Investigating the Effect of Diet on Human Sperm Parameters and Global Sperm DNA Methylation

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A thesis submitted in fulfilment of the requirements

of

Manchester Metropolitan University for the degree of

Master of Science (by Research)

School of Healthcare Science

Manchester Metropolitan University

2018

Declaration

With the exception of any statements to the contrary, all the data presented in this report are the result of my own efforts. In addition, no parts of this report have been copied from other sources. I understand that any evidence of plagiarism and/or the use of unacknowledged third-party data will be dealt with as a very serious matter.

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List of Abbreviations

ART Assisted Reproductive Technology

ATP Adenosine Tri-Phosphate

BMI Body Mass Index

CASA Computer Assisted Sperm Analysis

CGI CpG islands

CpG 5'-cytosine-phosphate-guanine-3'

DAZL Deleted in azoospermia-like

DGC density gradient centrifugation

DNA Deoxyribonucleic acid

DNMT DNA-Methyltransferase

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-Linked-Immunosorbent-Assay

ESC Embryonic stem cells

FFQ food frequency questionnaire

H3 Histone 3

H4 Histone 4

HAT histone acetyltransferase

HPG Hypothalamus-Pituitary-Gonadal

IC1 Imprinting centre 1

ICSI Intracytoplasmic sperm injection

IGF-2 Insulin-like growth factor 1

IVF *In Vitro* fertilisation

LSI Leisure Index Scores

MTHFR Methylenetetrahydrofolate reductase

NaCl sodium chloride

NICE National institute for Clinical Excellence

ns no significance

OAT Oligoasthenoteratozoospermia

OS Operating System

PBS phosphate-buffered saline

PCR Polymerase Chain Reaction

pH potential Hydrogen

SAM	S-adenosylmethionine-cycle
SAMi	Semen Analysis with Machine intelligence
SCL	somatic cell lysis
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane hydrochloride
UK	United Kingdom
WHO	World Health Organisation

Acknowledgements

First and foremost, I would like to thank my Director of Studies, Dr Michael Carroll for the continual support, for pushing me out of my comfort zone and always being there no matter the issue at hand. I'm glad we finally got there. Secondly, my supervisory team, Dr Chris Murgatroyd and Dr Jane Sarginson, for helping me navigate through such a niche area.

I would like to thank Dr Stéphane Berneau, for teaching me pretty much everything I know in the lab (something which requires patience I'm sure), for explaining statistical analysis to me at 2am in a library trip that I'm sure we're all still recovering from. For being there, regardless of whatever I need, you are always there to assist and guide me, and for that I'm truly grateful.

I would love to thank (almost) Dr Mia Tomova, for welcoming me into the world of research, assisting without judgement of some of my more hilarious mishaps (we all start somewhere), for being incredibly selfless and supportive no matter what, thankyou wonderful friend, without you I honestly don't think I'd have made it!

A special thanks to Dr Steven Bradburn, for navigating a molecular biology novice like myself through what seemed like endless PCR optimisation woes – and dignifying what must be the longest email thread on PCR with responses. We so nearly got there!

To Dr Maha Khalid Abdulkareem Al-Gazi, for being a wonderful friend and introduction into methylation.

Abstract

Male fertility has been declining over the past 50 years, vindicating demand for further research into the aetiological agent(s) responsible for this reduction in sperm count and quality. It is suggested that modern lifestyle factors including diet & sedentary lifestyle are culpable. Veganism has gained recent popularity by those conscious of ethical food sourcing and the impact of the meat industry upon the climate. In addition to these environmental benefits, adoption of a vegan diet also confers health benefits. There have been associations between a plant-based diet and reduced risk of prediabetes and type 2 diabetes and reduced body mass index (BMI). Abnormal BMI is already well established to have negative effects on reproductive health. However, a lack of research into the effect of vegan diet on sperm methylation is yet to be explored.

Male volunteers aged 22-40 years of age were recruited from the Manchester area, in reply to adverts placed around the university campus and through social media. Participants provided semen samples via masturbation and completed a food frequency questionnaire including questions regarding lifestyle, diet and biometrics. Participants were separated into cohorts according to self-reported dietary subscription. Omnivorous (n=5) and vegan (n=5). Sperm progressive motility, concentration, total sperm count, volume, pH and vitality were all measured. Sperm cells were isolated from semen via density gradient centrifugation; DNA was extracted from the sperm and an ELISA-based assay to assess sperm global methylation status was performed.

Means of sperm parameters were compared via unpaired t-tests for the two dietary cohorts, as were means of the global methylation data for the two dietary cohorts. Biometrics & Lifestyle factors were evaluated as potential confounding factors via unpaired t-tests and Fisher's contingency test. None were significant and therefore did not need to be controlled for. All data collected were also pooled into a combined cohort, and correlational analyses and linear regression performed to identify any associations between sperm parameters and global methylation, biostatistics & lifestyle factors and global methylation, sperm parameters and biostatistics & lifestyle factors, and interactions between sperm parameters all within the combined cohort.

Statistically significant positive correlations were observed between combined cohort sperm progressive motility and concentration (r=0.7766, p=0.0082**) and

sperm motility and vitality (r=0.6535, p=0.0404*). No other statistically significant correlations were identified in the analysis of this study.

The key aim of this pilot study was to determine whether subscription to an omnivorous or vegan diet has any significant effect on the methylation signature and quality of human sperm DNA. No such relationship was demonstrated in the results of this study, with no significant differences in sperm parameters or sperm DNA global methylation between omnivorous and vegan diets. This research alone suggests that subscription to an omnivorous or vegan diet has no bearing on the quality or methylation level of sperm DNA. Moving forward, further research should be carried out within a larger population to confirm these findings.

1.0 Introduction

1.1 Male Factor Infertility

Recent reports on declining male fertility have emphasized a demand for further research into the aetiologies behind this reduction in sperm count and quality (Stephen *et al,* 2006; Levine *et al,* 2017; Sengupta *et al,* 2018). This decrease in male fertility is possibly attributed to modern lifestyle factors, such as diet, smoking tobacco and drinking alcohol, sedentary behaviour, and the rise in health conditions such as obesity and diabetes (Magnusdottir *et al,* 2005; Virtanen *et al,* 2017).

In their systematic review and meta-regression analysis, Levine and colleagues reported an annual percentage decrease in sperm counts of 1.4%, an overall decrease of 52.4% in sperm count from 1973-2011 in males from Western countries unselected by fertility status (Levine *et al,* 2017). Comparably, Sengupta *et al,* (2018) reported a 32.5% decrease in mean sperm concentrations of European males over a 50-year period.

Male factor infertility is a complex, multifactorial disorder, compartmentalised into congenital, acquired, and idiopathic factors, currently affecting approximately 7% of the male population; with as many as 2% displaying sub-optimal sperm parameters (Kumar and Singh, 2015; Krausz and Riera-Escamilla*,* 2018). Congenital factors include both primary and secondary hypogonadism such as anorchia (absence of one or both testes), cryptorchidism (where one or both testicles fail to descend) and genetic abnormalities such as Klinefelter's syndrome and Prader-Willi syndrome. Acquired factors include testicular torsion, varicocele, erectile dysfunction and use of anabolic steroids (Ferlin *et al,* 2007; Masson and Brannigan, 2014; Osta *et al,* 2016; Carroll, 2019).

Idiopathic infertility accounts for ~40% of male factor infertilities (MFI), and relates to the aetiology of the infertility remaining unidentified following a full work-up including semen analysis and investigation of the ovulatory system and fallopian tubes of the female (Krausz *et al,* 2018). In the case of idiopathic infertility, where current routine investigations are insufficient to identify the cause, it is suggested that the infertility may be attributed to molecular, genetic or epigenetic changes in the individual (O'Flynn-O'Brien *et al,* 2010).

Idiopathic infertility is diagnosed in 15-30% of couples (Quaas and Dokras, 2008). In some cases, aberrant epigenetics may be the cause. Aberrant histone

modifications have been described in patients with consistently poor embryo development in embryos derived from *In Vitro Fertilisation* (IVF) (Hammoud *et al,* 2011; Aston *et al,* 2012). Sperm selected in the IVF clinic for Intracytoplasmic Sperm Injection (ICSI) based upon normozoospermic parameters have been shown to have aberrant methylation status and subsequent imprinting disorders (section 1.3) (Lazaraviciute *et al,* 2014). The ICSI-conceived offspring had higher rates of congenital abnormalities, despite the normozoospermic classification of sperm selected (Lazaraviciute *et al,* 2014).

1.2 Diet & Fertility

There are increasing numbers of individuals committing to a vegan lifestyle with 1.16% (600,000) of the UK population currently identify as vegan – quadrupling since 2014 at 0.25% of the UK population (The Vegan Society, 2018). Furthermore, this increase in demand of vegan alternatives has led to the transition of veganism from a niche dietary trend into the mainstream, prompting subsequent investment into animal free-alternatives via the food industry; thereby creating wider availability of vegan food, allowing a 'domino effect' of vegan converts, numbers of which are projected to continue increasing (The Vegan Society, 2018).

A plethora of preventative and therapeutic health effects of a vegan versus a more traditional, non-restrictive omnivorous diet has garnered much attention in recent research. Chen *et al* (2018) demonstrated an association between subscription to a plant-based diet and reduced risk of prediabetes and type 2 diabetes, in addition to reduced longitudinal insulin resistance. Spencer *et al* (2003) demonstrated an association between reduced body mass index (BMI) and reduced obesity levels compared to diets inclusive of particularly meat. A vegan diet has also been shown to reduce levels of low-density-lipoprotein (LDL); a risk factor in the development of atherosclerotic plaques, when compared to an omnivorous cohort (p=0.034) (Vinagre *et al,* 2013).

However, there is a lack of research into the effect of vegan diet on male factor infertility. Chavarro *et al* (2008) investigated the effects of isoflavone phytoestrogens present in soya products; widely consumed as dairy alternatives amongst those following a vegan diet, upon semen parameters, the results demonstrated those with higher consumption of foods containing soya and soya isoflavones had $41x10^6$/ml less sperm than those who consumed no soya-containing foods. The

lower production of sperm is speculated to be in part, due to the phytoestrogen content, which may interfere in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis responsible for the control of reproductive functions including spermatogenesis in males (Cederroth *et al,* 2012).

Afeiche *et al* (2013) demonstrated that a diet consisting of dairy (absent from vegan diets) had a negative impact upon sperm morphology. 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 (significant at p=0.0022), in addition to a 21% reduction in litter size in the high-phytoestrogen diet group (Cederroth *et al,* 2010).

1.3 Epigenetics

In 1942, Conrad Waddington coined the phrase 'Epigenetics' ("epi"= in addition to); now an umbrella term for what is widely accepted as heritable variances in genome function and phenotype that occur in the absence of DNA sequence alterations.

Epigenetic mechanisms are responsible for necessary biological processes, such as X inactivation, development, and repression of transposable elements within the genome, providing its stability.

Imprinting is an epigenetic mechanism characterised in mammals, occurring in <1% mammalian genes (Wilkinson *et al,* 2007). However, imprinting has also been documented in Sciaridae and Coccidae (invertebrate arthropods) (Barlow and Bartolomei, 2014) and is controlled via methylation or histone modifications at nearby imprinting centres (IC). Imprinting limits the phenotypic expression of a gene to either the maternal or paternal allele (monoallelic expression) and is often referred to as 'parent-of-origin inheritance'. For example, insulin-like growth factor II (IGF-2) necessary in embryonic development, is expressed paternally, and controlled by the imprinting centre 1 (IC1) (Barlow and Bartolomei, 2014; Genetics Home Reference, 2019).

Loss of methylation/histone modifications at the IC1 responsible for the appropriate control of IGF-2 expression and subsequent bi-allelic expression has been implicated in cancers and Beckwith-Wiedemann syndrome; characterised by overgrowth (Genetics Home Reference, 2019).

Epigenetic modifications include post-translational histone modifications such as histone acetylation; the addition of a negatively charged acetyl group (CH_3CO) to a lysine residue on a histone tail, via action of histone acetyltransferase enzymes (HAT), thus relaxing the histone structure, allowing active transcription of euchromatin (transcriptionally active lightly packed chromatin) of which, 92% of the genome is composed (International Human Genome Sequencing Consortium, 2004). Histone de-acetyltransferase enzymes remove the acetyl group, thereby condensing the chromatin, rendering it transcriptionally inactive heterochromatin (transcriptionally inactive densely packed chromatin) (**Error! Not a valid bookmark self-reference.**).

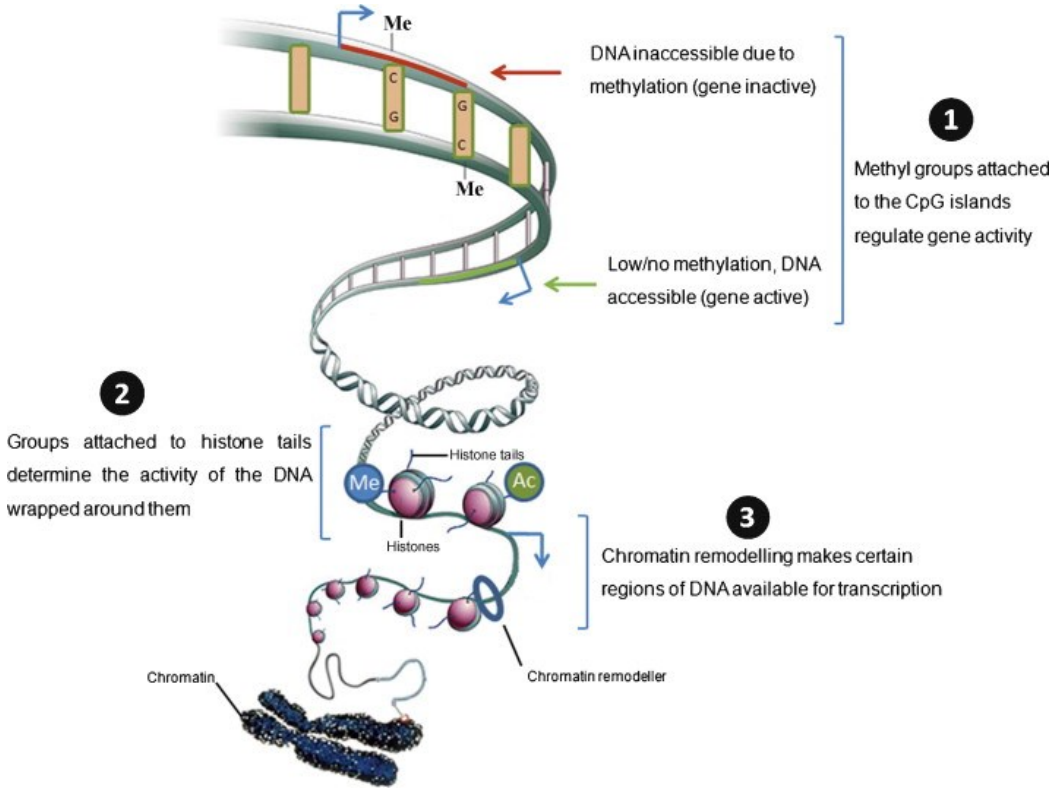

Figure 1. Summary of epigenetic mechanisms (Source: Rajender *et al,* 2011:64).

1.3.1 Methylation

Methylation occurs throughout the genome and is a vital process in ensuring tissue-specific expression/repression of appropriate genes. Methylation is also critical during embryonic development in ensuring appropriate imprinting (section 1.3).

Methylation of a 5'-cytosine-phosphate-guanine-3' site (CpG) (a cytosine-guanine dinucleotide in the 5' direction, separated by the phosphate backbone of the DNA

helical structure) via the addition of a methyl group (CH_3) to the 5th carbon in a cytosine pyrimidine ring, within a CpG site, forming 5-methylcytosine via covalent bonding through the action of the DNA-Methyltransferase enzyme family (DNMT). DNMT-3a and DNMT-3b control *de novo* methylation. DNMT-1 maintains these 5-methylcytosine patterns through subsequent cell divisions (Okano *et al,* 1999; Gujar *et al,* 2019).

Initiation of transcription occurs in promoter regions located at the 5' end of genes. Promotor regions are often rich in CpG dinucleotides and are subsequently termed CpG islands (CGI) (Khambata-Ford *et al,* 2003). Should methylation of a CpG site within the promoter region of a gene occur, the gene would become transcriptionally silent, or, 'off' (Jones, 2012).

Methylation is essential to life, as demonstrated by Li *et al* (1992) blastocyst-derived embryonic stem cells (ESC) without DNMTs are not viable past ~10 days' development.

The sum of deoxyribonucleic acid (DNA) methylation modifications within the genome is termed the methylome, which itself can display vast variation between cell types.

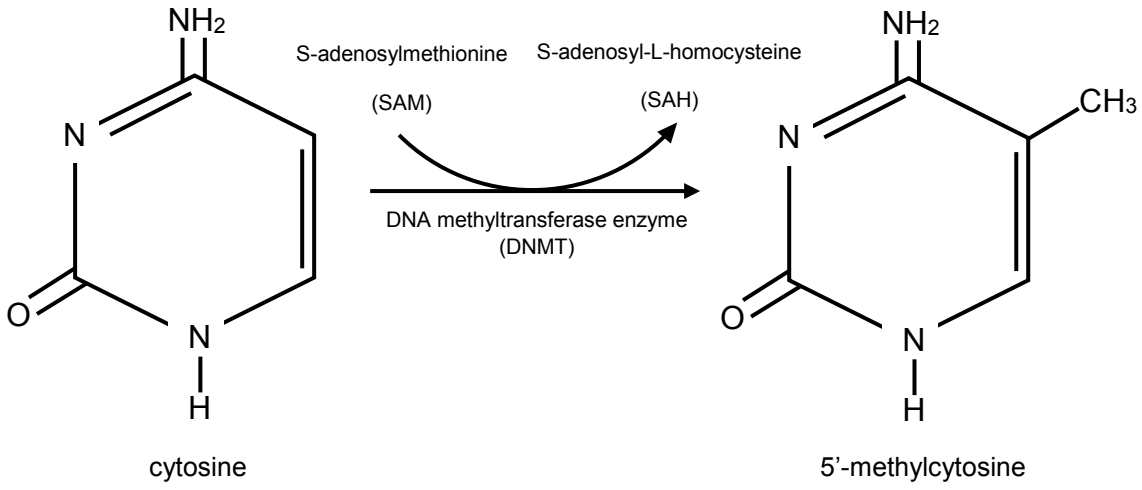

Figure 2. Schematic showing skeletal structure of cytosine and 5'-methylcytosine. S-adenosylmethionine acts as the methyl group donor, producing S-adenosyl-L-homocysteine as a by-product of the reaction. The reaction is catalysed by DNA methyltransferase enzymes. Adapted from source: Gibney and Nolan, 2010).

1.3.1.1 Methylation in Sperm

Sperm display a vastly different methylation signature to that of somatic cell DNA (therefore the methylome of the sperm only, is of interest in this particular research).

Sperm undergo transient epigenetic alterations throughout the stages of spermatogenesis (the production of sperm) including *de novo* methylation, demethylation and Histone 3 (H3) and Histone 4 (H4) (both implicated in chromatin structure) lysine residue acetylation and deacetylation. (Oakes *et al,* 2007; Stuppia *et al,* 2015).

It is important to note that mature sperm are a relatively hypermethylated cell type (Popp *et al,* 2010); approximately twice as methylated as the oocyte, with 40% and 89.4% global methylation respectively for the two cell types (Kobayashi *et al,* 2012).

Spermatogenesis occurs in the basal lamina of the seminiferous tubules in the testis, beginning at puberty and under 'normal' circumstances will continue throughout the male's adult life.

The process of spermatogenesis occurs over approximately 70 days (Kretser, 2000). Spermatogonia (primary cells) replicate via mitosis into primary spermatocytes, proceeding to meiosis I where crossover of homologous chromosomes occurs. At the conclusion of meiosis I these cells are known as spermatocytes and these divide again via meiosis II to produce spermatids (haploid round cells). Finally, spermatids undergo spermiogenesis, the process of specialisation of the round cell spermatid to a flagellated cell, where the development of the acrosome, tail and increased nuclear condensation occurs to produce elongated sperm. Though spermatogenesis is over at this stage these sperm are immotile and must further maturate in the epididymis (Gadea *et al,* 2013). The epididymis is responsible for the storage, maturation and transportation of the sperm produced in the testis to the vas deferens, whereby the mature sperm are prepared for ejaculation. This maturation and passage through the epididymis (~2 weeks) is where sperm are subjected to essential functional and morphological changes including the modification of chromatin. Sperm gain motility and the

capacity to fertilise an oocyte during this process (Sullivan and Mieusset, 2016; Zhou *et al,* 2018).

In terms of infertility, there is abundant evidence that epigenetics play a pivotal role. Many studies have linked disparities in methylation with aberrant sperm characteristics and infertile phenotypes (Montjean *et al,* 2015; Urdinguio *et al,* 2015).

However, there appears to be an inability within the field to agree whether abnormal sperm parameters are associated with hyper- or hypo- methylation.

Montjean *et al* (2015) found that oligozoospermic (0.2-15x10^6/ml) and asthenozoospermic (<20% progressively motile) males had significantly lower levels of mean global sperm methylation (hypomethylation) than normozoospermic males. Furthermore, those with elevated fragmentation (>30% sperm DNA denaturation index) also displayed significantly lower levels of mean global sperm methylation than those with 'normal' levels of sperm fragmentation.

Conversely, Houshdaran *et al* (2007) demonstrated significantly higher levels of methylation (hypermethylation) in sperm collected from males attending a fertility clinic.

Marques *et al* (2008) demonstrated aberrant methylation of imprinted genes H19 and MEST in individuals with sperm counts of <10x10^6/ml.

The methylome of sperm from a single ejaculate is highly variable. Jenkins *et al* (2014) investigated intra-individual methylation status of fractioned sperm. Following density gradient centrifugation, a technique used within the andrology lab to separate what are considered poorer sperm retained within the upper layer (35% density) from the bottom layer (90% density) containing high quality sperm (see section 2.5.3.2 for more on density gradient centrifugation). It was demonstrated that the poorer quality sperm exhibited a significantly more heterogenous methylation profile than the higher quality sperm (p=0.0083).

In addition to its variability, the sperm methylome responds to environmental factors. Denham *et al* (2015) demonstrated that global sperm methylation levels were modifiable by a 3-month period of exercise, compared to controls where global sperm DNA methylation levels remained constant (4.09%), the exercise group saw significant demethylation in sperm DNA (-6.63%) (p=0.006).

Low levels of sperm methylation (hypomethylation) have been implicated in poor assisted reproductive outcomes. Benchaib *et al* (2005) observed a disparity of

33.3% pregnancy rate for those with >555 arbitrary units of methylation (normal methylation) compared to just 8.3% pregnancy rate for those with <555 arbitrary units of methylation (hypomethylated). Interestingly, fertilization rate and quality of embryos were unaffected by methylation level of the sperm. This could be due to the intra-sample heterogeneity of the methylome. Bahreinian *et al* (2015) demonstrated a significantly lower mean percentage DNA methylation in men with varicocele compared with fertile individuals.

The number of genes involved in the careful coordination of spermatogenesis are perhaps somewhere in the thousands. However, these have not all been well characterised.

In terms of individual genes, deleted in azoospermia-like (DAZL) is a gene within the deleted in azoospermia (DAZ) gene cluster located on the Y chromosome. DAZL is an important candidate gene for methylation studies as its role is essential to spermatogenesis and is subsequently hypomethylated in healthy individuals. Any hypermethylation of DAZL would suggest poor spermatogenesis leading to azoospermia (low/absent sperm in ejaculate) (Zhang *et al*, 2016).

DAZL encodes an RNA binding protein essential to spermatocyte meiosis, a key step in spermatogenesis (Niu *et al,* 2014).

Aberrant DAZL methylation has been observed in oligoasthenoteratozoospermic (OAT) individuals (Navarro-Costa *et al,* 2010). Loss of genes within the DAZ cluster results in azoospermia and oligospermia (Reijo *et al,* 1995). Furthermore, those with azoospermia possess fewer transcripts of the DAZL gene within the testis in contrast to normozoospermic individuals (Lin *et al,* 2001).

Methylenetetrahydrofolate reductase (MTHFR) codes for an enzyme of the same name, essential to folate (vitamin B9) metabolism by conversion of 5,10-methylenetetrahydrofolate reductase to 5-methyltetrahydrofolate. Part of the process of the regeneration of homocysteine to methionine – an amino acid crucial in the production of S-adenosyl-methionine – the methyl group donor critical to the methylation of DNA (Genetics Home Reference, 2017). Any disruption of the regulation of MTHFR can have downstream implications and subsequently result in the hypomethylation of DNA.

Wu *et al* (2010) reported MTHFR promotor region hypermethylation in the sperm of 41 out of 94 men with idiopathic infertility. Similarly, Karaca *et al* (2017) reported an association between MTHFR promotor region hypermethylation and increased risk

of infertility. This suggests that MTHFR plays an important role in spermatogenesis and is an important candidate gene in the investigation of idiopathic infertility.

1.3.2 Dietary Compounds Involved in the Control of Methylation

1.3.2.1 Vitamin B12 (Cobalamin)

Vitamin B12 is a crucial nutrient, aiding in many biochemical processes such as erythropoiesis, DNA synthesis, synthesis of myelin, and is critical in the SAM cycle (S-adenosylmethionine-cycle) as a co-factor for the re-methylation of homocysteine to methionine which then combines with adenosine triphosphate to form S-adenosylmethionine; the methyl group donor involved in the methylation process (Figure 3).

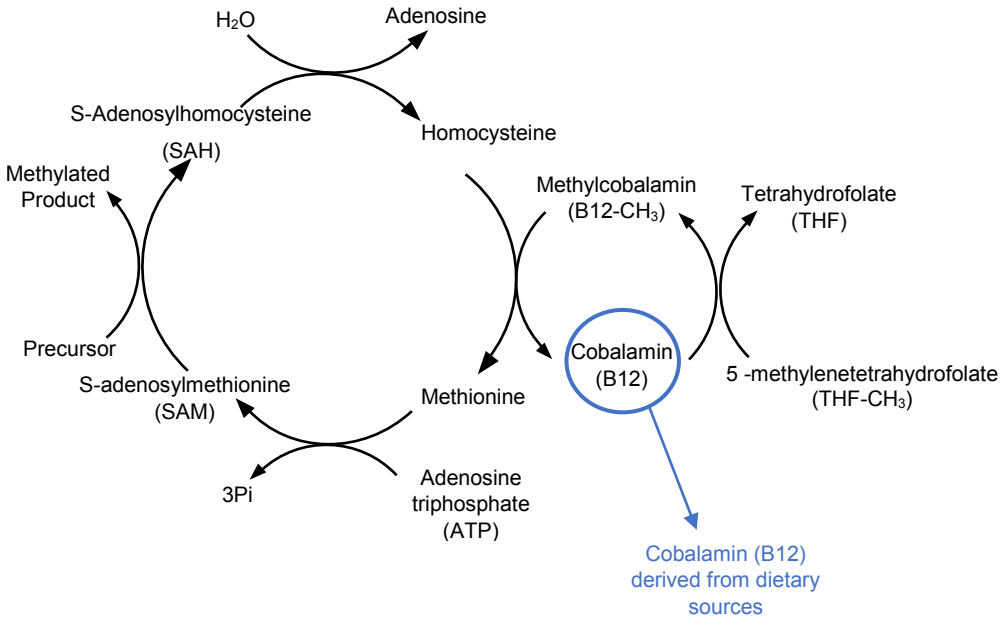

Figure 3 Schematic showing the S-adenosylmethionine cycle, and the role of dietary cobalamin (B12). Adapted from (Source: BioCat, No Date:Online).

Therefore, a deficiency in the vitamin can lead to hyperhomocysteinemia (high homocysteine levels) due to the lack of methionine available to bind. Hyperhomocysteinemia can have a plethora of health implications as an independent risk factor in the development of disease, including Alzheimer's and

cardiovascular disease, in addition to global hypomethylation (Morris, 2003; Ganguly and Alam, 2015).

Individuals adhering to a strict vegan diet often become deficient in B12 due to the predominantly animal-based nature food sources high in the vitamin. The average vegan diet provides less than the average daily requirement of B12, compared to the average omnivorous diet which provides approximately double the average recommended daily intake of 2.4µg (average vegan B12 intake of 0.0 ± 0.1µg for females, and 0.1 ± 0.03µg for males compared to average omnivore B12 intake of 5.0 ± 2.5µg for females, and 5.9 ± 1.5µg for males) (Larsson and Johansson, 2002; The National Institutes of Health Office of Dietary Supplements, 2018).

Table 1 Selected food sources of vitamin B12 with micrograms per arbitrary serving and subsequent percentage of daily recommended intake. The two items suitable for those subscribing to a vegan diet are highlighted in yellow. Foods suitable for a lacto-ovo vegetarian diet are highlighted green. All foods listed are suitable for the typical omnivorous diet. (The National Institutes of Health Office of Dietary Supplements, 2018).

Food	**Micrograms (µg) per serving of B12**	**Percent DV***
Clams, cooked, 3 ounces	84.1	1,402
Liver, beef, cooked, 3 ounces	70.7	1,178
Breakfast cereals, fortified with 100% of the DV for vitamin B12, 1 serving	6.0	100
Trout, rainbow, wild, cooked, 3 ounces	5.4	90
Salmon, sockeye, cooked, 3 ounces	4.8	80
Trout, rainbow, farmed, cooked, 3 ounces	3.5	58
Tuna fish, light, canned in water, 3 ounces	2.5	42
Cheeseburger, double patty and bun, 1 sandwich	2.1	35
Haddock, cooked, 3 ounces	1.8	30
Breakfast cereals, fortified with 25% of the DV for vitamin B12, 1 serving	1.5	25
Beef, top sirloin, broiled, 3 ounces	1.4	23
Milk, low-fat, 1 cup	1.2	18
Yogurt, fruit, low-fat, 8 ounces	1.1	18
Cheese, Swiss, 1 ounce	0.9	15
Beef taco, 1 soft taco	0.9	15
Ham, cured, roasted, 3 ounces	0.6	10
Egg, whole, hard boiled, 1 large	0.6	10
Chicken, breast meat, roasted, 3 ounces	0.3	5

Of the foods listed 2 out of the 18 (both cereals fortified with varying % daily recommended intake of vitamin B12) are suitable for a vegan diet (yellow) in stark contrast to the wide range of high-B12 foods available to omnivores and vegetarians who consume animal products such as eggs and milk (green) (See Table 1).

Due to this primarily animal-based nature of this vitamin, it is difficult to obtain sufficient B12 through plant based diet alone.

Boxmeer *et al* (2013) demonstrated a significant positive correlation between B12 seminal plasma concentration and sperm concentration in a cohort undergoing IVF/ICSI, in addition to an association between low seminal folate levels and DNA damage in sperm from fertile males.

1.3.2.2 Zinc

Zinc is a co-factor essential to the action of DNA Methyltransferase-1 (DNMT-1) enzymes (responsible for the addition of methyl groups to cytosine residues in predominantly CpG dinucleotides by covalent bond to the 5th carbon atom in the pyrimidine ring). Common dietary sources of zinc include; red meats, seafood, dairy, fortified cereals, beans and nuts. However, many plant based sources of this mineral also contain phytates which inhibit zinc absorption, subsequently diminishing its' bioavailability; therefore, many vegan and vegetarian diets may be lacking in this mineral/unable to absorb it, and may show subsequent hypomethylation (Wise, 1995).

In addition to its' role in the support of DNMT-1 enzyme function, zinc has been implicated in maintenance of clinically 'normal' sperm parameters. Zhao *et al (*2016) demonstrated a positive correlation between zinc supplementation and percentage morphologically 'normal' sperm forms, motility, and increased semen volume.

1.4 Aims & Objectives

The overall aim of this research is to investigate the effects of vegan and omnivorous diets respectively on sperm parameters and the methylation status of sperm DNA.

This will be executed via the following objectives:

1. Investigate any differences in semen parameters between omnivorous and vegan cohorts.
2. Quantify total sperm DNA 5-methylcytosine levels (a marker of global methylation) via colorimetric Enzyme-Linked-Immunosorbent-Assay (ELISA) and investigate any differences in global methylation between omnivorous and vegan cohorts.
3. Investigate any effect between lifestyle/biometrics and semen parameters.
4. Investigate any interactions between semen parameters.

The null hypothesis (H_0): Dietary subscription has no significant effect on the methylation signature and quality of human sperm DNA.

The alternate hypothesis (H_1): Dietary subscription has a significant effect on the methylation signature and quality of human sperm DNA.

2.0 Materials and Methods

Table 2 Materials used.

Equipment	
-80°C Freezer	Eppendorf CryoCube™F570
Agarose Gel Electrophoresis Tank	Bio-Rad SubCell® GT Cell
Agarose Gel Imager	Bio-Rad ChemiDoc™ Touch Imaging System
Brightfield Microscope Imager	Zeiss Axio Imager M1
CASA Slides, 2 Chamber 20µm	CellVision Technologies
Cell Culture Incubator	NUAIRE™ Autoflow Direct Heat CO_2 Incubator
Centrifuge	Sigma 3-16KL Refrigerated Centrifuge
Class II Laminar Flow Cabinet	NUAIRE™ Biological Safety Cabinet
Cryogenic Storage Vials	Greiner Bio-One™ Cryo.s™ Conical Bottom 1mL Polypropylene Tubes with Internal Thread Cap
Falcon Centrifuge Tubes 15 & 50ml	Corning™Falcon™ 15ml/ 50ml Conical Centrifuge Tubes
Flat Cap PCR Tubes 0.2ml	STARLAB
Light Microscope	Leica DM500 Light Microscope
Microcentrifuge	Labnet Prism Air-Cooled Microcentrifuge
Neubauer Improved Haemocytometer Rhodium Coated	Hawksley Medical and Laboratory Equipment
Pasteur Pipettes	SLS Select PIP4204 Polyethylene Pasteur Pipette, Individually Wrapped, Sterile, 1 mL
pH Electrode	Mettler-Toledo InLab® Micro
pH Meter	Mettler Toledo™ FE20 FiveEasy™ Benchtop pH Meter
Pipette Tips	STARLAB TipOne® Pipette Tips
Pipettes	Gilson
Plate Reader	Synergy HT plate reader, BioTek® Instruments
Power Supply for Gel Electrophoresis	Bio-Rad PowerPac™ Basic Power Supply
Pyrosequencer	Qiagen PyroMark Q24 Pyrosequencer
Sample Collection Pots	Sterilin UK
Shaking Incubator	Labnet AccuTherm™ Microtube Shaking Incubator
Slides	ThermoScientific™ SuperFrost™ Plus
Spectrophotometer	ThermoScientific NanoDropOne
SAMi	Pro-Creative Diagnostics
Thermal Cycler	Agilent Technologies SureCycler 8800
Vortexer	SLS Lab Basics Vortexer

Consumables, Chemicals and Reagents	
25bp Ladder	Bioline Hyperladder™ 25bp
5-methylcytosine Detection Kit	Epigentek MethylFlash™ Methylated DNA Quantification Kit (Colorimetric)
Agarose	Bio-Rad Certified™ Molecular Biology Agarose
Bisulphite Conversion Kit	Qiagen EpiTect Bisulphite Conversion Kit
Boric Acid	Fisher-Scientific
Chloroform	VWR Chemicals
Eosin-Y Colour	Sigma-Aldrich
Ethanol	Fisher Scientific
Ethylenediaminetetraacetic acid	Fisher Scientific
Isoamyl Alcohol	Sigma-Aldrich
Isopropanol	Fisher Scientific
Nigrosin	Sigma-Aldrich
PCR Kit	Qiagen Pyromark PCR Kit
Phenol	Sigma-Aldrich
Predesigned Primers	Qiagen CpG Assay
Pyrosequencing Kit	Qiagen PyroMark Gold Q24 Reagents
Sodium Chloride	Sigma-Aldrich
Sodium Dodecyl Sulphate	Sigma-Aldrich
Tris(hydroxymethyl)aminomethane hydrochloride	Sigma-Aldrich
Colloidal Suspension for Density Gradient	Nidacon PureSperm 100
Phosphate-Buffered-Saline	Invitrogen™ by ThermoFisher Scientific
Water for Molecular Biology	Acros Organics
Software	
CASA Data Capture	SAMi, Procreative Diagnostics
Plate Reader Data Capture	Gen5™ software version 2.05.5
Data Processing	Microsoft® Excel for Mac Version 16.18
Statistical Analysis	GraphPad Prism® 7.0e for Mac OS X

2.1 Participant Recruitment and Sample Procurement

Semen samples were obtained from consenting donors aged 22-40, covered by faculty ethical approval at Manchester Metropolitan University (EthOS Reference Number: 0482; SE1617127)(Ethical approval in Appendix 7.2). Donors were verbally informed of the study details and given a participant information sheet with details of the study (Appendix 7.3). Participants consented (Appendix 7.4) and completed a medical screening questionnaire upon their first visit (Appendix 7.5). All participants were self-reported non-smokers per the food frequency questionnaire (FFQ) (Appendix 7.6), and were selected based upon exhibition of normozoospermic parameters including; total sperm count, concentration, progressive motility and vitality according to World Health Organisation (WHO) reference ranges (WHO, 2010).

Each participant completed the food frequency questionnaire on the day of donation, detailing the 3 months' prior diet and biometrics (age, height, weight).

Food consumption frequency was measured on a 7- point descriptive scale. 0- Never, 1-Less than once a month, 2- 1-2 times a month, 3- Once a week, 4- 2-3 times a week, 5- 4-6 times a week, 6 – Every day.

Participants were assigned to one of two groups according to their self-reported diet (vegan, omnivorous) (5 participants per dietary group). All participants had followed their current diet for a minimum of 3 months (1 cycle of spermatogenesis ~70 days) (Kretser, 2000) prior to donation. Participants were pseudonymised by the following system: VGANXX/ OMNIXX for example; VGAN01, OMNI01, VGAN02, OMNI02 etc.

All semen samples were produced on site at Manchester Metropolitan in a designated, private room for sperm donation. Participants were verbally provided with information on how to produce the sample and given a sample collection pot (Sterilin, UK).

All neat semen samples underwent standard semen analysis according to the WHO 5th edition manual for the examination and processing of human semen before processing. Semen was allowed for liquefication at 37°C for 30 minutes in a direct heat 5% CO_2 incubator. The total volume of semen was measured and recorded. Semen pH was measured immediately after the 30 minute liquefaction period using a pH meter & probe (Mettler – Toledo).

2.2 Sperm Motility and Concentration

Sperm motility and concentration was assessed using Computer Assisted Sperm Analyser (CASA – Sami®, Procreative). Briefly, 5 µl of semen was applied to a cell counting slide (Vitrolife, UK) and analysed by CASA. Using a heated stage (37.5°C) concentration, speed, and percentage motility (A= progressively motile B= Non-linear motility C= progressively immotile D=Immotile) were calculated by software (SAMi®, Procreative Diagnostics) and noted in addition to the presence of debris, round cells and agglutination. Samples were snap frozen in liquid nitrogen and stored at -80°C until required.

2.3 Vitality Assay

Eosin-nigrosin stain was prepared per the WHO laboratory manual for the examination and processing of human semen (5th Edition, 2010) by dissolving 0.67g of eosin Y and 0.9g of NaCl in 100ml purified water with a magnetic stirrer. Nigrosin (Sigma-Aldrich) (10g) was added to the solution, which was heated to 60°C then left to cool to room temperature, and filtered to remove impurities. The resulting suspension was stored at room temperature in a sealed dark glass bottle.

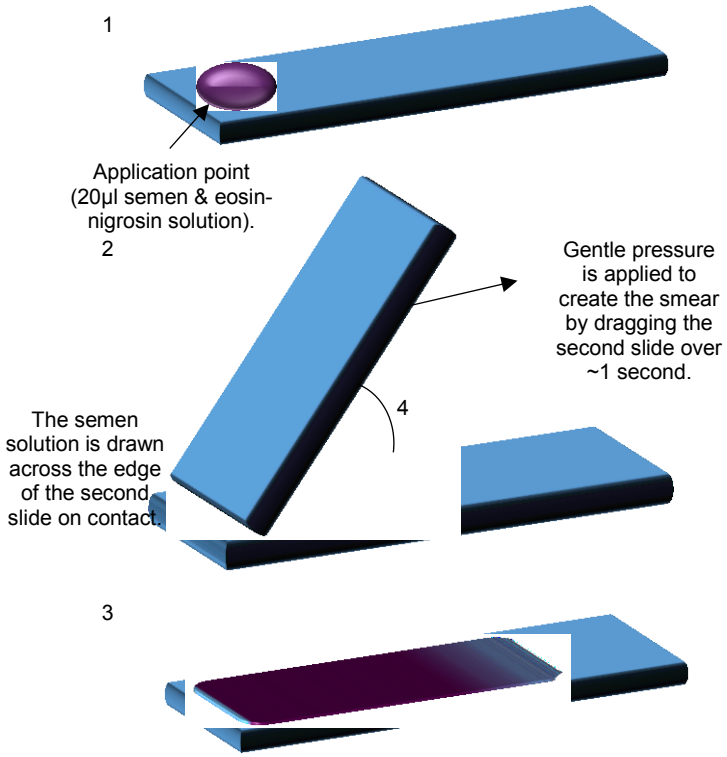

Figure 4 Schematic demonstration of the feathering technique used for vitality assay. (1) Application. (2) Direction of drag used to create smear. (3) Completed vitality slide left to air dry.

Vitality slides were produced in duplicate using eosin-nigrosin dye-exclusion assay. A total of 20µl semen and 20µl eosin-nigrosin stain (10µl/10µl per slide) was mixed using a pipette to ensure homogeneity in 1.5ml Eppendorfs, and the stain produced by the feathering technique recommended by WHO (WHO, 2010) (Figure 4). Slides were examined using brightfield optics at x1000 magnification with oil immersion. A live-dead percentage score was obtained from 200 cells per slide (each slide counted in duplicate) obtaining an overall average live-dead score per participant. Dead sperm stain dark pink due to the influx of dye, live sperm remain white due to their intact membrane (Figure 5).

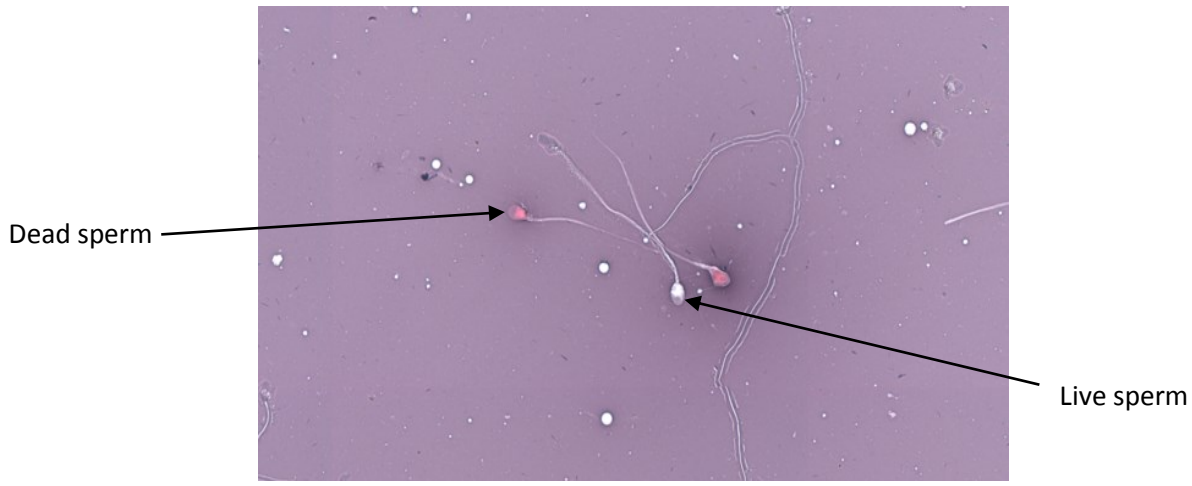

Figure 5 Eosin-Nigrosin stain showing 2 dead sperm with permeabilised membranes (stained dark) and one live sperm with intact membrane (centre) (white, no stain). Taken using Zeiss Axio imager M1 at 63x/1.4 objective with oil immersion (Zeiss).

2.4 Sperm Preparation

2.4.1 Separation of Sperm from Seminal Plasma

Sperm were isolated from the seminal plasma via centrifugation at 300g for 10 minutes. The supernatant will be divided into 0.5ml Eppendorfs and snap-frozen in liquid nitrogen prior to transfer to a -80°C freezer pending further analysis. The resulting sperm pellet will be transferred to a cryogenic vial (Greiner BioOne™) snap-frozen and stored at 80°C.

2.4.2 Separation of Germ and Somatic cells

In order to ensure sperm samples were free of somatic (round) cell contamination – methods of somatic cell eradication was explored. This was important for downstream sperm DNA methylation analysis.

'Round cells' is an umbrella term referring to the presence of somatic cells such as epithelial and white blood cells in the semen, in addition to immature germ cells (spermatids), all of which are commonly found in the clinically 'normal' ejaculate. The most predominant round cell type in the ejaculate is the leukocyte. The World

Health Organisation (WHO) defines leukocytospermia as $1x10^6$ /ml leukocytes in the ejaculate (WHO, 2010).

Both Density Gradient Centrifugation (DGC) and somatic cell lysis detergents were assessed.

2.4.3 Sperm Isolation Techniques

In recent years, there has been a drive for researchers to develop and diversify sperm isolation techniques in order to accommodate and reflect the vast range of infertility aetiologies presenting in the clinic. A patient presenting with oligozoospermia or cryptozoospermia would not be a suitable candidate for procedures based upon migration such as swim-up or migration-sedimentation due to the low yields obtained through these methods. In this case, separation techniques such as density gradient centrifugation or glass-wool filtration would be a more beneficial method of isolation, delivering a higher yield.

Behind this drive is the shift from the antiquated belief that, infertility is a predominantly female issue. Male factors are the exclusive cause of infertility in 20-30% of cases, furthermore; male factors contribute alongside female factors to 50% of cases (Agarwal *et al,* 2015).

As participants were not recruited from an IVF clinic and were selected based upon samples meeting the WHO semen characteristics reference range, it is known that they are normozoospermic samples.

Density gradient centrifugation and somatic cell lysis methods were assessed as these are suitable for normozoospermic samples, provide high yields, and are among the most popular techniques documented in recent literature.

2.5.3.1 Initial Sample Preparation

Following liquefaction, the fresh sample was centrifuged at 300g for 10 minutes in order to artificially concentrate the sperm & round cell populations (all centrifugation steps are performed at ambient temperature unless stated otherwise). The pellet was then re-suspended in 2ml phosphate-buffered-saline (PBS) pre-warmed at 37°C.

Baseline sperm count and round cell count was assessed via a haemocytometer count according to WHO procedures (WHO, 2010).

The remainder of the sample was divided equally into 2x clean centrifuge tubes (Corning™Falcon™), pending exposure to the 2 sperm isolation techniques. Results of these techniques are included in the appendix (Appendix 7.1).

2.5.3.2 Density Gradient Centrifugation

A discontinuous, two-step gradient was used (Figure 6). Silane-coated silica particles in differing concentrations suspended in PBS provide the density of both media (PureSperm 100 (Nidacon) was diluted appropriately to obtain the 40% and 80% suspensions used). The principal of density gradient centrifugation (DGC) is that progressively motile sperm are capable of crossing the interphase between the two media faster, travelling through the denser media (lower phase, 80%) in the direction of the centrifugal force. Progressively immotile, immotile and decapitated sperm, round cells and debris are maintained within the lighter (upper phase, 40%) of the gradient (Figure 7).

This upper phase was discarded, leaving the lower phase containing a pure population of progressively motile sperm.

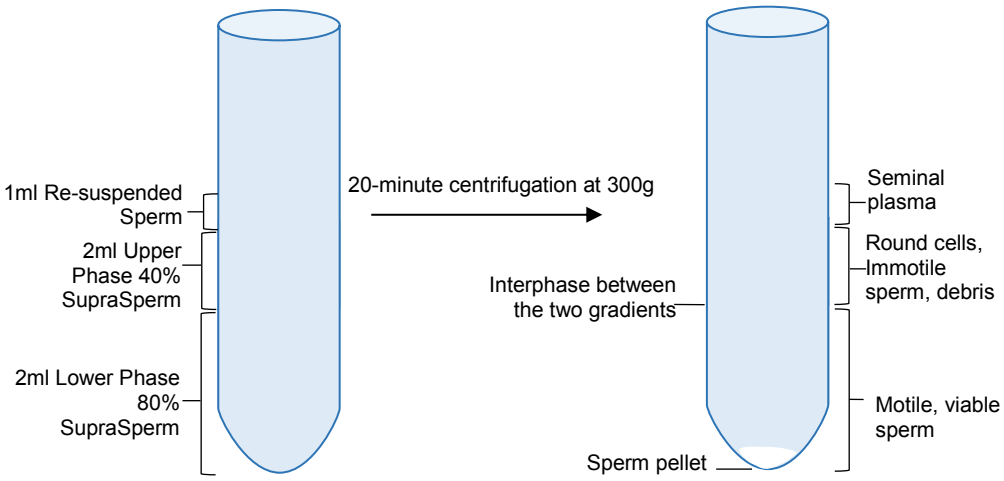

Figure 6 Schematic showing the principle of density gradient centrifugation. A two-step discontinuous gradient was used.

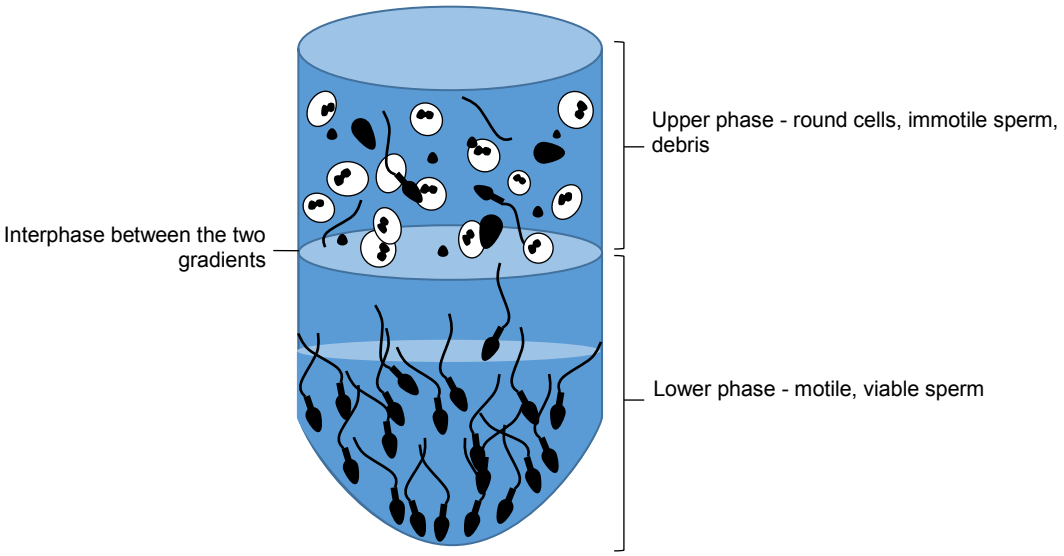

Figure 7 Schematic demonstrating the cellular components and separation post- density gradient separation.

Density gradient centrifugation was performed on one remaining aliquot of washed sperm, using the PureSperm system (Nidacon). PureSperm 40% and PureSperm 80% were pre-warmed at 37°C in a water bath prior to gradient preparation. In a 15ml Falcon tube, 2ml PureSperm 40% was underlaid with 2ml 80% PureSperm. Re-suspended sperm (1ml) was pipetted to ensure homogeneity before being carefully dispensed on top of the prepared gradient using a Pasteur pipette; being sure not to disrupt the layers. At this point, clear definition between the layers should be visible (Figure 6). After centrifugation at 300g for 20 minutes, the supernatant was discarded and the pellet transferred to a clean 15ml Falcon tube, re-suspended in 2ml pre-warmed PBS and centrifuged at 300g for 10 minutes. This washing step was repeated and the resulting pellet re-suspended in 2ml pre-warmed PBS.

To ensure sperm samples were free from round cell contamination prior to DNA extraction – both density gradient and somatic cell lysis (SCL) was carried out (see Appendix 7.1 for SCL method).

Density gradient centrifugation was found to be more effective in eliminating round/somatic cells.

2.5.3.3 Application of DGC to Experimental Samples – Optimisation

It was observed that no cellular components of any kind formed a pellet, nor did they migrate past the interphase of the traditional two-step discontinuous gradient.

All sperm were retained at the interphase, within the upper 40% suspension. This was presumably due to the sperm's immotility post-thaw.

Therefore, the method for post-thaw density gradient centrifugation was modified (Figure 8) to a simple one-step gradient using only 40% PureSperm, overlaid with the thawed pellet (reconstituted in an equivolume of PBS).

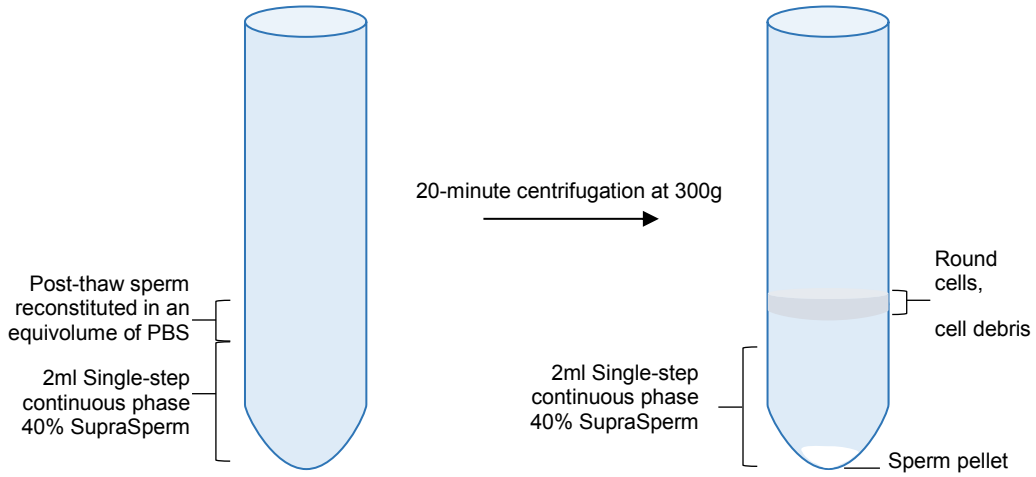

Figure 8 Density gradient centrifugation modified to a one step continuous gradient consisting of 40% PureSperm media.

Following a 20 minute centrifugation at 300g, the supernatant containing round cells and debris (Figure 8) was aspirated and discarded in addition to the media, leaving the sperm pellet. The pellet was then washed in 2ml PBS and centrifuged at 500g for 5 minutes. Aliquots (12.5µl) were taken from each sample at this point, and added to 50µl PFA fixative, and a haemocytometer count taken to establish whether each sample contained the minimum cellular threshold for a suitable DNA yield for later analysis. Total sperm count at initial semen processing and total sperm count post-density gradient centrifugation are displayed in Table 3.

The supernatant was discarded and the pellet transferred to a cryogenic vial (Greiner BioOne™) prior to flash-freezing in liquid nitrogen. The pellets were then returned to a -80°C freezer pending further processing.

Table 3 Total sperm count at initial semen processing ($x10^6$) and post-density gradient centrifugation, analysed by computer assisted sperm analysis for omnivorous and vegan diets.

Sample	Total Sample Sperm Count at Initial Semen Processing ($x10^6$)	Total Sperm Count Post-Density Gradient Centrifugation ($x10^6$)
VGAN01	252.48	76.75
VGAN02	223.42	13.6
VGAN03	71.65	28.5
VGAN09	397.34	85
VGAN10	419.98	81.75
OMNI02	562.41	30
OMNI05	231.93	31
OMNI08	125.19	14.6
OMNI09	122.48	30
OMNI11	564.68	65.5

2.5 DNA Extraction & Purification

Phenol-chloroform method of DNA extraction was optimised for maximum recovery of sperm DNA. Optimisation was necessary to obtain the correct yield of DNA consistently per sample, in order to satisfy minimum working concentrations of DNA necessary for the subsequent global methylation assay (a minimum working concentration of 50ng genomic DNA per sample).

Semen samples were also procured for this purpose per section 2.1.

Phenol-Chloroform method of DNA extraction was selected due to its high yield and popularity in literature.

Sperm pellets were removed from -80°C storage and allowed to thaw at ambient temperature prior to transfer to sterile 1.5ml Eppendorfs. 500µl lysis buffer containing 10% SDS (sodium dodecyl sulphate), 0.5M EDTA (Ethylenediaminetetraacetic acid), 1M Tris(Tris(hydroxymethyl)aminomethane hydrochloride) (pH 8) and 5M NaCl (sodium chloride was added, in addition to 25µl proteinase K. Eppendorfs were vortexed and incubated at 50°C for 20 minutes with agitation.

Following incubation, 500µl 25:24:1 phenol:chloroform:isoamyl alcohol was added, and the samples vortexed prior to centrifugation at 12,000g, 4°C for 15 minutes.

Being sure not to disrupt the bottom layer, the supernatant was transferred to new Eppendorfs, and an equivolume of chloroform added, vortexed and centrifuged at 12,000g, 4°C for 5 minutes. Supernatant was transferred to new Eppendorf, and an equivolume of cold isopropanol added prior to inversion by hand and centrifugation at 12,000g, 4°C for 10 minutes. Following centrifugation, the supernatant was discarded, and 500µl 70% cold ethanol was added, the tubes inverted by hand, and centrifuged at 12,000g, 4°C for 5 minutes. The supernatant was discarded and the step repeated by addition of 500µl 70% cold ethanol, inversion and centrifugation at 12,000g, 4°C for 5 minutes. The supernatant was discarded and any remaining ethanol removed using a p10 pipette, with all samples pulse-spun in a microcentrifuge (Labnet) to remove any residual ethanol. Tubes were allowed to air dry for 1 minute under the fume hood and eluted with 100µl TE buffer prior to DNA quantification using a nanodrop spectrophotometer. (See Figure 9 for a schematic overview of the 5 main steps in phenol-chloroform extraction).

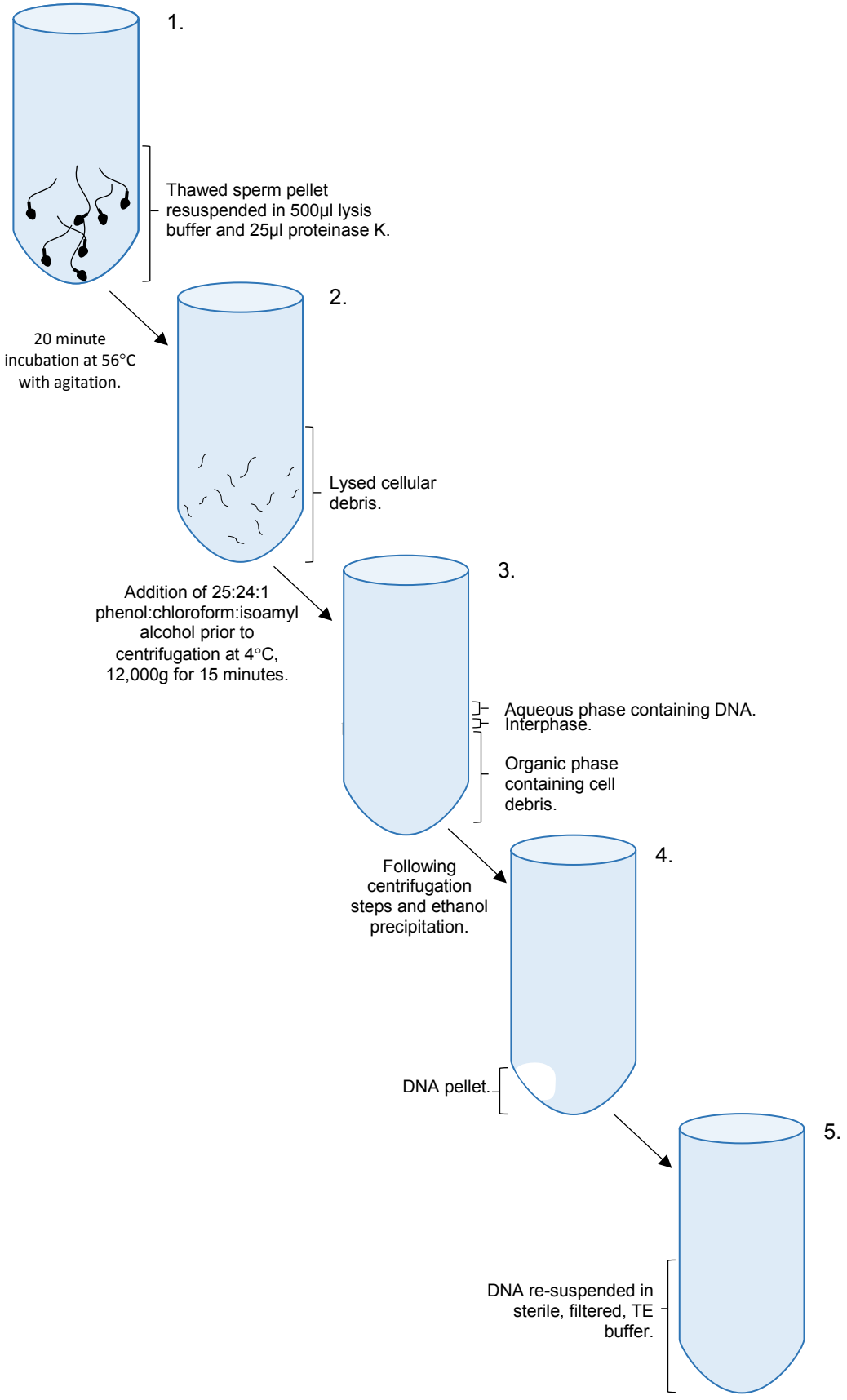

Figure 9 Schematic of the main 5 steps of phenol chloroform extraction and ethanol precipitation.

DNA was quantified using NanoDropOne (ThermoFisher). The nanodrop was blanked using 1µl TE buffer (10mM Tris-HCl 1mM EDTA Na_2, pH 8) prior to testing each sample using 1µl volumes. Quantity (ng/µl) and purity (A260/A280, A260/A280) of DNA recovered per sperm sample is displayed in Table 4 below.

Samples were then stored at -20°C until further use.

Table 4 DNA quantities (ng/µl) and purity ratios at A260/A280 and A260/A230 per sample using NanoDropOne spectrophotometer (Thermoscientific).

Sample	DNA (ng/µl)	Purity A260/A280	Purity A260/A230
VGAN01	28.8	1.85	2.16
VGAN02	3.6	1.76	1.79
VGAN03	10.0	1.61	1.61
VGAN09	53.2	1.77	1.03
VGAN10	7.1	1.53	1.75
OMNI02	36.3	1.83	2.26
OMNI05	11.2	1.89	1.42
OMNI08	13.2	1.66	1.08
OMNI09	10.5	1.74	1.37
OMNI11	19.0	1.88	1.49

2.6 Quantification of Global Methylation Levels

Epigentek MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric) (Epigentek Group Inc) was used to quantify levels of 5-methylcytosine% and thereby quantify global methylation per sample (Vryer and Saffery, 2017).

Recommended genomic DNA input was 50ng-200ng, 50ng was used due to the limitations of sample size. This allowed the experiment to be run in duplicate. Experimental setup is shown in Figure 10.

2.6.1 DNA Binding

Binding solution (80µl) was added to each of the 24 wells using a multi-channel pipette. Negative control (1µl), positive control (1µl) and varying volumes all constituting 50ng genomic DNA from each sample, equilibrated to 8µl with nuclease free water were added in duplicate to corresponding wells shown in Figure 10. The

plate was then covered using plate seal, and incubated at 37°C for 90 minutes to facilitate DNA binding.

2.6.2 Methylated DNA Capture

The solution was removed from all wells, which were then washed 3 times with 150µl wash buffer, which was then discarded from the wells. Diluted capture antibody (50µl) was then added to each well, and the plate covered and incubated at room temperature for 60 minutes. Capture antibody was then discarded, and all wells washed 3 times using 150µl wash buffer. Diluted detection antibody (50µl) was added to each well, and the plate covered and incubated at room temperature for 30 minutes. Detection antibody was then discarded and each well washed 4 times using 150µl wash buffer. Enhancer solution (50µl) was then added to each well, and the plate covered and incubated at room temperature for 30 minutes. Enhancer solution was then discarded and each well washed 5 times using 150µl wash buffer.

2.6.3 Signal Detection

Developer solution (100µl) was added to each well, and the plate incubated in the absence of light at room temperature for 12 minutes. Stop solution (100µl) was added to each well to stop the enzyme reaction. Absorbance was measured at 450nm after 2 minutes as suggested by the manufacturer's guidelines.

Synergy HT plate reader was used (BioTek® Instruments) was used to read the strip-well plate, and Gen5™ software version 2.05.5 used for data capture.

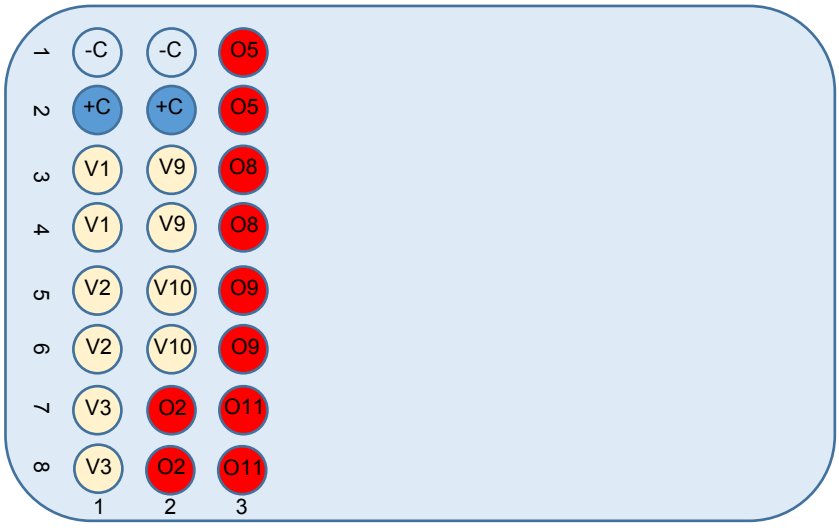

Figure 10 Schematic Representation of the strip-well plate used, with duplicate negative & positive controls, and samples.

2.6.4 Relative Quantification of 5-methylcytosine

Relative quantification using single point control to determine the relative methylation status of the omnivorous and vegan cohorts was carried out. The equation below, provided by the manufacturer (Epigentek) was used to quantify the percentage of 5-methylcytosine% in the total sperm DNA of each sample:

$$5 - Mc\% = \frac{(Sample\ OD_{450} - Negative\ Control\ OD_{450}) \div S}{(Positive\ Control\ OD_{450} - Negative\ Control\ OD_{450})\ x\ 2 \div P}\ x\ 100\%$$

Where:

S is the amount of sample DNA input in ng

P is the amount of positive control input in ng

2.7 Data Collection & Statistical Analysis

Microsoft® Excel for Mac Version 16.18 was used for all data collection and processing. GraphPad Prism® 7.0e for Mac OS X was used for all statistical analysis. The Shapiro-Wilk test for normality was used to identify Gaussian distribution of all data sets and identify subsequent appropriate tests. Potential confounding factors including age, BMI and alcohol intake were all tested for normality. Age was the only factor that was not normally distributed and was therefore tested for any correlations with all other datasets. No associations were found, therefore there was no need to control for age as a confounding factor.

Unpaired t-tests were used to calculate any significance in sperm parameters between the two dietary groups, except pH data which was not normally distributed; where a Mann-Whitney test was used.

Associations between various parameters (with datasets from both dietary groups combined as a whole) were calculated.

Correlation for nonparametric data (global methylation) was calculated via Spearman correlation and linear regression analyses, and for parametric data

correlation was calculated via Pearson correlation coefficient and linear regression analyses.

GraphPad software was also used to produce graphs; each plotted as mean± standard error of the mean (SEM).

Self-reported biometrics were used to calculate body mass index scores (BMI) via the equation:

$$Body\ Mass\ Index = \frac{Weight\ (Kilograms)}{Height(Metres)^2}$$

Self-reported exercise frequency and level of strenuousness was used to calculate Godin Leisure Index Scores (LSI) via the equation:

$$Weekly\ Leisure\ Activity\ Score = (9xStrenuous) + (5xModerate) + (3xLight)$$

3.0 Results

3.1 Part 1 – Impacts of Omnivorous and Vegan diets

3.1.1 Sperm Parameters

Sperm parameters were measured for each dietary cohort (total sperm count, motility, concentration, vitality and pH). Red dotted lines signify WHO (2010) lower reference ranges for each of the sperm parameters used in the selection criteria for normozoospermic samples (Section 2.1). The data were analysed for normality using Shapiro-Wilk test, normally distributed data were analysed via parametric tests (t-test) and non-normally distributed data were analysed via non-parametric tests (Mann-Whitney test). All parameters were normally distributed except for pH values.

There were no statistically significant difference between the total sperm count, motility, concentration, vitality or pH between vegan diets and omnivorous diets (Figure 11).

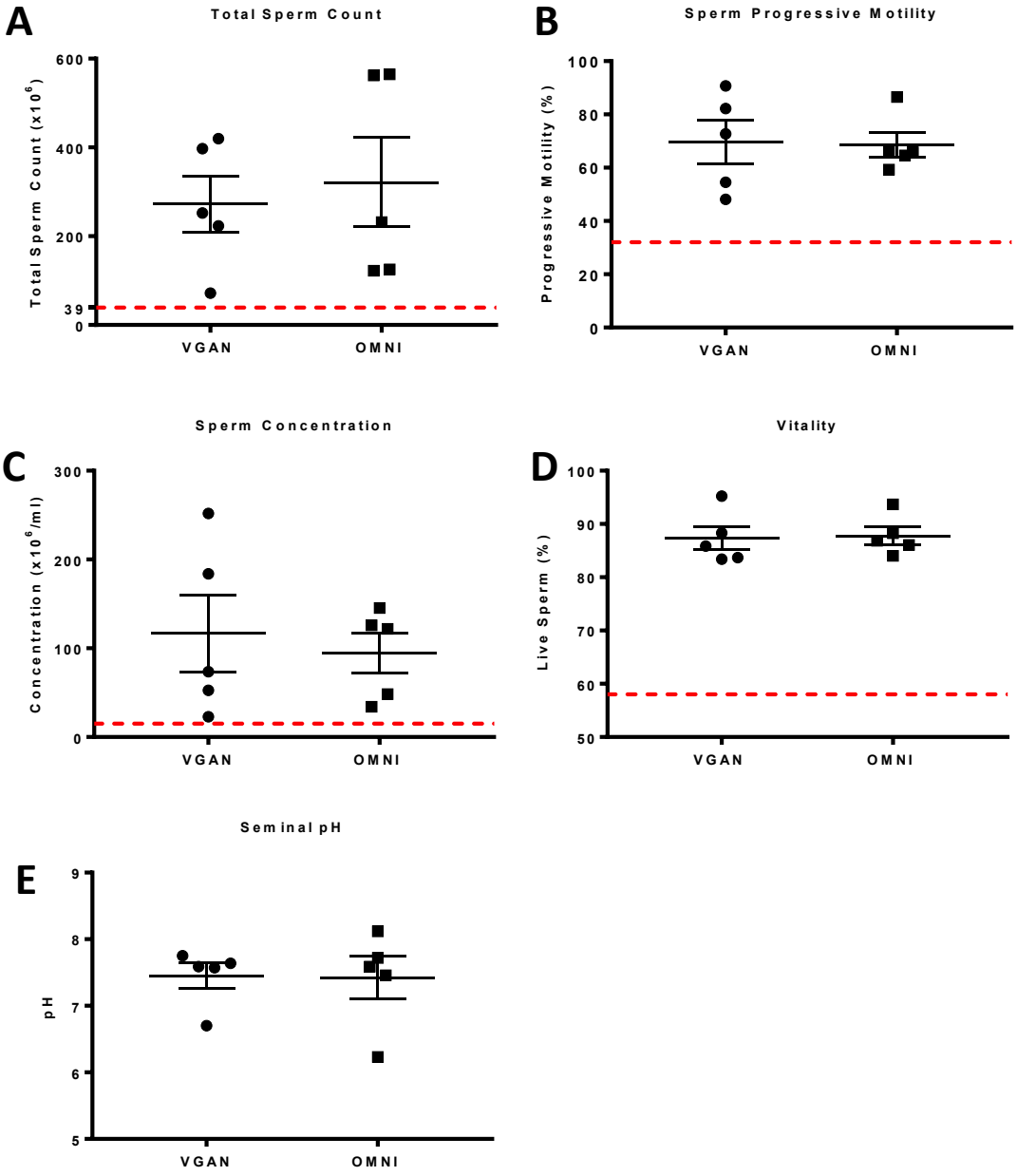

Figure 11 **Sperm parameters for omnivorous(OMNI) and vegan (VGAN) diets.** WHO lower reference ranges for the respective parameter represented as red dotted lines on each graph (WHO, 2010). **A**– Total sperm count for omnivorous and vegan diets calculated using concentration (x10^6/ml) and ejaculate volume (ml) (p=0.6954)(unpaired t-test). **B** – Sperm percentage progressive motility (fast progressive + slow progressive(A+B)) analysed via computer assisted sperm analysis for omnivorous and vegan diets (p=0.9129)(unpaired t-test). **C**– sperm concentration (x10^6/ml) analysed via computer assisted sperm analysis(p=0.6668)(unpaired t-test). **D**- sperm vitality (%live) Duplicate eosin-nigrosin dye exclusion assay quantified via duplicate examination of slides using 1000x magnification with oil immersion (p=0.8606)(unpaired t-test). **E**- Seminal pH measured by pH probe (p>0.9999)(Mann-Whitney test). Error bars represent mean ± standard error of the mean (SEM). No significant difference in any sperm parameters was observed between the dietary cohorts p>0.05, n=5 per dietary cohort. *P*-values are reported at 95% confidence intervals.

3.1.2 Global methylation

The primary aim of this research was to establish any significant differences in the global methylation of total sperm DNA for those subscribing to a vegan or omnivorous diet. This was measured by 5-methylcytosine% (an indicator for global methylation) percentage in total sperm DNA (Vryer and Saffery, 2017).

No significant difference in percentage 5-methylcytosine% was found between the vegan and omnivorous diet cohorts (Figure 12), however the range of percentage total DNA 5-methylcytosine% within the vegan cohort (4.06, *n*=5) was larger than that of the omnivorous cohort (0.94, *n*=5).

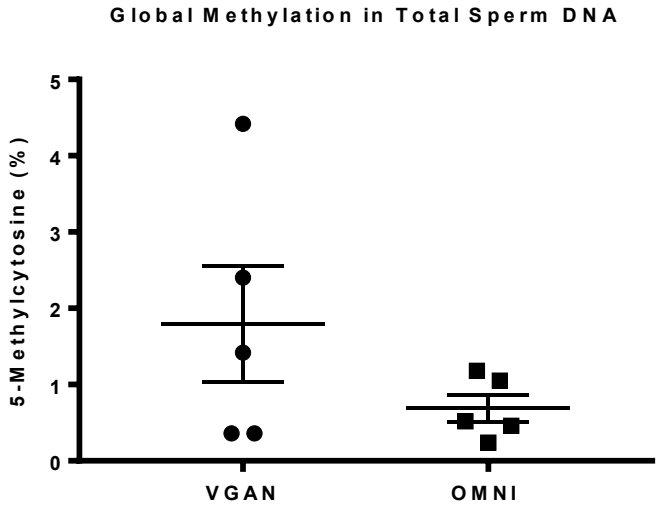

Figure 12 Global methylation (5-methylcytosine% in total DNA) between the vegan and omnivorous diets (*p*=0.1954)(unpaired t-test) (*n*=5 per dietary group). Error bars represent mean ± SEM. Measured using MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek).

3.1.3 Evaluation of potential confounding factors from biometrics and lifestyle

The possible confounding factors were analysed via unpaired t-tests to identify if there was a difference between any potential confounding factors (age, BMI and alcohol consumption) for the vegan and omnivorous dietary cohorts. No significant differences were found (Table 5).

The leisure score index (section 2.7) is an arbitrary measurement of weekly exercise used to identify insufficiently active (≤23) and active (>24) lifestyles (Godin and Shephard,1985). The LSI classification could also be a potential confounding factor however; there was no significant difference between the score for vegan and omnivorous dietary cohorts (Table 6).

As no differences were found, no confounding factors needed to be accounted for in statistical analyses.

Table 5 Biometrics & Lifestyle factors between dietary cohorts. *[a] Median (IQR) *[b]Mean ±SEM. P-values calculated via unpaired t-tests.

Biometrics & Lifestyle factors			*p*-values	
Age[a] (years)	VGAN OMNI	33 (26 – 34), *n*=5 23 (22.5 – 32.5), *n*=5	0.5317	ns
BMI[b] (kg/m^2)	VGAN OMNI	23.38 ± 0.9265, *n*=5 24.18 ± 1.345, *n*=5	0.6374	ns
Alcohol consumption[b] (units/week)	VGAN OMNI	8.8 ± 4.841, *n*=5 5.6 ± 2.315, *n*=5	0.5675	ns

Table 6 LSI partition between dietary cohorts for insufficiently active (≤23) and active participants (>24). Calculated via Fisher's contingency test (*p*>0.999).

Leisure Score Index	≤23	>24	Significant
OMNI	0	5	ns
VGAN	1	4	

3.2 Part 2 – Investigation of Global Methylation and Sperm Parameters and Lifestyle and Biometric Factors

Due to the lack of significance between the vegan and omnivorous dietary cohorts (Figure 11;Figure 12;Table 5;Table 6) the dietary datasets were combined. This was statistically valid as neither of the dietary cohorts demonstrated any significance in any areas, and therefore the combined datasets are representative of the population. This allowed further correlational analysis into the effects of lifestyle and biometrics on sperm parameters and any interactions between sperm parameters, irrespective of dietary subscription.

3.2.1 Sperm parameters

Table 7 demonstrates mean sperm parameters of the combined cohort (*n*=10).

Table 7 Sperm parameters (*n*=10) displayed as mean±SEM.

Sperm parameters	Mean ±SEM
Total Sperm Count ($x10^6$) [a]	297.2 ± 56.73
Sperm Concentration ($x10^6$/ml) [a]	106 ± 23.29
Progressive Motility (% A+B) [a]	69.12 ± 4.34
Vitality (% live) [a]	87.54 ± 1.28

Subsequently, any relationship between sperm parameters and global methylation of total sperm DNA of the combined cohort was investigated via correlational analysis (Pearson's Correlation). No significant correlation was identified between the sperm parameters and global methylation of total sperm DNA (Table 8).

Table 8 Correlation between sperm parameters and global methylation using Pearson's correlation (displayed as *r*=coefficient value(*p*-value) (ns=no significance).

Sperm parameters	***r* (*p*-values)**	**Significance**
Total Sperm Count (x10^6)	0.1945 (0.5889)	ns
Sperm Concentration (x10^6/ml)	0.3587 (0.3063)	ns
Progressive Motility (% A+B)	0.1945 (0.5889)	ns
Vitality (% live)	-0.1824 (0.6136)	ns

3.2.2 Lifestyle and biometrics

Table 9 demonstrates biometrics & lifestyle factors of the combined cohort (*n*=10). (Parametric data shown as mean ± SEM, nonparametric data shown as median (interquartile range(IQR)).

Table 9 Biometrics & Lifestyle factors *[a]mean ±SEM *[b]median(Interquartile Range).

Biometrics & Lifestyle factors	Mean±SEM/Median(IQR)
Age (years)[a]	28.6 ± 2.088
Body Mass Index (kg/m^2)[a]	23.78 ± 0.7814
Alcohol (units/week)[b]	5 (0-12)
Leisure Score Index[b]	47.5 (34.75-75.25)

A relationship between biometrics & lifestyle factors and global methylation of total sperm DNA of the combined cohort was investigated via correlation analysis (Pearson's Correlation for age and BMI (parametric), Spearman's Correlation for alcohol and LSI(nonparametric)). No significant correlation was identified between the biometrics & lifestyle factors and global methylation of total sperm DNA (Table 10).

Table 10 Correlation of Biometrics & Lifestyle factors *[c] Pearsons Correlation *[d] Spearmans Correlation (ns=no significance).

Biometrics & Lifestyle factors	***r* (*p*-values)**	**Significance**
Age (years)[c]	0 (>0.9999)	ns
Body Mass Index (kg/m^2)[c]	-0.0581 (0.8727)	ns
Alcohol (units/week)[d]	0.0432 (0.9074)	ns
Leisure Score Index[d]	0.2067 (0.5651)	ns

3.3 Part 3 – Investigation of the Impact of Biometrics & Lifestyle Factors on Sperm Parameters and Their Interaction

Any relationship between biometrics & lifestyle factors and sperm parameters of the combined cohort were investigated via correlation analysis.

3.3.1 Sperm parameters and lifestyle

To investigate any relationship between biometrics & lifestyle factors and sperm parameters of the combined cohort, correlation analysis was performed using Pearson's correlation for age and BMI (parametric) and Spearman's correlation for alcohol and LSI (non-parametric). No significant correlation was found (Table 11).

Table 11 Associations between biometrics & lifestyle factors and sperm parameters. Displayed as *r*=correlation coefficient(*p*-value) *[a]Pearsons correlation *[b] Spearmans Correlation.

	Age (years)[a]	Body Mass Index (kg/m^2)[a]	Alcohol (units/week)[b]	Leisure Score Index[b]
Total Sperm Count(x10^6)	-0.3294 (0.3527)	-0.2828 (0.4286)	-0.1108 (0.7615)	0.5152 (0.1334)
Sperm Concentration(x10^6/ml)	-0.5589 (0.0930)	-0.0810 (0.8240)	0.0554 (0.8819)	0.2 (0.5837)
Progressive Motility (% A+B)	-0.3806 (0.2779)	0.1084 (0.7657)	0.4062 (0.2420)	0.0667 (0.8651)
Vitality(% live)	-0.4519 (0.1898)	0.1823 (0.6142)	0.0492 (0.8970)	0.297 (0.4069)

3.3.2 Dependence of sperm parameters

Any relationship between the sperm parameters in the combined cohort was investigated via Pearson's correlation analysis.

Progressive motility and sperm concentration in the combined cohort were found to be significantly positively correlated (r=0.7766, p=0.0082). The progressive motility and vitality in the combined cohort were also found to be significantly positively correlated (r=0.6535, p=0.0404) (Table 12).

Table 12 Interaction between semen parameters. *[a]Pearsons Correlation data presented as r=correlation coefficient(p-value).

Sperm parameters	Total Sperm Count (x10^6)	Sperm Concentration (x10^6/ml)	Progressive Motility (% A+B)	Vitality (% live)
Total Sperm Count (x10^6) [a]		0.6308 (0.0505)	0.288 (0.4197)	0.5688 (0.0862)
Sperm Concentration (x10^6/ml) [a]			**0.7766 (0.0082)****	0.6038 (0.0645)
Progressive Motility (% A+B) [a]				**0.6535 (0.0404)***

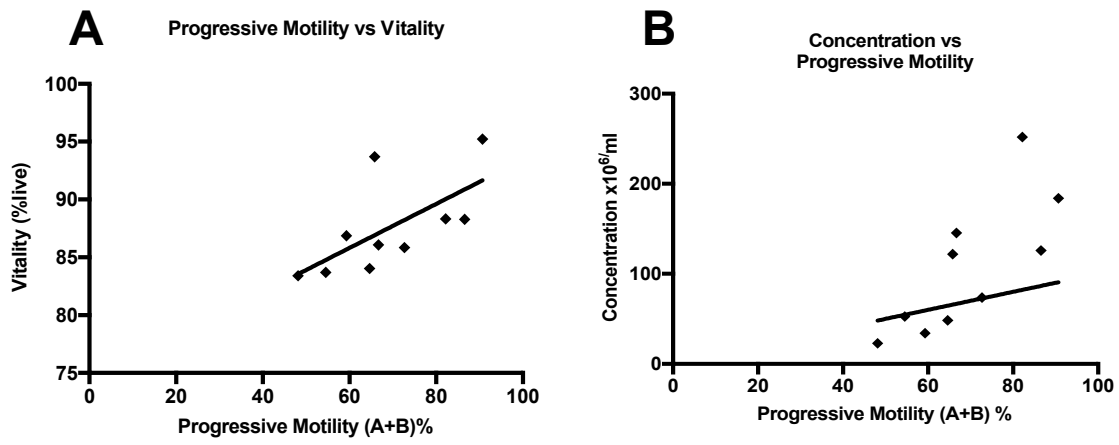

Figure 13 **A** - Pearson correlation of progressive motility (A+B)% vs vitality (%live) r=0.6535 p=0.0404* in the total dataset. **B** - Pearson correlation of concentration (x10^6/ml) vs progressive motility (A+B)% r=0.7766 p=0.0082** in the combined cohort.

3.3.3 Independence of Biometrics & Lifestyle Factors

Any relationship between the biometrics & lifestyle factors was investigated via Pearson's correlation for age and BMI (parametric) and Spearman's correlation analysis for alcohol and LSI (nonparametric). There was no statistically significant correlation found between biometrics & lifestyle factors in the combined cohort (Table 13).

Table 13 Interaction between biometrics and lifestyle factors. Displayed as *r*=correlation coefficient (*p*-value) *[a]Pearsons correlation *[b]Spearmans Correlation.

Biometrics & Lifestyle factors	Age (years)[a]	Body Mass Index (kg/m^2)[a]	Alcohol (units/week)[b]	Leisure Score Index[b]
Age (years)[a]		-0.3689 (0.2941)	0.3684 (0.2899)	-0.3171 (0.3693)
Body Mass Index (kg/m^2)[a]			-0.1579 (0.6593)	0.3049 (0.3887)
Alcohol (units/week)[b]				-0.2585 (0.4674)
Leisure Score Index[b]				

4.0 Discussion

Lifestyle and environment are important influences in human health. There are many aspects of lifestyle that are known to affect reproductive health such as alcohol intake, recreational and pharmaceutical drugs, smoking cigarettes and a BMI outside of the healthy range. Poor dietary choices in combination with sedentary lifestyle likely lead to obesity, which in turn, is implicated as a risk factor in diseases such as stroke, diabetes, cancer (Dixon, 2010) and well documented negative effects on reproduction and fertility outcomes (Hammoud *et al,* 2008a; Broughton and Moley, 2017).

The epigenome responds to its environment. Lifestyle factors such as diet and obesity could have an impact in inducing aberrant DNA methylation, which has been associated with male infertility and poor semen parameters.

Global DNA methylation has previously been shown to be a poor marker for infertility, although global DNA methylation has been shown to decline with age (Nevin and Carroll, 2015). However, decreased global methylation has been shown to be associated with male infertility and poor sperm quality (Benchaib *et al,* 2003).

Global DNA methylation assays are a cost-effective method to analyse DNA methylation, particularly in cases where the candidate genes are unknown. Global DNA methylation analysis is therefore a good method for discovering unknown epigenetic changes.

As decreased global methylation could be associated with infertility, this research investigated if diet, lifestyle or biometrics had any correlation with global methylation.

As a pilot study, the aim of this study was to investigate any potential relationship between dietary choice, semen parameters and sperm DNA methylation status. Global methylation levels were estimated via percentage of total DNA 5-Methylcytosine present using an ELISA based assay (Epigentek).

All participants self-reported as non-smokers and any other potentially confounding factors were investigated to ensure that they were accounted for in the statistical analysis (Table 5; Table 6**).**

4.1 Part 1 – Impacts of Omnivorous and Vegan diets

4.1.1 Sperm parameters

Diet and sperm parameters have long been associated in terms of obesity or malnourishment. Previous research indicates that any dietary effects on sperm parameters for those with a healthy BMI are often due to a deficiency in minerals and/or vitamins such as zinc, which is heavily implicated in male infertility. Abbasi *et al,* (1980) demonstrated a significant (p<0.05) reduction in total sperm count following restriction of zinc to 2.7-5.0mg daily for a 24-40 week period. Interestingly, total sperm count returned to the baseline level 6-12 months following intervention.

Datillo *et al* (2014) demonstrated that a 4-month period of daily supplementation with multivitamins (B vitamins, vitamin E, zinc) resulted in 18 (21%) spontaneous pregnancies & births in couples with ≥2 failed assisted reproductive technology (ART) attempts. Compounds derived from plants such as genistein, present in soya are demonstrated to have a negative effect upon sperm count and motility, in addition to playing a role in an aberrant transcriptome in testicular germ cells (Mendiola *et al,* 2010). Veganism (a restrictive diet absent of all animal products) is becoming increasingly popular over a traditional omnivorous diet for a variety of social, economic and environmental reasons (section 1.2).

In this study, no significant relationship was established between the sperm parameters and diet choice (Figure 11). There are many cofounding factors that could affect this such as the multivitamin supplements self-reported by the entire vegan cohort compared to none of the omnivorous cohort. Not all confounding factors are easily identifiable as levels of vitamins and minerals were not measured in this study and therefore could not be accounted for. Any study investigating diet is extremely variable as caloric intake, macronutrient levels, time that meals are eaten, and fasted periods could all affect results.

Further research is necessary to thoroughly investigate any effect diet choice may have on sperm parameters.

4.1.2 Global Methylation

Despite the inability within the field to agree whether global methylation is a direct measure for infertility or not, it is nevertheless a useful, cost effective measurement of total DNA methylation when searching for novel candidate genes for investigation. Vitamin B12 is a dietary compound which is essential to the methylation process. B12 assists in the re-methylation of homocysteine to methionine, which in turn

combines with ATP to form S-adenosylmethionine; the primary methyl donor in the methylation process (section 1.3.2.1). B12 is normally deficient in vegan diets, which could potentially result in hypomethylation due to the lack of methyl donor production.

There was no statistically significant difference in levels of 5-methylcytosine% between the vegan cohort and omnivorous cohort (p=0.1954). This could potentially be due to B12 supplementation of the entire vegan cohort and no supplementation of the omnivorous cohort. However, this could not be controlled for and was not possible to measure or account for supplementation via any other method except the food frequency questionnaire (Appendix 7.6).

4.1.3 Evaluation of Potential Cofounding Factors from Biometrics and Lifestyle

A food frequency questionnaire was used primarily to assess whether participants were following their self-reported diet (vegan, omnivorous), quantify weekly alcohol unit intake, weekly exercise and biometrics.

This approach can come with caveats with limitations to self-reporting; accurate memory of events decreases with time, the requested information is usually inaccurately reported, increasing age also increases the inaccuracy of self-reporting. Education level also affects accuracy of self-reporting, those with a higher education provided more accurate information than those without (Short *et al,* 2009).

This adds further confounding factors; however, the participants were only asked to recall the previous 3 months, and of the cohorts none of the participants were of advanced age, therefore dietary recall can be considered relatively accurate.

In order to exercise more control and minimise confounding factors, participants could be asked to follow strict calorie-matched diet plans using a food diary or food tracker mobile app. Alternatively, to maintain an observational approach, vegan and omnivorous cohorts could be further subdivided into lacto-omnivorous, lacto-ovo-omnivorous, vegan, raw-vegan etc and diets and caloric intake tracked via food diary or food tracker mobile application in order to pinpoint which foods, if any, have an effect upon the epigenetic signature of sperm DNA.

Furthermore, compounds involved in the methylation process such as phytoestrogens, B12, zinc, were not directly measured in this research. The level of

compounds was assumed on the basis of self-reporting in the food frequency questionnaire and dietary subscription, vegan diets are generally deficient in B12 due to the lack of meat, and high in phytoestrogens due to the adoption of soya as a dairy alternative, and *vice versa* in omnivorous diets. For example, questionnaire data demonstrated that all 5 vegan participants consumed soya products on at least a weekly basis, whilst none of the 5 omnivorous participants consumed soya, research has previously indicated that soy isoflavones can have an effect on methylation by reactivating methylated genes partially via direct DNA methyltransferase inhibition (Fang *et al*, 2005).

Measuring of such compounds within the blood and semen would assist in the quantification, and correlation with methylation data.

Despite this, when analysing the potential confounding factors in the cohorts of vegan and omnivorous diet choices, the biometrics and lifestyle factors were not statistically significant and therefore did not need accounting for in further analysis (section 3.1.3).

4.2 Part 2 – Investigation of global methylation and sperm parameters and lifestyle and biometrics

4.2.1 Sperm parameters and global methylation

Sperm parameters are a useful tool for assessing fertility. Due to debate in the field about how accurate global methylation of total sperm DNA is as a predictor of infertility. This study used correlation analysis to investigate the relationship between global methylation of total sperm DNA and sperm parameters which would have given an indication as to whether this study found global sperm methylation to be related to other parameters that are implicated in male infertility. This study found that global methylation was not correlated to any of the sperm parameters, this is in line with previous studies suggesting the same (Nevin and Carroll, 2015).

4.2.2 Lifestyle and biometrics

Diet has often been implicated in reproductive health, particularly in the case of an abnormal BMI. A BMI of >30kg/m^2 (obese) or <18.5kg/m^2 (underweight) as defined by WHO (World Health Organisation, 2018) contributes to poor reproductive

outcomes (Qin *et al,* 2007; Ramlau-Hansen *et al,* 2007; Zain and Norman, 2008;Gaskins *et al,* 2015). Obesity is associated with hypogonadism and further disrupts the HPG axis negatively affecting both male and female fertility (Chambers and Anderson, 2015; Corona *et al,* 2015). Obese women respond poorly to assisted reproductive technologies (Kasum *et al,* 2018), have increased complications during pregnancy, increased miscarriage, increased blood pressure, increased incidence of gestational diabetes as well as larger foetus' which can lead to complications during parturition (Poston *et al,* 2016). Obesity in males disrupts the HPG axis and is associated with poor sperm parameters (Du Plessis *et al,* 2010). Studies have demonstrated increasing prevalence of low progressively motile sperm with increasing BMI, in addition to a positive correlation between BMI and oligozoospermia (Hammoud *et al,* 2008b). Petersen *et al* (2018) observed a significant increase in sperm DNA fragmentation and percentage mitochondrial membrane potential damage with increasing age. However, in the cohort of males with both vegan and omnivorous diets that were used in this pilot study the BMI was within the normal range and sperm parameters were all non-significant.

4.3 Part 3 – Investigation of the impact of lifestyle on sperm parameters and their interaction

4.3.1 Sperm parameters and lifestyle

This study found no correlation between sperm parameters and lifestyle and biometrics (section 3.3.1). This is potentially due to the chosen cohort being selected for normozoospermia. All participants lead relatively healthy lifestyles, their BMI was normal, alcohol intake was not above the recommended average, they did not smoke, all (except one) were sufficiently active and none of the participants were of advanced age.

4.3.2 Dependence of sperm parameters

Progressive motility and sperm concentration were positively correlated in the combined cohort, as was progressive motility and vitality, this is in agreement with what is well established in previous literature. This is a useful result as it indicates that despite the small sample size the cohort seems to still be relatively representative of the average population due to the data's predictable behaviour.

4.3.3 Independence of lifestyle and biometrics

There was no significant correlation between any of the lifestyle and biometric factors. This indicates that the lifestyle and biometrics were not confounding factors and therefore did not need accounting for in the statistics.

4.4 Limitations

Limitations of sample size (*n*= 5 for vegans; *n*= 5 for omnivores) may have biased these data. Interestingly, the vegan cohort showed a much higher variation of percentage total DNA 5-methylcytosine than the omnivorous group (range of 4.06 5-methylcytosine% in total DNA, *n*=5 and 0.94 5-methylcytosine% in total DNA, *n*=5, respectively) (Figure 12). Further research should be conducted within a larger cohort to confirm disparities between 5-methylcytosine% range in between dietary exposure groups. Due to the reliance on the self-report method, it was difficult to assess any nutrient deficiencies, furthermore, caloric intake was not tracked.

All the participants recruited on to this study had normozoospermic semen parameters (count, concentration, motility and vitality) according to WHO guidelines (WHO, 2010) however, their fertility could not be confirmed as none of the participants had confirmed offspring. Per the questionnaire, it was confirmed that none of the 10 participants had any children, nor were they actively attempting to conceive, therefore had no proven fertility status.

This research comprises a predominantly white demographic of volunteers. Hsuing and colleagues observed a 1.26% (*p*=0.03) higher global methylation levels of leukocyte LINE-1 in non-Caucasians compared to Caucasians (Hsuing *et al,* 2007). Zhang and colleagues demonstrated a 2.2% reduction in mean global methylation of LINE-1 in non-Hispanic Blacks, and 1.3% reduction of leukocyte LINE-1 methylation in Hispanics when compared to non-Hispanic Whites (Zhang, 2011). Recruitment of exclusively Caucasian participants in this particular research eliminates the confounding factor of these racial disparities in methylation described by Zhang (2011), however may limit extrapolation of results to any racial group other than Caucasian.

Sample size (*n*=10) may have also occluded the extrapolation of results to the general population. Larger sample size, a longitudinal approach and measurement of additional genes involved in spermatogenesis and methylation would assure

more validity. A control group of healthy, normozoospermic males and a group of those attending fertility clinics may give a clearer indication as to whether fertility status has any effect upon the sperm methylome, and therefore whether aberrant epigenetics play a role in idiopathic infertility.

The analysis of a single sample per participants provided a snapshot of the current effect of recent dietary exposure upon sperm parameters and total 5-Methylcytosine and was practical given the timescale of this research. Collection and analysis of multiple samples at 3-month intervals would provide a transient perspective upon the effects of diet upon the sperm methylome over 3 cycles of spermatogenesis (perhaps with recruitment of participants undergoing a change in diet from omnivorous to vegan).

4.5 Further Research

Whilst results of methylation analysis were not significant. This pilot study warrants further exploration into the relationships between dietary exposures and the sperm-specific methylome; including potential subsequent implications of diet upon fertility and offspring health as a transgenerational consideration.

Initial experimental design included investigation into the methylation status of specific CpG sites within promotor regions of sperm and methylation specific genes; DAZL (Deleted in azoospermia-like) and MTHFR (methylenetetrahydrofolate reductase), in addition to quantification of 5-methylcytosine as a marker of global methylation. Following optimisation, PCR products were obtained for the regions of interest for subsequent use in pyrosequencing analysis, however repeated contamination diminished the validity of the experiment. These results were anticipated to correlate with the differences in 5-methylcytosine% and confirm the role of diet as a modifier of methylation, and would certainly be an interesting future avenue of research.

5.0 Conclusion

Differences in sperm parameters and global methylation between dietary cohorts were assessed. No significance was found, only larger variation of 5-methylcytosine% within the vegan cohort (range of 4.06, n=5), than that of the omnivorous cohort (range of 0.94, n=5). This indicates that perhaps the sperm methylome of those following a vegan diet may be more heterogenous than the sperm methylome of those following an omnivorous diet.

Correlation between sperm parameters (within the combined cohort, disregarding dietary subscription) was demonstrated, however this is not a new finding within the field. Progressive motility and sperm concentration were found to be significantly positively correlated (r=0.7766, p=0.0082). The progressive motility and vitality were also significantly positively correlated (r=0.6535, p=0.0404).

This project aimed primarily to investigate potential differences in sperm global methylation levels, in addition to sperm count, concentration, motility and vitality, between those adhering to an omnivorous diet or a vegan diet.

Based upon results of this pilot study, it can be concluded that diet has no effect upon sperm parameters or the sperm methylome.

The paucity of research in this particular area necessitates further investigation. Future research designed with the considerations described in the discussion must be conducted in order to better elucidate any differences between the sperm methylation status of those subscribing to a vegan or omnivorous dietary pattern, and whether either an omnivorous or vegan diet confers a methylome beneficial to fertility status and offspring health.

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7.0 Appendices

7.1 Somatic Cell Removal

As mentioned in section 2.5.3.1, during optimisation of somatic cell removal, sperm were exposed to 2 techniques; density gradient centrifugation, and somatic cell lysis using detergent. This was to determine a suitable technique capable of total round cell removal, to then use on the experimental samples.

Samples for this purpose were procured per section 2.1.

Following liquefaction, the fresh sample was centrifuged at 300g for 10 minutes in. The pellet was then re-suspended in 2ml phosphate-buffered-saline (PBS) pre-warmed at 37°C.

Baseline sperm count was assessed via a haemocytometer count and round cells counted according to WHO procedures (WHO, 2010).

The remainder of the sample was divided equally into 2x clean falcon tubes (Corning™Falcon™), pending exposure to the 2 sperm isolation techniques.

Density gradient centrifugation was performed as described in section 2.5.3.2.

Sperm and round cells were counted using a haemocytometer, according to WHO guidelines (WHO, 2010).

7.1.2 Somatic Cell Lysis Detergent

$5x10^6$ sperm were added to the following 8 conditions and incubated on ice for the indicated timepoints (Table *16*).

After the indicated time had passed, the samples were immediately centrifuged at 300g for 3 minutes. The supernatant was discarded, and re-suspended in 100µl PBS. Sperm and round cells were counted using a haemocytometer, according to WHO guidelines (WHO, 2010).

Table 14 Original baseline concentration of sperm and round cells ($x10^6$) post-wash counted using a haemocytometer following WHO guidelines (WHO, 2010).

Baseline Concentration of Sperm & Round Cells Post-Wash ($x10^6$/ml)	
Sperm	**Round Cell**
50.5	1.6

Table 15 Concentration of sperm and round cells post-density gradient centrifugation (x10^6/ml) counted using a haemocytometer following WHO guidelines (WHO, 2010).

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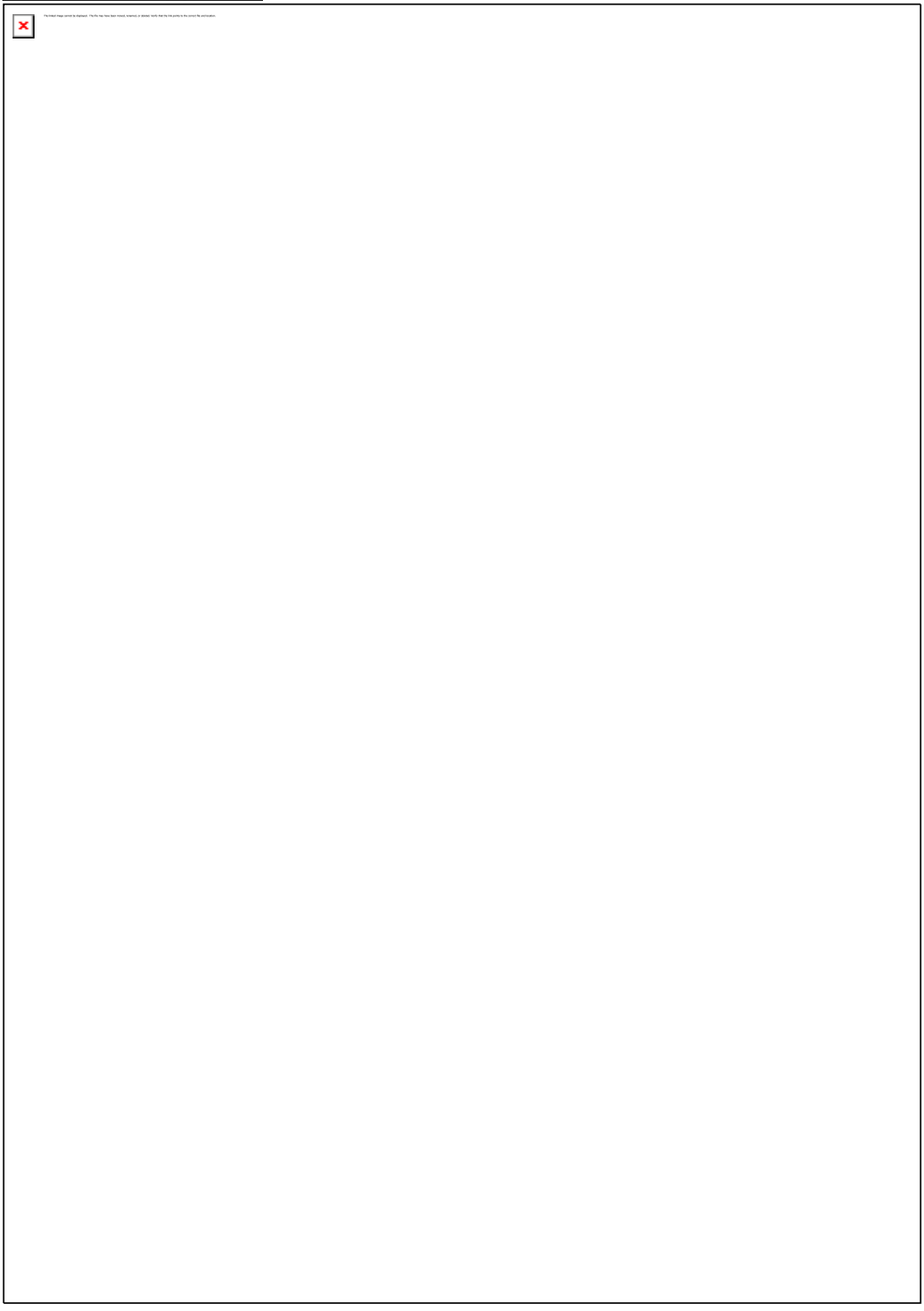
Concentration of Sperm & Round Cells Post-Density Gradient Centrifugation (x10^6/ml)	
Sperm	**Round Cell**
21.7	0

Table 16 Concentration of sperm and round cells (x10^6) post-detergent treatment using increasing concentrations of Triton-X detergent with phosphate buffered saline control. Counted using a haemocytometer following WHO guidelines (WHO, 2010).

Concentration of Sperm & Round Cells Post-Somatic Cell Lysis Buffer Treatment with Increasing Concentrations of Detergent at Increasing Exposure Times (x10^6/ml)								
Concentration of Triton-X detergent used								
Incubation Time (Min)	0% Triton-X (PBS Control)		0.05% Triton-X		0.1% Triton-X		0.5% Triton-X	
	Sperm	**Round Cell**	**Sperm**	**Round Cell**	**Sperm**	**Round Cell**	**Sperm**	**Round Cell**
5	13.75	2.3	21.6	1.5	21.03	0.8	14.98	0.4
30	20	0.9	23.35	1.2	14.45	0.3	12.22	0.2

Total removal of round cell population (0x10^6/ml) demonstrated in the density gradient centrifugation condition (Table 15). Therefore this method was used in the methodology.

7.2 Ethical Approval



7.3 Participant Information Sheet

Participant information sheet SE1617127 v.2

Title of Study: *Investigating the effect of diet choice 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. A change in lifestyle can positively or negatively affect sperm parameters (number, motility and morphology, sperm DNA integrity). This study will investigate if a vegan, vegetarian or pescetarian diet can modify some of the human sperm parameters compared to omnivore diets. This information will offer potential therapeutic options that may improve male infertility.

Who can take part?
Any male aged over 18 years old, not taking medication.

What is involved?
You will be required to provide blood and semen samples (or optionally 3 repeated visits) and complete lifestyle and diet questionnaires.
All samples and questionnaires will be confidential and anonymised via an ID code in accordance with our ethics approval.

- For semen samples: you will be required to provide a semen sample (via masturbation, after a 2-5 days of abstinence) either at home or within a secure room at the school of healthcare science. If you prefer to provide the semen sample at home you will be given a sample pot and instructions. A full sample is required and it will have to be brought in to the laboratory for analysis within an hour of production. 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 to assess DNA damage levels and any alterations in DNA. The samples will be stored at -80°C for further analysis. Sperm cells are not viable upon thawing. Protein, DNA and RNA will be stored for further analysis relating to this study only. 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.

  Your semen sample may also be used in training research staff or teaching purposes. Anonymity is assured, no identifying details will be available where used in these cases. All samples will be destroyed after use.

Please note that this is not a diagnostic test and you will not be informed of the quality of your semen/sperm. Recompense is not given for this study.

- For blood samples: You will also be asked to provide a blood sample (5cl). The blood will be taken by an experienced phlebotomist and is a quick and painless procedure. The blood parameters will be assessed, prepared and stored at -80°C until required. These samples will be used for biochemical and molecular analysis (such as hormone levels, proteins and DNA (no drugs or their metabolites will be measured).

- At each visit, a questionnaire will be provided. This questionnaire will ask for information concerning environmental, diet, lifestyle exposures, which will include some sensitive personal and lifestyle questions such as sexual abstinence and drug use. If you decide not to answer a question – please cross the question with a line (this will inform the researcher that the question was not missed accidently).

7.4 Participant Informed Consent Sheet

Participant informed consent. ID code ……

Name: ……

Date of Birth: ……

Project title:

Investigating the effect of diet choice on human sperm.

Principal Investigator:
Dr Michael Carroll

Investigator/Collaborators:
Dr Stephane Berneau
Dr Christopher Murgatroyd
Dr Andy Povey (external collaborator)
Prof. Daniel Brison (external collaborator)

Ethics approval number: SE1617127 (v.1)

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. I also understand that no personal identifying information will be attached to any data derived from this sample and all data presented or published will be anonymised.

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 stored at -80°C at MMU and used for retrospective biochemical and molecular biology analysis in this study.

  (*please circle*) **YES NO**

- ☐ I give my consent for blood collected from me during the course of this study to be stored at MMU for retrospective biochemical and molecular biology analysis

  (*please circle*) **YES NO**

- ☐ I give my consent for samples to be donated to MMU for use in this study and afterwards for future scientific / medical research and/or teaching purposes. I understand that my sample will not be used for any reproductive activities.
  (*please circle*) **YES NO**

Signed …… Date ……

Name (Print)……..

Witnessed …… Date ……..

Name (Print) ……..

7.5 Medical Screening Questionnaire

Medical Screening Questionnaire SE1617127 (v.1) **Participant code:**

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.

Date of Birth: _____

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**

Do you smoke cigarettes / e-cigarettes? **YES/NO**
If yes, how many per day? _____

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. diabetes 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:

7.6 Food Frequency and Lifestyle Questionnaire

Donor ID

Diet and lifestyle Study

Sperm Quality

This study has been set up to investigate what factors may affect male fertility. Please work through the questionnaire filling in each question that applies to you, by ticking (✔) the box or by writing in the space provided.

Date questionnaire filled in: ____day _____month _____ year

Please complete the details above, then turn over and answer the following questionnaire. This questionnaire will ask information concerning environmental, diet, lifestyle exposures, which will include some sensitive personal and lifestyle questions such as sexual abstinence and drug use.

If you decide not to answer a question – please cross the question with a line (this will inform the researcher that the question was not missed accidently).

Please read the questions carefully and give attention to the **bold** words. Answering this questionnaire should take about 10-20 minutes. Most of the questions have YES or NO answers, or questions where you need to tick a box ✔ or write a number or a word. There is one question about your job where we will ask you to answer in detail.

General questions:

Age

1. What is your date of birth? day_____month _____ year

2. What is your **height** and your **weight** now? (material available &in the room if necessary)

Height ☐ cm or ☐☐ inches

Weight ☐ Kg or ☐☐ ounces

Ethnicity

3. Which ethnic group do you consider you belong to?

☐ White British

☐ Other White Background, Please describe _____

☐ Black-Caribbean

☐ Black-African

☐ Other Black Background, Please describe _____

☐ Indian

☐ Pakistani

☐ Bangladesh

☐ Malaysian

☐ Chinese

☐

Other Ethnic Group, Please describe _____

4. Are you …

☐ Married ☐ In relationship

☐ Single ☐ Widower

☐ Divorced ☐ Other

5. Do you have any children?

☐ Yes ☐ No

6. Are you attempting to conceive a baby?

☐ Yes ☐ No

7. Have you ever had your semen analysed?

☐ Yes ☐ No

Your job during the last three months:

Please write in the table in the next page the type of work you have done in your job during the ***last three months***, please, if you have been in two or more jobs during the last three months, mention all of them. To help you, we have given some examples of what other men have written in a previous study. Please answer this question even if you were not exposed to any hazard in you job (for example office job).

Examples	**Job Title**	**Type of company** (what did they do)	***During the last three months,* What were the main tasks you did in your job*? And what were the hazards (chemicals, radiation,....) you were exposed to (if applicable)?***	**Type of job (full or part time)**	**Did you have to wear any sort of personal protection during your job**
1	Telephone account manager	Airline	Meetings with clients over telephone, lots of sitting down.	Full-time	None
2	Sales assistant	Garden centre (Gardening)	1- Selling pesticides, weed killers, fertilisers. 2- Making up pesticides, weed killers, fertilisers for use in garden centre. 3- Cleaning up spillage.	Part-time	Protective clothing, respirator, gloves, coverall
3	Pipefitter	Plumbing firm	Installing industrial pipe work, gas, water, steam, compressed air and this includes soldering. Currently I use flux which is lead free solder.	Full-time	None

4	Electrical engineer	Nuclear power planet	Executing, evaluating, delivering and managing complex engineering and / or cross functional tasks or programmes of work, recognising stakeholder and customer requirements.	Full-time	Protective clothing, gloves……
5	Researcher	University	Working in the lab and dealing with chemicals and biological materials.	Full-time	Protective clothing, gloves
6	Decorating	Domestic decorating	1- Wallpaper decorating: I used adhesives, fillers, putty. 2- Painting: oil paint, white spirit. 3- Decorating involves lacquers and silicones.	part-time	Gloves

Please complete the table below and write as much as details to describe ***your jobs in the last three months***.

If you had more than one job *during the last three months*, please mention all of them	**Job Title**	**Type of company (what did they do)**	***During the last three months,* What were the main tasks you did in your job*? *And what were the hazards (chemicals, radiation,....) you were exposed to (if applicable)?***	**Full or part time**	**Did you have to wear any sort of personal protection during your job**

During the last 3 months, at your job and/or at home, have you worked with, or been exposed to any of the chemicals or physical agents in the table below?

Chemicals or Physical agents at *job/home*	**Please put ✔ in shaded boxes for Yes or NO**		
	Yes ✔		**No ✔**
Metal Dust or Fumes			
Pesticides			
Weed killers (herbicide)			
Fertilisers			
Oils or Greases			
Detergents or Soaps (at job only)			
Glues, Adhesives or Resins (for example, decoration)			
Paints, Varnishes or Lacquers			
Printing Inks or Dyestuffs			
Dry Cleaning Fluids			
PVC or plasticisers (at job only)			
Other Solvents (at job only) **(For cleaning, degreasing, thinning, lubricating, mixing, etc.)**			
Extremely Hot Environments (at job, Sauna, bathing in hot water for long time)			
Heavily Vibrating Machinery, Equipment or Vehicles			

Non-Ionizing Radiation (microwaves, lasers, or high voltage electrical power)			

Health

Have you ever had x-rays? Y N

7.1. **If yes,** was it for the pelvic region? Yes No

8. Have you ever had chemotherapy? Yes No

9. In the last 3 months, have you had any fever or flu? N

9.1. **If yes,** how long did it last? Days

10. In the last 3 months, have you had any illnesses? Yes No

11.1. **If yes,** how would you describe this illness? Please tick

A) Breathing or lung problems.

B) Skin problems.

C) Hearing problems.

D) Stress, depression or anxiety.

E) Headache and/or eyestrain.

F) Heart disease / attack, other circulatory system.

G) Infectious disease (virus, bacteria).

H) Bone, joint or muscle problems which mainly affect:

H1) Arms, hands, neck or shoulder.

H2) Hips, legs or feet.

H3) Back. ☐

I) Other, please specify: ☐

11.2. Were any of these made worse by your work? Yes ☐ No ☐

11. In the last 3 months, have you used any medications? Yes ☐ No ☐
[such as antibiotics, pain killers, antihistamines etc]

11.1. **If yes,** please specify: ☐
[name of medicine, dose and duration (if known)]

12. Do you currently have a varicocele? ☐ No ☐ Don't know ☐
(A varicocele is a medical condition when
veins become enlarged inside the scrotum)

12.1. **If No**, Have you ever had a varicocele? ☐ Yes ☐ No

Lifestyle

13. ***Smoking***

Are you : please tick
All appropriate answers

A ***current*** smoker (*at least* ***one cigarette per day***) ☐

Currently using e-cigarettes ☐

An ***ex-smoker*** ☐

A ***non-smoker*** (***never*** smoked) ☐

If you are a current smoker, please answer questions **14.1** **14.3** then go to question **15**

If you use e-cigarettes, please answer questions **14.4** **14.6** then go to question **15**

If you are an ex-smoker, please answer questions **14.7** **14.14** then go to question **15**

If you are a non-smoker, please answer questions **14.11** **14.14** then go to question **15**

If you are a current smoker

13.1. What do you smoke? (please tick the boxes for all that apply)

☐ Cigarettes ☐ hand rolled cigarettes ☐ cigars ☐ pipes

☐ Other please specify: ……

13.2. How much do you smoke ***a day on average*** (cigs/day, oz/day,other…)?

_____

13.3. At what age did you **start** smoking regularly? _____

If you use e-cigarettes

13.4. How often do you currently use an e-cigarette? (Please tick only one box)

☐ Daily ☐ Less than daily, but at least once a week

☐ Less than weekly, but at least once a month ☐ Less than monthly

13.5. ***If daily***, on average how many times per day do you use an e-cigarette? ☐

13.6. At what age did you **start** smoking regularly? _____

If you are an ex-smoker

13.7. What did you smoke? (Please tick the boxes for all that apply)

☐ Cigarettes ☐ hand rolled cigarettes ☐ cigars ☐ pipes

☐ Other please specify: …….

13.8. At what age did you **start** smoking regularly _____

13.9. At what age did you **stop** smoking? _____

13.10. How many cigarettes (etc) did you smoke ***a day on average*** (cigs/day, oz/day,other…)

_____

If you are an ex-smoker or non-smoker

13.11. Are you exposed to cigarette smoke of other smokers at home, work, club…? ☐ Yes ☐ No

If yes:

13.12. ***During the last three months,*** How many hours a week (on average) have you been exposed to the cigarette smoke of other people? ☐

13.13. Are you exposed to e-cigarette vapor of others at home, work, club…? ☐ ☐

If yes:

13.14. ***During the last three months,*** How many hours a week (on average) have you been exposed to the e-cigarette vapor of other people? ☐

Drinking Alcohol:

 ☐ ☐

14. Do you drink alcohol regularly? Yes No
(***at least one drink per week***)

If yes:

14.1. ***During the last three months***, how much alcohol did you consume on average ***every week*** (please write a number in the boxes when applicable)?

☐ Pints of Lager ☐ Pints of Beer ☐ Measures of spirits (singles)

☐ Bottles of alcopops ☐ Glasses of wine ☐ Measures of fortified wines
(WKD, Smirnoff Ice…) *(Port, Sherry, Martini …)*

Other, please specify ☐

Drug consumption:

15. Have you ever consumed a recreational drug? ☐ ☐

15.1. **If yes**, which of the following drugs have you used in the past year:

☐ Methamphetamines (speed, crystal) ☐ Cocaine

☐ Cannabis (marijuana, pot) ☐ Narcotics (heroin, oxycodone, methadone, etc)

☐ Inhalants (aerosol, glue) ☐ Hallucinogens (LSD, mushrooms)

☐ Tranquilizers (valium) ☐ Other (please specify ……..)

15.2. How often have you used these drugs? ☐ Monthly ☐ Weekly ☐ Daily or almost daily

16. Have you consumed a recreational drug in the last 3 months? ☐ Yes ☐ No

16.1. **If yes**, which of the following drugs have you used:

☐ Methamphetamines (speed, crystal) ☐ Cocaine

☐ Cannabis (marijuana, pot) ☐ Narcotics (heroin, oxycodone, methadone, etc)

☐ Inhalants (aerosol, glue) ☐ Hallucinogens (LSD, mushrooms)

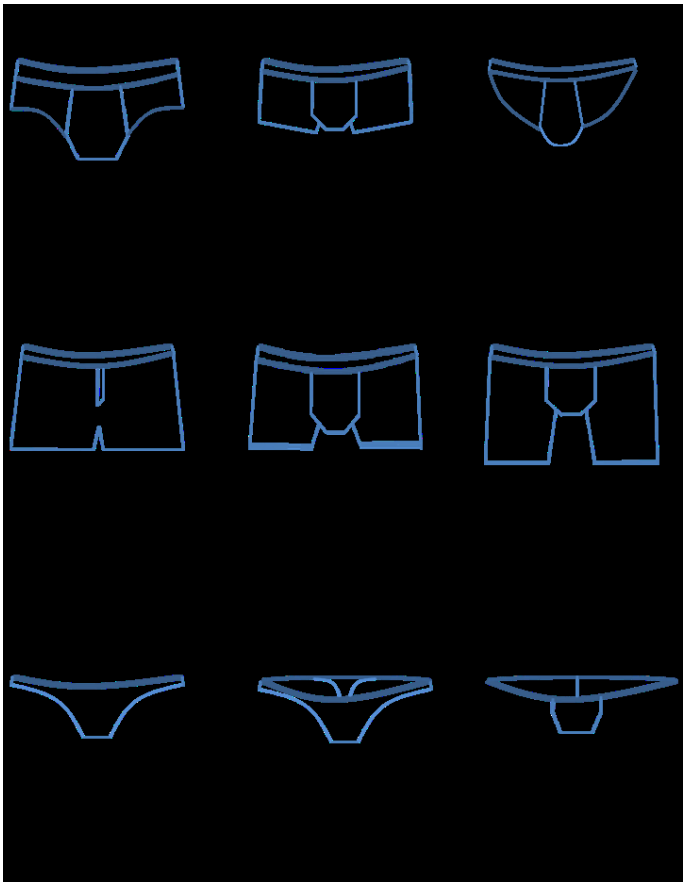
☐ Tranquilizers (valium) ☐ Other (please specify ……..)

Clothing:

17. Do you usually wear underwear? ☐ Yes ☐ No

17.1. **If yes**, Please indicate the style of underwear you typically wear (tick all that are appropriate)

c



17.2. At night, do you usually wear underwear? ☐ Yes ☐ No

Exercise:

18. ***In a typical week*****,** how many times did you do the following kinds of exercise for **more than 15 minutes**? And how **many hours (in total)** per week?

18.1. **Strenuous** exercise ☐ times ☐
Hours ☐
(such as swimming laps, aerobics, calisthenics, running, jogging, basketball, cycling on hills or faster than 12mph, and racquetball)

18.2. **Moderate** exercise ☐ times ☐
Hours ☐
(such as brisk walking, golf, volleyball, cycling on level streets, recreational tennis and softball)

18.3. **Mild** exercise ☐ times ☐
Hours ☐
(*exercise which requires a minimum of effort, such as slow walking, billiard, croquet, putting away groceries*)

19. ***during your leisure-time*****,** how many hours did you do strenuous activity for long ***enough to work up a sweat*** (so that your heart beats rapidly)? ☐
Hours

Dietary Supplements:

20. In the last 3 months, have you taken dietary or exercise supplements (Including protein shakes)? Yes ☐ No ☐

If Yes, Please indicate which of the below are being used and how often ☐

Whey Protein Yes ☐ No ☐ How many Days per Week

Pea Protein Yes ☐ No ☐ How many Days per Week ☐

Hemp Protein Yes ☐ No ☐ How many Days per Week ☐

Soy Protein Yes ☐ No ☐ How many Days per Week ☐

Brown Rice Protein Yes ☐ No ☐ How many Days per Week ☐

Other Protein Yes ☐ No ☐ How many Days per Week ☐

Creatine Yes ☐ No ☐ How many Days per Week ☐

Zinc Yes ☐ No ☐ How many Days per Week ☐

Selenium Yes ☐ No ☐ many Days per Week ☐

Arginine Yes ☐ No ☐ How many Days per Week ☐

Carnitine Yes ☐ No ☐ How many Days per Week ☐

☐ ☐

Calcium Yes No many Days per Week

Vitamin C Yes No How many Days per Week

Vitamin D Yes No How many Days per Week

Vitamin E Yes No How many Days per Week

Vitamin B_6 Yes No How many Days per Week

Vitamin B_{12} Yes No How many Days per Week

Multivitamins Yes No many Days per Week

Glutathione Yes No many Days per Week

Lutein Yes No How many Days per Week

Diet:

21. How would you describe your diet ***during the last three months***?

Vegan eat eater Pe an

Vegetarian Meat and fish eater her

……..

21.1. Would you describe your diet as:

☐ Raw (not boiled) ☐ lant-based ☐ an only

☐ Dairy-free ☐ egg ☐

☐ Wheat-free ☐ luten-free ☐ these

21.2. How long have you been following this diet? ……

21.3. Have you been previously been vegetarian or pescetarian ☐ Yes ☐ No

21.4. **If yes,** please specify diet and how long have you followed this diet?

Previous diet: ……

Duration: ……..…

22. On average, how many portions of FRUIT do you eat a day? ☐
(examples include a handful of grapes, an orange, a glass of fruit juice, a handful of dried fruit)

23. On average, how many portions of VEGETABLES do you eat a day? ☐
(examples includes 3 heaped tablespoons of carrots, a side salad, 2 spears of broccoli)

24. On average, how many times do you eat per day? ☐
(examples, breakfast, lunch, diner, afternoon tea, snacks)

24.1. Please describe briefly your typical daily diet:
(example, "no breakfast" but lunch and dinner with 2 snack breaks)

25. Please answer the following questions (**Table in the next page**) about what you usually eat and drink. Although the list looks a little bit long, it should not take more than 10-15 minutes to complete. Please read the instructions below.

- Please tick (*Never*) column if you have not eaten the food during the last three months.
- If you tick the wrong box by mistake or you want to change your answer, please put a ✕ on the wrong answer and a ✔ in the right box.
- In the last column *("How much do you normally eat **per serving**?")*, you can answer by using the number of unit or number of pieces. For example, 3 apples, 5 tablespoons, 3 sausages, 2 cup of tea, 1 bottle (500ml) of orange juice, 1 pint of milk, 5 slices of bread or toast, large, medium or small serving of broccoli, chicken, beef, ……. .

FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Chicken or other poultry ***(white meat)***					✔			large, medium or small serving
Beef including mince	✔✕			✔				250g or large serving

FOODS	How many times did you eat these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**? Small, Medium or Large
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Chicken or other poultry ***(white meat)***								

Smoked chicken or other poultry								
Beef including mince								
Beefburgers								
Lamb								
Ham								
Bacon								
Sausage or chorizo								
Hot dogs or frankenfurter								
Liver								
Smoked red meat (red meat includes beef, lamb, ham, bacon,…)								

Very fried meat, barbeque, grilled meat (***red and white meat***)								
Pizza								
Pizza (veggie or vegan)								
Fish (dried, frozen)								
Oily fish (fresh or canned e.g. tuna, salmon, sardines,…)								
FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**? Small, Medium or Large
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Fried fish								
Smoked fish								
Oysters								
Shrimps								

Broiled or grilled squid								
Sauerkraut								
Crisps								
French fries, fried potatoes, chips								
Spicy food (medium/hot)								
Vegetable oil								
Olive oil								
Soy flour, Soybeans, boiled, Tempeh, Soy meat.								
Tofu								
Soy Milk								
Almond milk								
Coconut Milk								
Full fat milk								
FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	

		mon th						Small, Medium or Large
Semi-Skimmed milk								
Skimmed milk								
Dried Milk								
Yoghurt (Low fat)								
Yoghurt (Natural)								
Soy Yoghurt								
Dairy-free Yoghurt								
Cottage cheese								
Cheese (all types)								
Halloumi								
Butter / margarine								
Low fat spread or half fat ghee								
Cholesterol lowering spread								
Eggs								
Orange (fruit, juice)								
Blackberry (fruit, juice)								

Blueberry (fruit, juice)								
Cherry (fruit, juice)								
Elderberry (fruit, juice)								
FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**? Small, Medium or Large
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Kiwi (fruit, juice)								
Grape (black/red/blue) (fruit / juice)								
Mango (fruit, juice)								
Pomegranate (fruit, juice)								
Strawberry (fruit, juice)								
Raspberry (fruit, juice)								
Aubergine								

Onion								
Kidney Beans, Black Beans								
Butternut & winter Squash								
Asparagus								
Leek								
Lettuce								
Chinese Cabbage								
Cabbage (other types)								
Beet red (red beet or beetroot)								
FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**? Small, Medium or Large
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Tomatoes								
Carrots								
Broccoli								

Kale								
Spinach								
Parsley (dried)								
Clove								
Cumin								
Basil								
Thyme								
Cinnamon								
Oregano								
Ginger								
Bok choy (Pak choi)								
Chick peas, dried								
Breakfast cereal								
Wheat bran								
Wheat germ (whole wheat bread)								
Biscuits or Cookies								
	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per**

FOODS	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	**serving**? Small, Medium or Large
Weetabix Protein & other protein bars								
Sweet potatoes								
Pumpkin seeds								
Sesame seeds								
Multi-grain bread								
Bread (other)								
Pies, cakes and puddings								
Brown Rice								
White Rice								
Quinoa								
Ice cream								
Dark Chocolate								
Cocoa Power								
Breakfast tea (cups)								
Black tea (cups)								
Green tea (cups)								

Coffee (cups, Not decaffeinated)								
Coffee (cups, decaffeinated)								
Coca-Cola (or non brand coke) Not decaffeinated								
Coca-Cola (or non brand coke) Decaffeinated								
FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**? Small, Medium or Large
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Coca-Cola (or non brand coke) Low sugar								
Other Soda								
Other soda Low sugar								
Red Bull (or non brand) Not decaffeinated								
Other energising drink								
Walnut								
Pecan nuts								

Peanut								
Cashew Nut								
Almond								
Hummus								
Garlic								
Dried Apricots, dates and prunes								
Yeast (nutritional) Such as marmite								

26. Where do you get most of your drinking water at home?

☐ Mains tap water ☐ Bottled water ☐ Other eg well or spring

27. How is your milk usually packaged?

☐ Plastic ☐

☐ Carton ☐ please state ☐

28. How is your vegetable/olive oil usually packaged?

☐ Plastic Gl☐ Other, ☐ state ☐

29. How are your beverages (juices, fizzy soft drink) packaged?

☐ Mostly glass M☐ plastic Mostly ca☐

☐ No packaging Ca☐ Other, ☐ state ☐

Travel:

30. In the last three months, have you been travelling?

☐ Yes ☐ No

30.1. **If yes,** where about?

☐ UK ☐ Another European country ☐ North America

☐ South America ☐ Africa ☐ Asia

☐ Oceania

30.2. **If yes,** which of the following modes of transport have you used to travel:

☐ Car ☐ Bus ☐ Train

☐ Plane ☐ Other, please state …….

30.3. **If you went outside UK**, where did you go? Please specify the duration

Please answer the following questions **about the last 24 hours:**

31. Smoking:

31.1. Have you smoked **in the last 24 hours**? ☐ Yes ☐ No

31.2. **If yes,** what did you smoke? (Please tick the boxes for all that apply)

☐ Cigarettes ☐ hand rolled cigarettes ☐ cigars ☐ pipes

☐ Other please specify: …….

31.3. How much of the above did you smoke (e.g. 5 cigarettes)? ☐

bstin

If No,

31.4. **In the last 24 hours**, were you exposed to other people's cigarette smoke? ☐ Yes ☐ No

31.5. **If yes**, how long were you exposed for? ☐ Hours

32. Use of E-Cigarettes:

32.1. **In the last 24 hours**, have you used e-cigarettes? ☐ Yes ☐ No

32.2. **If Yes** how many times? ☐

32.3. **In the last 24 hours** were you exposed to other people's e-cigarette vapor? Ye ☐ No ☐

32.4. **If Yes** for ☐ long? Hours

33. Have you consumed a recreational drug in the last 24 hours? Yes No

33.1. **If yes,** which of the following drugs have you used:

☐ Methamphetamines (speed, crystal) ☐ ine

☐ Cannabis (marijuana, pot) ☐ tics (heroin, oxycodone, methadone, etc)

☐ ☐

Inhalants (aerosol, glue) Hallucinogens (LSD, mushrooms)

☐ Tranquilizers (valium) ☐ (please specify ……..)

34. How long have you been abstinent?

35. Have you provided the semen sample in MMU? ☐ N☐

36. Attendance at Work

4.1. What is your current job title (e.g. Decorator)?

4.2.. Have you been at work during the last 24 hours ☐ Yes ☐ No

4.3.. If 'yes', was this typical of your normal work ☐ Yes ☐ No

If No, please give more details:

37. In the last 24 hours did you have any of these foods?

37.1. Chicken or other poultry ☐ Yes ☐ No

If yes, please state how cooked (fried, smoked, barbeque, grilled, other …)

37.2. Red meat (beef, lamb, ham, sausages ……) ☐ Yes ☐ No

If yes, please state how cooked (fried, smoked, barbeque, grilled, other …)

37.3. Fish ☐ Yes ☐ No

If yes, please state how cooked (fried, smoked, barbeque, grilled, other)

If there is any information about your job, lifestyle, diet or habit that you think may be of relevance to this study, please use the space below.

Many thanks for filling in the questionnaire.