0	55.7	72.3	
4	2.0	42.9	85.7	35.5	53.1	78.6	
5	2.5	20.9	52.2	28.8	63.7	82.5	
6	3.0	16.2	48.5	26.0	69.9	76.5	4
7	2.8	33.5	93.8	32.9	54.4	75.7	
8	1.8	21.3	38.3	22.7	56.0	84.8	
9	1.8	10.5	19.0	24.8	65.9	67.4	
10	2.8	8.7	24.2	31.7	58.5	79.3	
11	4.0	8.1	32.5	30.0	60.0	76.5	
12	0.8	31.3	25.1	42.0	44.3	81.3	2
L13							
1	4.0	43.4	173.8	74.2	20.4	89.3	5
2	4.2	11.0		68.3	20.5	90.2	
3	4.2	22.6	94.8	57.2	36.2	87.6	
4	4.4	61.5	270.4	69.2	23.6	89.6	
5	4.4	14.5	63.8	48.2	49.8	80.3	
6	4.7	45.8	215.3	70.0	23.9	89.9	5
7	4.2	20.5	85.9	44.4	42.2	80.9	
8	4.2	49.9	209.7	68.1	22.5	87.6	
9	3.3	29.9	98.5	47.0	44.6	84.0	
10	3.8	39.7	151.0	61.0	32.7	83.8	
11	4.2	26.5	111.5	46.7	43.3	78.6	
12	3.2	44.6	142.8	61.3	30.5	85.1	1
L14							
1	4.2	5.9		55.9	29.6	91.3	2
2	3.5	51.7	181.0	54.0	35.2	83.3	
3	3.2	18.8	60.3	42.0	52.9	83.2	
4	3.5	18.5	64.9	47.8	49.0	84.2	
L15							
1	1.5	30.1	45.2	54.4	34.3	77.8	6
2	3.2	4.4	13.9	15.5	73.3	83.8	
3	2.8	23.3	65.1	41.1	49.2	86.2	
4	3.2	24.5	78.5	44.2	48.6	86.3	
5	3.6	31.6	113.8	41.6	49.1	83.3	
6	3.8	27.1	102.9	27.6	59.6	80.0	5
7	1.8	15.3	27.5	35.3	54.1	75.6	
8	2.6	38.4	99.9	43.9	48.1	78.5	
9	2.9	25.5	74.0	49.0	43.2	84.3	
10	1.6	35.5	56.7	15.8	82.9	69.3	
11	4.0	47.0	187.9	56.9	30.8	92.0	
12	3.7	45.1	166.7	52.4	31.7	83.1	3
L16							
1	2.6	20.0		77.7	13.6	94.7	6

2	2.5	81.6	203.9	63.8	23.9	91.3	
3	3.1	130.7	405.0	76.7	14.1	93.5	
4	3.7	68.0	251.5	64.8	27.4	94.4	
5	2.3	30.0	69.0	63.6	30.5	81.2	
6	2.0	85.7	171.4	79.7	13.1	89.5	6
7	2.7	72.4	195.5	57.2	22.9	92.5	
8	1.5	140.7	211.0	77.6	13.2	89.2	
9	1.1	126.8	139.4	71.3	21.0	90.4	
10	0.8	115.3	92.2	66.9	23.1	86.4	
11	1.7	99.6	169.3	71.2	11.9	89.4	
12	1.8	90.7	163.3	68.1	20.9	93.1	7
L17							
1	3.2	4.9	15.6	24.7	58.4	72.1	1
2	2.5	6.6	16.4	28.8	60.4	58.4	
3	2.3	22.5	51.6	42.1	46.5	72.8	
4	4.0	35.0	140.1	37.6	55.3	53.7	
5	2.5	27.4	68.5	24.5	48.9	78.6	
6	2.2	60.3	132.6	39.2	54.9	55.8	4
7	1.3	19.9	25.9	20.3	72.8	62.0	
8	3.5	14.3	50.1	18.2	73.6	63.4	
9	4.0	9.9	39.4	9.5	88.5	78.6	
10	4.0	29.7	118.8	18.4	75.6	70.0	
11	3.5	19.9	69.7	11.4	79.7	62.9	
12	1.8	25.3	45.6	35.8	56.0	59.4	2
L18							
1	1.8	49.9	89.7	54.2	30.1	85.0	5
2	2.0	76.5	153.1	66.4	26.6	76.2	
3	2.0	125.9	251.8	76.5	17.1	85.2	
4	1.7	75.9	129.1	78.2	16.0	85.3	
5	1.7	67.7	115.0	72.9	18.8	87.0	
6	1.0	46.3	46.3	69.7	11.8	89.0	2
7	1.5	35.8	53.6	21.1	69.0	72.4	
8	2.5	49.6	124.1	77.4	12.8	87.7	
9	1.8	79.2	138.6	74.6	14.2	89.3	
10	1.7	68.9	117.1	73.0	17.2	90.0	
11	1.0	78.9	78.9	61.8	29.6	85.6	
12	2.2	161.6	138.3	66.0	26.7	78.2	1
L19							
1	1.6	5.6	8.9	44.9	50.2	80.8	3
2	0.9	4.4	4.0	19.1	78.0	84.2	
3	1.6	9.2	14.7	47.4	47.9	82.8	
4	0.9	11.9	10.7	53.5	38.6	87.9	
5	1.0	6.5	6.5	50.7	41.6	87.8	

6	1.7	5.1	8.7	51.9	41.8	87.1	6
7	1.8	1.4	2.5	59.2	35.3	84.2	
8	1.3	19.7	25.6	67.5	26.0	87.4	
L20							
1	3.6	65.0	234.1	60.9	26.6	89.9	2
2	1.1	67.4	74.1	62.7	31.1	71.6	
3	4.0	55.1	220.5	53.3	41.0	84.7	
4	2.0	73.9	147.8	68.8	22.4	84.5	
5	3.0	59.7	179.1	66.3	25.7	79.4	
6	5.6	78.0	436.9	68.2	21.6	90.3	2
7	1.0	98.7	98.7	57.8	32.3	79.0	
8	2.5	64.4	161.1	74.8	13.8	86.7	
9	3.4	46.0	156.2	46.9	42.1	79.2	
10	1.7	104.6	177.8	61.0	30.2	77.4	
11	3.4	62.8	213.5	57.7	33.9	78.5	
12	3.0	44.3	133.0	51.3	36.0	84.5	4

L. Longitudinal; L01 to L20: the participants from 1 to 20. Number indicates visit number.