Exploring RNA Expression in Spermatozoa

M.K.A. Al-Gazi

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Maha K.A. Al-Gazi

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Department of Life Sciences,

Faculty of Science and Engeering,

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We must

make “A” choice To take “A” chance

Or

Our life will never “change”

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List of Abbreviation

5hmc	5-hydroxymethylcytosine
AI	Artificial insemination
ART	Assisted reproductive techniques
ATP	Adenosine Tri Phosphate
BAM	Binary Alignment Map
CASA	Computer assisted semen analysis
cDNA	Complementary DNA
cmyc	transcriptional regulator Myc-like
CpG	Cytosine-phosphate-guanosine motif
CRL	Custom RNA Ladder
DGCR8	dsRNA binding protein
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
dsDNA	double strand DNA
eNOS	endothelial nitric oxide synthase
ERK5	extracellular signal regulated kinase5
FTH1	Ferritin heavy chain1
HML	Ligation Buffer
hsa-miRNA	*Homo sapiens*-miRNA
HSP90A	Heat shock protein 90A
HRL	High-Resolution Ladder
HYA	Hyaluronoglucosaminidase
IVF	in vitro fertilization
JARIDID	Jumonji AT rich interactive domain 1D

KDM5D	lysine demethylase 5D
KLK2	Kallikrein-related peptidase2
miRNA	MicroRNA
MMU	Manchester Metropolitan University
MiRNA	*Mus musculus*- miRNA
mRNA	messenger RNA
NaOAc	Sodium acetate
NGS	next generation sequencing
NKX3-1	NK3 homeobox1
PAR masked	Pseudo autosomal regions masked
PCR	Polymerase chain reaction
piRNA	Piwi-interacting RNA
PML	PCR mix
PAR masked	Pseudoautosomal regions on chromosome Y
PPARγ	Peroxisome proliferator-activated receptor gamma
PrM	Protamines
QC	quality control
qRT-PCR	Quantitative Real-time PCR
RA3’	RNA3’ Adaptor
RA5’	RNA5’ Adaptor
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RP1	RNA PCR Primer

RPI	RNA PCR Index
RPL26	ribosomal protein L26
RPL5, L3	ribosomal protein L5, L3
RPL7	ribosomal protein L7
RPLP0	ribosomal protein lateral stalk subunit
RPS27	ribosomal protein S24
RPS4X	ribosomal protein S4, X-linked
RPS4X	ribosomal protein S4, X-linked
rRNA	ribosomal RNA
RT	reverse transcription
RT	room temperature
RTP	RNA RT primer
RT-PCR	Real-time PCR
SAM	Sequence Alignment Map
siRNA	Small interference RNA
SMCY	Lysine demethylase 5D
sncRNA	small noncoding RNA
SP17	Sperm protein 17 transcript
SPM	Sperm preparation medium
ssDNA	single strand DNA
STAR aligner	Spliced Transcripts Alignment to a Reference aligner
STP	Stop solution
TBE	Tris-buffered saline
TIMP3	metallopeptidase inhibitor3
TPT1	Tumour protein transitionally-controlled 1

tRNA	transfer RNA
UCSC	University of California, Santa Cruz
UHR	Universal Human Resource
UV	Ultraviolet
VmP1	Vacuole membrane protein1
WHO	World Health Organization
ZFD	Zinc finger domain

Abstract

Spermatozoa are known to be a carrier of genetic materials and serve no other function; However, it appears that it has a complex population of RNA including small noncoding RNA (sncRNA) that can deliver to the oocyte during fertilization. sncRNAs contribute to cellular gene regulation, in terms of their role in pre and post-fertilization genomic code, which in turn participate in the embryonic development process and any deviations in the gene expression pattern may lead to development retardation or early embryonic death. Moreover, phenotypic or environmental changes of parents can alter the phenotype of the next generation via specific miRNA expression regulation changes.

The present research investigated the profile of sperm mRNA and miRNA expression in both motile and immotile human sperm, in order to understand the role of sperm epigenetics in regulating genes that are involved in spermatogenesis and sperm function, in specific sperm motility, that has an impact on male fertility. Semen samples were collected from normo-spermic participants. RNA was extracted, and the mRNA profile in motile and non-motile sperm was investigated by Next Generation Sequencing. Results found that most transcripts expressed in motile sperm belonged to ribosomal mRNA.

Secondly, the thesis investigated and the miRNA expression changes in motile and immotile sperm. Preamplification of miRNAs with miscript PreAmp PCR kit before quantitative RT-PCR was performed using misprint PCR custom plate for 84 different sperm-specific miRNAs, in order to establish the miRNA profile in different motility grades.

The results suggested that miRNAs were differentially expressed in different sperm activity groups of the same sample and confirmed the role of miRNA in the physiological process of the spermatozoa. Data revealed that the miRNA expression profile in the sperm could serve as biomarkers for male fertility assessment.

To further, elucidate the relative expression and the epigenetic control of the sperm specific microRNAs in high-fat diet (HFD) mice and age-matched controls (AMC) by study the miRNA expression via qRT-PCR and DNA methylation of the most significant miRNA through finding its promoter region. The aim of this study was to explore the link between obesity and miRNA profile expression in a mouse model. We found that sperm-specific microRNAs from HFD mice were upregulated, with miR-21a-5p expression being highly significant and was regulated by methylation of the CpG islands on VMP1 promoter.

In conclusion, the current study demonstrated a differential expression of miRNA in sperm from motile and immotile populations. In addition, this study revealed links with obesity and altered expression of sperm miRNAs in a mouse model. There was a change in the methylation status and expression of miR-21a-5p, which may indicate the impact that paternal high fat diet has on sperm miRNA expression and DNA methylation.

Publications from this work

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M. Carroll, MK. Al-Gazi, C. Nevin. MicroRNA expression, and sperm motility. Association of Clinical Embryologist Annual Meeting [2015].

Chapter 1: General Introduction

1.1 Male Infertility

Infertility is a complex condition affecting around 15% of couples worldwide. It is well defined as an unsuccessful clinical pregnancy after 12 months or more of regular and unprotected intercourse (WHO, 2010; Hwang et al., 2011; Mascarenhas et al., 2012; HFEA, 2018). The main reasons for infertility in both male and female are varied due to endocrinopathies, infections, obesity, and genetic causes (for review see (O'Brien et al., 2010). Recent studies have reported a decline in male fertility and in particular sperm count (Levine et al, 2017).

Male infertility is due to sperm production or function defects. The main known causes could be a result of tubular obstruction, illness or injuries, cancer, mumps or inherited factors including Y chromosome microdeletions or Klinefelter (XXY) syndrome, and many other idiopathic aetiologies that cause genital tract disorders (Ferlin et al., 2006; Coward and Wells, 2013). Environmental and lifestyle factors also contribute to male infertility such as obesity that causes hormonal imbalance and sperm function defects (Vander et al., 2018). Low sperm count, poor sperm motility (asthenospermia), and high abnormal morphology percentage in the ejaculate are the most likely indicator for low fertilization rate, with an emphasis of the motility importance than other semen criteria (Donnelly et al., 1998; Shen et al., 2019).

Another cause of decreasing sperm quality is obesity, which can alter sperm production and quality through increases scrotal temperature and increased oxidative stress, resulting in reduced sperm motility and increased sperm DNA

damage (Katib, 2015). The prevalence of obesity has increased substantially globally with the increased burden of obesity-related complications. In men, obesity in addition to being a major risk factor for serious chronic diseases, there is a growing concern on the affects of ferritility (Rybar, R., et al., 2011) and more particularly, the long term health of the offspring. Obesity-related impaired spermatogenesis is associated with a decrease in microscopic and molecular sperm characteristics and pregnancy success. Much work is needed to unravel the link between obesity and the impact on sperm molecular andrology, including changes to the epigenome, which may translate to the offspring.

There has been a growing interest in the role of miRNAs and sperm function, in terms of sperm capacity to fertilize the oocyte. It is further suggested, that such sperm-specific miRNAs also drive the developing embryo and its differentiation (Ghorbian, 2012); although still relatively little is known about the mechanisms of action, as to how miRNAs exert their function. Regarding male infertility, several studies have found that some transcripts of miRNAs differ in expression between sperm from fertile and infertile men (Abu-Halima et al., 2013).

1.2 Spermatogenesis

Spermatogenesis is the process of sperm development within the seminiferous tubules in the testis after puberty. During embryogenesis, the activation of SRY gene (sex reversal Y) expression leads to the differentiation of seminiferous tubule from the genital cords in the gonadal medulla accompanied by differentiation of Sertoli cells from the epithelium layer of the tubules (Gilbert,

2000). The primordial germ cells then migrate from the genital ridge of the male embryo to enter the gonads under the control of TGF- beta 1, OCT 4, and alkaline phosphatase pathways and then give rise into sperm by spermatogenesis (Wilhelm et al., 2007).

The testis has a dual function that is regulated by the hypothalamus and pituitary glands: The first function is an endocrine function to produce the androgen (male sexual hormone) by the interstitial Leydig cells. The second function of the testis is the sperm production in the seminiferous tubules from the undifferentiated spermatogonial stem cells (SSCs), these cells are capable of self-renewal and can generate the same cells to produce the spermatogenic lineage via mitotic division, SSCs migrate to the adlumin for cytodifferentiation or spermiogenesis (the last stage of spermatogenesis).

Spermatogenesis requires 64 days and includes three phases:

1. Mitosis: the proliferation and differentiation of the spermatogonium (diploid germ cells, 2n) into type A1…A4 spermatogonium then divided into type B spermatogonium. B cells are the last precursor cells undergoing mitosis to form the primary spermatocytes.

2. Meiosis: the division of each primary spermatocytes to form two haploid secondary spermatocytes (n) in the first meiotic division, and the production of four spermatids in the second meiotic division.

3. Morphogenesis, in which the development and formation of the final elongated haploid spermatozoa with a condensed nucleus that is capable of fertilization within the testis.

Before the final stage, the spermatids are connected by cytoplasmic bridges that will be lost after moving toward the tubule's lumen to form mature sperm. All developmental stages occur in close contact and support of Sertoli cells (Gilbert, 2000). The testis is capable to produce about 300-600 cell per second per gram of testis (Cooke and Saunders, 2002; Coward and Wells, 2013).

At the molecular level, genetic and epigenetic, transcription and post-transcription mechanisms are actively involved in the regulation of spermatogenesis. Perturbations in molecular control are known to be one of the critical causes of male infertility (Khalil and Wahlestedt, 2008; Zamudio et al., 2008; Bettegowda and Wilkinson, 2010).

Transcription in sperm is repressed due to chromatin remodeling and mRNA translation inhibition, the chromatin packaging changes of the round nuclear shape to an elongated one with a trace of cytoplasm content that accompanied by ribosome disappearance, as well as, 60% of histone is replaced first by intermediary transition proteins, then into protamines 1 and 2. In spite of chromatin loss and histone replacement, 15% of the chromatin stayed functional and provides a sperm transcriptional activity (McLay et al., 2003).

1.3 Sperm structure:

Sperm is a Greek word origin "sperma" (means 'seed'). Sperm is a unique, highly specialized and differentiated cell, 60-70 µm in length with the principal oocyte fertilization purpose (Georgadaki et al., 2016). Sperm comprises of basic structures of the followings:

The sperm head, which is typically void of cytoplasm, contains a nucleus filled with the haploid paternal DNA securely twisted around protamine. The acrosome

cap, which lies at the front of the sperm head, contains proteolytic enzymes including acrosin, trypsin, and hyaluronidase that aids in oocyte penetration and fertilization. The sperm-oocyte interaction is facilitated by acrosin through the process of sperm capacitation and acrosomal reaction, which is essential to support the sperm-oocyte penetration reaction (Schill et al., 1988; Harper et al., 2008).

The midpiece holds the centriole with the fundamental purpose for mitosis and meiosis, while the mitochondria are required for sperm survival and the spermatozoa locomotion by oxidative phosphorylation of ATP and energy production.

The flagellum, or tail, that whips in motion to drive the sperm towards the oocyte. The tail functions through the central axoneme surrounded by two central singlet microtubules and nine doublet microtubules that are responsible for the motion by sliding past each other in the presence of ATP (Rauber, 2008; Chavarria et al., 1997), (Figure 1.1).

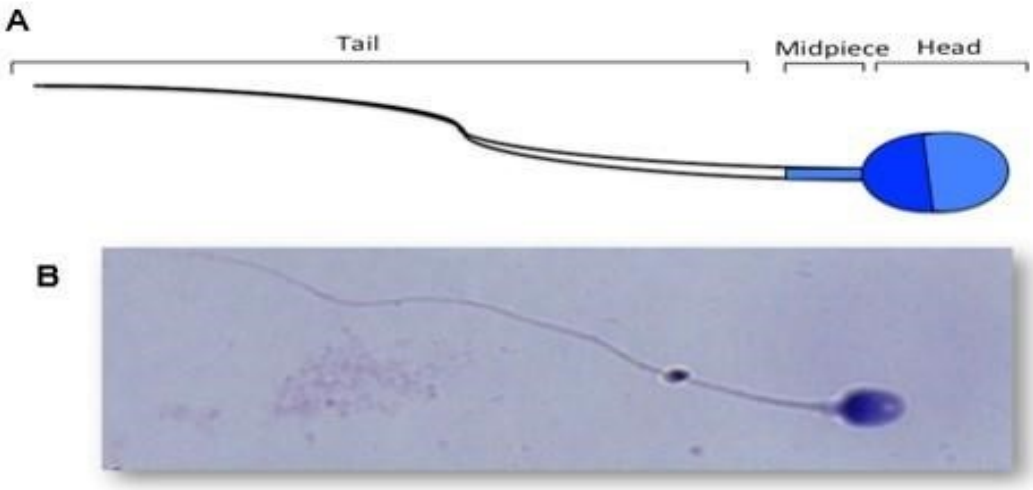

Figure 1.1 Structure of human sperm.

Schematic represents the human sperm head (containing the acrosome), midpiece and tail (principal piece). (B) Sperm morphology stain (Papanicolaou stain, 1000 X oil).

The sperm completes the first maturation process in the epididymis and acquires motility post emerging from the testis, with the aid of secondary glands secretion, which protects the sperm from the vaginal acidic environment. The second process called sperm capacitation in the female reproductive tract and acrosomal reaction activities when the two male and female pronuclei merged (Gervasi et al., 2018). The transport of a sperm-specific phospholipase C-ζ (PLC-ζ) to the female gamete during fertilization and initiation of the PLC-ζ signal and increase Ca++ influx which is essential for embryonic development (Saunders et al., 2002; Boerke et al., 2007).

The molecular changes of the mature haploid sperm cells are regulated by gene expression and accompanied by biochemical and physiological changes such as

the formation of disulfide bonds and the increased level of saturated fatty acids, as well as Ca++ signaling pathway that promotes sperm capacitation and oocyte fusion (Carvacho et al., 2018). Transcription in sperm is repressed due to the general concept that has been established previously of chromatin is an extremely compacted component and the protamines replace the histone content (chromatin remodeling process) and mRNA translation inhibition. The chromatin packaging changes of the round nuclear shape to an elongated one with a trace of cytoplasm content that accompanied by ribosome disappearance, as well as, 60% of histone is replaced first by intermediary transition proteins, then into protamines 1 and 2. In spite of chromatin loss and histone replacement, 15% of the chromatin stayed functional and provides a sperm transcriptional activity (McLay et al., 2003).

The protamine-DNA binding and compaction allow the DNA to fit into the relatively small spermatozoa head (Schagdarsurengin et al., 2012; Kanippayoor et al., 2013; Brunner et al., 2014). The interest of researchers in sperm biology has developed recently to decline the notion that sperm is the paternal genetic carrier only and serve no other function after fertilization, However, sperm molecular composition approved its importance post-fertilization and early embryonic development (Lin et al., 2013; Al-Gazi and Carroll 2015; Guo et al., 2017). A series of small non-coding RNAs (snc-RNAs) is One of the biological key regulator molecules for male fertility, which include the microRNAs (miRNAs) that are found in sperm and seminal plasma (Peña 2015).

1.4 Assessing male infertility

1.4.1 Semen Analysis

Semen analysis is an essential diagnostic assay in the evaluation of male reproductive health. There are a number of specific parameters important in evaluating male fertility according to WHO 2010 criteria:

Sperm concentration and sperm count: Normal sperm concentrations and counts range from 20 million/ml to over 200 million/ml. Concentrations lower than 15 million/ml are referred to as oligozoospermia and azoospermia refer to a lack of sperm in the ejaculate.

Sperm motility refers to the percentage of progressively motile sperm that is traveling in a straight-forward path or large circle with reasonable velocity. The immotile percentage describes sperm that shows no signs of movement; while non-progressive motility denotes an absence of progression, for example, swimming in small circles where the head is barely moving as the result of the flagellar movements (WHO, 2010), (Figure 1.2).

Normal levels of sperm motility are defined as levels higher than 40% of sperm in a sample showing progressive motility. The term asthenozoospermia is used to describe samples in which there is reduced sperm motility below 32 %.

Earlier editions of the WHO manual classified sperm motility into 4 grades:

Grade A (A): Rapid progressive motility (i.e. ≥25 μm/sec at 37°C, which is approximately equal to five heads length or half a tail length).

Grade B (B): Slow or sluggish progressive motility.

Grade C (C): Non-progressive motility (<5 µm/sec).

Grade D (D): Immotile sperm that fails to move at all.

A high percentage of active sperm is obviously important to ensure the transport of sperm and achieve successful fertilization (Agarwal and Allamaneni, 2011; WHO, 2010). The sperm reaches to the female ovulation tract with the aid of specific molecules that act as chemotactic agents for the sperm, in the presence of Ca++ ions, to enhance sperm hyperactivation and penetrates the oocyte cumulus oophorous. The sperm that is aggressively motile can resist the female oviduct turbulences close to cilia and enter the lumen of fallopian tubes, hence stimulates the prostaglandins secretion and utero muscular contraction that are vital for fertilization (Kölle et al., 2015).

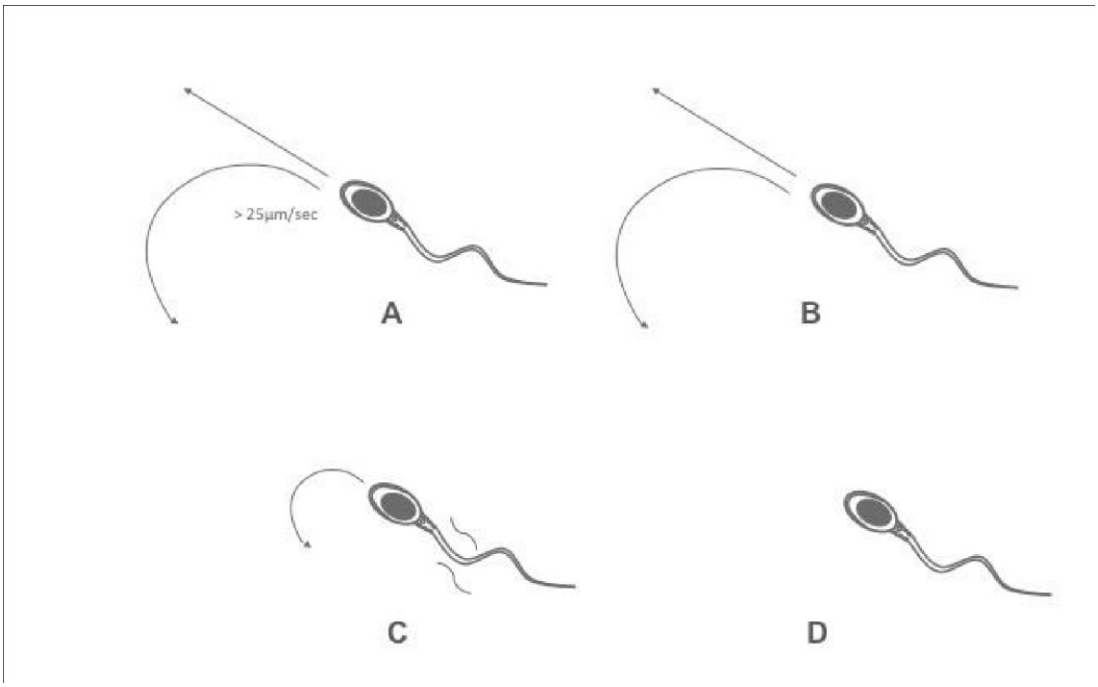

Figure 1.2 Sperm motility groups.

A – fast progressive sperm, moves linearly or in large circles quicker than 25 µm/sec. B – slow progressive sperm, moves linearly or in large circles slower than 25 µm/sec. C – nonprogressive sperm, moves in small circles or 'twitches'. D – immotile sperm, no movement.

Sperm morphology: The presence of a wide range of morphological discrepancies between sperm in a population can make it difficult to judge the sample provided *in vitro*. Although the level of the clinical importance of morphology to fertility is debated, the normal morphology percentage that correlates with the success of fertilization *in vivo* or *in vitro* defined as over 4%. The term teratozoospermia describes the presence of a high percentage of abnormal spermatozoa in the semen sample, that were classified into three different categories depending on the abnormality location: head (acephalic, giant head, or multiple head), midpiece (presence of cytoplasmic droplet) and/or tail (short, long, or double tail). The main abnormality causes may be due to genetic, high-temperature exposure to the testes, and chemical or toxic substances contact, as well as lifestyle, and infections (Coutton et al., 2018).

Aspermia is another term that describes the lack of semen completely from the ejaculate as a result of the passing of semen into the bladder at ejaculation (WHO, 2010).

1.4.2 Sperm RNA

The concept that sperm carries only paternal DNA and the paternal RNA content in mature sperm were lost during spermatogenesis, and therefore served no function during fertilization has been challenged (Kramer and Krawetz, 1997). However, it is now established that spermatozoa contain a wide spectrum of RNA species including mRNAs, tRNAs and various non-coding RNAs (ncRNAs) such as miRNAs (Boerke et al., 2007). Studies have found that sperm RNA regulation provides a critical role in the process of fertilization when they are utilized to the

oocyte and participate in regulating the paternal genome and various biological events involved in early embryonic development (Miller et al., 2005; Lalancette et al., 2009; Kildemo, 2012). A cascade of paternal pathways may involve in regulating the events of postfertilization such as AKAP-4 and FOXG1B, and provide a level of control of the maternal genome transition into embryonic status (Ostermeier et al., 2005; Boeker et al., 2007).

Importantly, sperm does not contain ribosomal (r)RNA (18S and 28S rRNA), which are essential for protein synthesis, and they were depleted during spermiogenesis. Therefore, sperm cells are transcriptionally and translationally quiescent (Johnson et al., 2011).

mRNA and miRNA are RNAs that participate in gene regulation of sperm post-transcriptionally by controlling cellular fate and development and can be transferred through germ cells to the next generation (Krawetz, 2005; Ivey and Srivastava, 2010).

1.4.3 Sperm messenger RNA

Pessot 1989, was first who described the Messenger RNA (mRNA) in transcriptional inactive sperm (Pessot et al., 1989). mRNA is one kind of long RNA (comprises about 5% of total RNA) that is transcribed from DNA strand to form antisense strand that is used as a template for translation and the encoding proteins through genetic codes (Kukurba and Montgomery, 2015).

The translational ability of mRNA comes from the structure that contains methylated 5’cap end and 3’ polyA end units of non-coding sequence make it more stable and capable of translation into proteins. The coding region is the unit

of protein synthesis while the UTR region is for the expression regulation (Figure 1.3), (Kozak, 2005). During transcription, the mRNA contains exon regions only, while the intron regions are removed from the primary transcript during the silencing process of gene transcription (Keren et al., 2010).

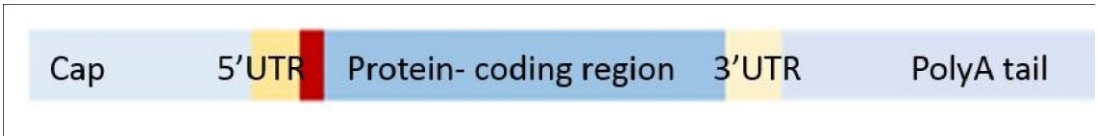

Figure 1.3 mRNA regions. The figure is representing the five and three prime untranslated regions of mRNA transcript.

Mature sperm contains more than 3000 kinds of functionally viable mRNAs that play a critical role in gene expression during spermatogenesis, capacitation, acrosomal reaction, and onwards when has been delivered to the oocytes post fertilization (Ostermeier et al., 2004; Gur and Breitbart, 2006). Spermatozoal mRNA are not all located in the nucleus, some of them are present in the flagellar fibrous sheath like SP17 transcript (ChirivaInternati et al., 2009), or on the sperm surface like SMCY (also known as KDM5D, HYA or JARIDID), which have a major role after fertilization (Anderson, 2013). SMCY encodes a protein of ZFD, from this protein is a minor histocompatibility antigen for a graft rejection of sperm donor in an oocyte recipient (Dhanoa, Mukhopadhyay et al., 2016). SMCY is mainly functioned during sperm prophase stage of meiosis and in chromatin remodeling, any mutation in this transcript can lead to sperm maturation arrest and chromosome condensation during meiosis (De Jonge and Barratt, 2006).

Sperm becomes incapable of transcription during the second meiosis of spermatogenesis and decreases mRNA transcription together with a decrease in a repertoire of mRNA content and 18S and 5S rRNA in the nucleus or present in

a negligible amount and is not enough to support cytoplasmic mRNA translation (Miller and Ostermeier, 2006). These events include histone hyperacetylation subsequent by replacement of histones by transition proteins mainly TNP1/2 then by protamines (PRM1/2) that condensed in the sperm nucleus that results in RNA content reduction and loss of transcription in spermatids which demand mRNA transcription in a high level but translationally delayed to another period of genesis (Cullinane, 2014). New proteins are needed for sperm morphological changes during maturation. rRNA is depleted in sperm during spermatogenesis, however, 95% of RNA in other cells are containing rRNA, which needs rRNA depletion step to get rid of this kind of RNA during the mRNA sequencing process.

1.5 Gene expression

Regulation of gene expression is imperative for the control of cell function and fate (Smorag, 2013). The processes of gene expression are comprising of transcription, RNA splicing, protein synthesis or translation, and post-translational modification of proteins. Gene expression may be modulated to alter the mRNA sequence level, which results in different protein structure, and can be studied to find out which genes are turned “on” to produce mRNA or “off” and enables to know the mRNA concentration can manipulate various cellular development phases. Different methods were used in studying the gene expression like Northern Blotting, SAGE (serial analysis of gene expression), microarrays, RT-PCR and RNA-Seq (Perdacher, 2011; Su et al., 2011).

This can explain how similar cells can behave differently and explains the phenotypic changes between species. Apparently, proteins have got an important

characterization in regulating gene expression, however, the discovery of ncRNA including miRNA has found that they are the most abundant RNA in gene regulation process post-transcriptionally by repressing mRNA (Bartel, 2004).

1.5.1 Sperm Epigenetics:

Epigenetics (“epi” from the Greek: means outside or over) or non-Mendelian para-mutation inheritance describes the alteration of gene expression without affecting the coding DNA sequence of the cell; therefore, it is relatively transient and potentially revised if the cause is corrected (Rassoulzadegan and Cuzin, 2015). Lifestyle and environment signals can promote diseases and health derivation via epigenetic mechanisms, such as histone modification, chromatin remodeling, DNA methylation, and non-coding RNA including miRNA expression (Lujambio and Esteller, 2009; Marczylo et al., 2012). There is growing evidence indicating that epigenetics could have a significant role in subfertility and the link between fertility and health (Dada et al., 2012), and any epigenetic modification during spermatogenesis can cause deleterious effects on sperm epigenome and affect its function and the subsequent progeny health (Schagdarsurengin et al., 2012; Marshall, 2015).

Small ncRNAs are epigenetic small molecules with a vast impact in regulating biological processes during mammalian development, non-coding refers to non-translated transcripts and most of them are cellular homeostasis regulators substances that are classified to be epigenetic regulators at a transcriptional and translational level in general. In sperm, miRNA, Piwi-interacting RNA (piRNA) and siRNAs are some sorts of short snRNAs (Santosh et al., 2015).

siRNAs (20-30 nt length) are endogenous molecules resulted from the cleavage of the dsRNAs by Dicer enzyme and regulating cell functions by RNA interference phenomenon or RNAi to initiate gene silencing by destabilisation of mRNAs, hence controlling cellular growth and development as well as the formation of heterochromatin (Dana et al., 2017). siRNAs have been implicated therapeutically for some diseases like cancer through its antiapoptotic and anti-proliferative action (Phillips, 2008).

piRNAs (24-31 nt length) have been counted as the largest class of small RNAs in mammalian tissues, it has been reported that they have an RNA silencing regulating function in germ cells especially spermatogenesis and sustain male fertility via their interaction with piwi protein (Tosar et al., 2018; Siomi et al., 2011; Capra et al., 2017).

Finally, miRNAs are considered as key regulators of all cellular functions. Regarding male gametes, a small amount of miRNA has been retained in sperm and are vital in the development of both sperm and embryos (Krawetz et al., 2011).

Another functional class called long noncoding RNA (lncRNA) which is known to have a function in chromatin remodeling, controlling the transcription and post-transcription events (Guttman et al., 2009).

1.5.2 miRNA

miRNAs are a novel class of endogenous ~17-26 nucleotide length, which control post-transcriptional gene expression by targeting 3'UTR mRNA resulting in its degradation or later protein expression inhibition, thus involved in all biological

process of living organisms. miRNA was first discovered in *C.elegans* in 1993 when lin-4 miRNA was detected with a conserved complementary site on the lin-14 mRNA transcript (Lee et al., 1993; Wightman et al., 1993; Schickel et al., 2008). Later on, let-7 has been found to target the lin-41 and they have crucial effects on the developmental timing of *C.elegans* larvae (Reinhart et al., 2000). Lin4 and let-7 are highly conserved and have the same function in other species (Lagos-Quintana et al., 2001).

It has been well established that miRNAs have an impact on the pathogenicity of common diseases such as cardiovascular diseases, tumourigenesis, immune-inflammatory diseases, and metabolic disorders (Mogilyansky and Rigoutsos, 2013).

Studies on the relationship between miRNA and stress, which cause up or down-regulation of miRNA expression have revealed the mRNA targets effects and cellular response. However, the absence of miRNA function can cause impairment in the whole process of cellular development (Mendell and Olson, 2012).

Thousands of miRNA have been discovered, and an online database with analysis was established first by Ambros laboratory 2003 (Ambros et al., 2003). miRNA annotation and registry including all information about published miRNAs data can be explored via miRBase database system (http://www.mirbase.org/) by Sanger Institute. miRBase contains more than 25141 mature miRNAs in about 193 different organisms (Griffiths-Jones 2004) or miRNA visa system database (www.cbrc.kaust.edu.sa/mirnavisa.org), (Kamanu et al., 2013). Development of deep sequencing techniques opened a new way of research of the miRNAs and the studying of the profile and function in normal and aberrant conditions as well

as prediction of new miRNAs which gave a hallmark of the miRNA importance (Kong et al., 2012).

1.5.2.1 miRNAs families and functions

miRNAs are situated in polycistronic (within one locus) miRNA “clusters” that have a similar functional role and are suitable for disease biomarkers such as cancer and cardiovascular disease (CVD). miRNAs families that have perfectly matching nucleotides in the seed region (2-7 nt) from 5’ end, as well as the complementary site for 3’UTR mRNA in this region, are highly conserved across species, therefore they are generated from a single primary transcript and regulating the same target genes (Guerra-Assunção and Enright, 2012). For example, miR 17~92 cluster, also known as oncomiR-1 because they are dysregulated in solid cancers, is located on chromosome 13 in human and include a range of different miRNAs that are important in normal development (Table 1.1), has been included a range of different miRNAs that are important in normal development. Also known as oncomiR-1 because they are dysregulated in solid cancers. miR-17-5p is highly expressed in some tumours while showing low expression pattern in the blood of non-small lung cancer patients. This family of miRNAs is also implicated in age-related conditions (Heegaard et al., 2012; Mogilyansky and Rigoutsos, 2013). A wide range of functions of miR-17 have been revealed such as enhancement of the prostate tumour invasion and growth through cellular proliferation exaggeration of tumour cells has approved by targeting TIMP3 (Concepcion et al., 2012). Furthermore, these miR-17 clusters have been involved in adipogenesis and found to have an inhibitory role and

adipogenesis promotion by targeting BMP2 mRNA along with miR-106a (Xu and Wong, 2008).

Table 1.1 miR-17 family.

The seed regions of 2-8 from 5' end of miRNA sequences of miR-17 family are having the perfectly matching nucleotide (Hausser et al., 2013; Mogilyansky and Rigoutsos, 2013).

miRNAs	miRNA sequences and the shaded seed region
hsa-miR-17-5p	5'CAAAGUGCUUAGUGCAGGUAGU 3'
hsa-miR-20A-5p	5'UAAAGUGCUUAUGUGCAGGUAG 3'
hsa-miR-20b-5p	5'CAAAGUGCUCAUAGUGCAGGUA 3'
hsa-miR-106b-5p	5' UAAAGUGCUGACAGUGCAGAU 3'
hsa-miR-93-5p	5'CAAAGUGCUGUUCGUGCAGGUAG 3'
hsa-miR-106a-5p	5'CAAAGUGCUAACAGUGCAGGUA 3'

Another example, lethal-7 (let-7) miRNA family has similar sequences and only one nucleotide differs in order. To determine this difference, a letter at the end was added as let-7a, let-7b, let7c, etc. (Table 1.2). The most important function of let-7 family is to promote differentiation and timing of the development of organisms (Schulman et al., 2005; Roush and Slack, 2008). Let-7 dysregulation can cause cellular growth and development retardation and promotes diseases like cancer (Roush and Slack, 2008).

Table 1.2 Lists of isoform sequences of let-7 miRNAs family, the shaded area is representing the miRNA seed region

miRNAs	miRNA sequences and the seed shaded region
hsa-let-7a	5' UGAGGUAGUAGGUUGUAUAGUU 3'
hsa-let-7b	5' UGAGGUAGUAGGUUGUGUGGUU 3'
hsa-let-7d	5' UGAGGUAGUAGGUUGUAUGGUU 3'

hsa-let-7e	5’ UGAGGUAGGAGGUUGUAUAGUU 3’
hsa-let-7f	5’ UGAGGUAGUAGAUUGUAUAGUU 3’

1.5.2.2 miRNA biogenesis: synthesis and functions

miRNAs are transcribed from specific genes by RNA polymerase II in the nucleus. They are first formed as primary transcripts (pri-miRNA) about 70nt, then folded into hairpin structures that are successively processed by several enzymes in the nucleus called Drosha and DGCR8 then transported to the cytoplasm by Expotin5 to be processed by Dicer enzyme (Almeida et al., 2011; Al-Gazi and Carroll, 2015), (Figure 1.4). A 22 nucleotide duplex, mature miRNA is then produced in which one strand assembles into a protein-RNA complex called RISC (RNA induced silencing complex) as a part of gene silencing process called interference RNA (RNAi) (Leung and Sharp, 2010). The small size of miRNA made them less prone to degradation and more stable than other types of long RNAs and mediates post-transcriptional gene suppression (MacRae et al., 2008; Zubakov et al., 2010). A single miRNA species can base pair to its target on multiple sites within a single mRNA transcript to cause their degradation. Additionally, one miRNA can regulate the expression of multiple gene targets and furthermore a single mRNA transcript can be targeted by more than one miRNAs (Smorag, 2013). miRNA and protein-coding genes expression regulation occur post-transcriptionally. Basically, DNA binding proteins like P53 and other transcription factors bind to the miRNA promoter region and regulate their expression (Boominathan, 2010). miRNA can reduce the protein output by inhibiting their translation without changes in the level of mRNA targets (Bagga et al., 2005; Curry et al., 2011).

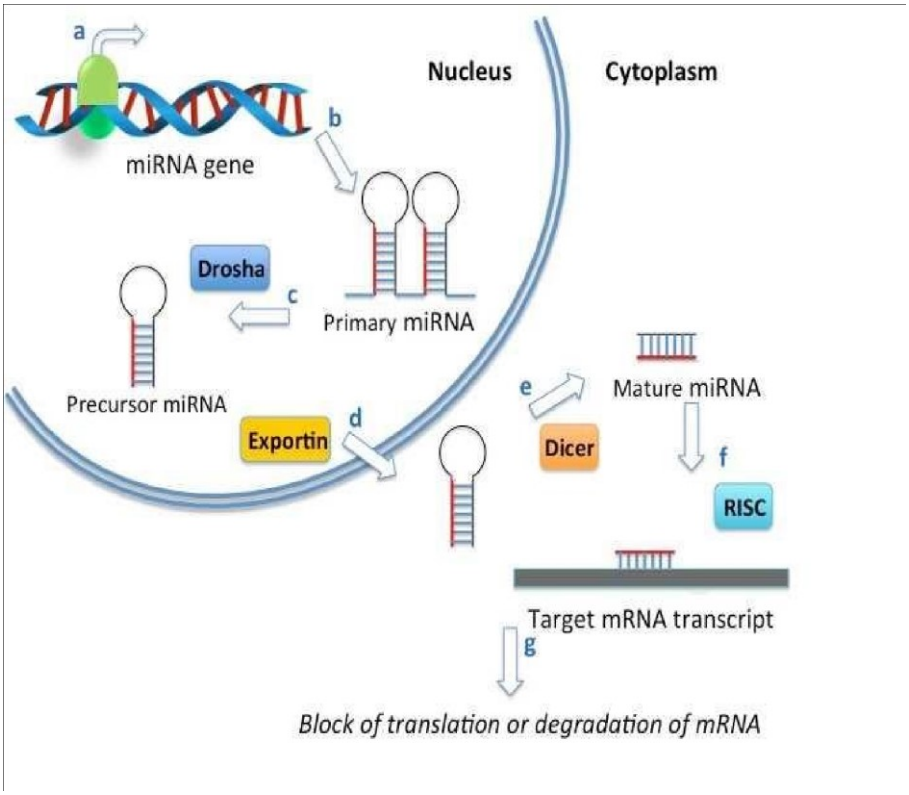

Figure 1.4 Schematic representation of miRNA biogenesis.

a) miRNAs transcription is conducted via RNA polymerase II; b) A double-stranded hairpin called primary miRNA (pri-miRNA) is then is formed. c) pri-miRNA then cleaved by the help of Drosha, a member of superfamily RNase III endonuclease to form pre-miRNA molecule. d) exportin-5 is then assisted in pre-miRNA transport to the cytoplasm. e) Another enzyme called Dicer is then involved in processing pre-miRNA in the cytoplasm to produce short and double-stranded miRNA. f) The pre-miRNA act together with AGO (Argonaute) and other proteins to form the RISC component, and finally, the formation of a single-stranded mature miRNA. and g) mature miRNA targets the mRNA (adapted from (Al-Gazi and Carroll, 2015).

miRNAs have a critical biological function upon binding at nucleotides 2-8 seed region to a complementary 3'UTR sequence of specific mRNA, leading to either mRNA degradation or protein translation arrest thus, influencing cell functions including gene expression mediation during development, differentiation, cell proliferation, cell fate decision, and stress response, apoptosis and death (Yerramilli et al., 2013). The half population of miRNA is encoded independently within noncoding gene transcripts while the others within intronic protein-coding genes (Guerra-Assunção and Enright, 2012). An individual miRNA could target

more than one protein coding mRNA post-transcriptionally through multiple pathways or act upon a single target to mediate a disease phenotype (Aurora et al., 2012). Nevertheless, until now little is known about their mechanism of action and how they exert their function. Regarding male infertility, the sperm contains an abundant profile of miRNA together with a set of mRNA targets also expressed in fertilized metaphase II oocyte, suggesting miRNA regulatory functions (Amanai et al., 2006). For instance, miR 143 (Esau et al., 2004), miR-27b (Karbiener et al., 2009), miR-375 (Ling et al., 2011), and miR-14 were involved in adipogenesis in mice. They act as a modulator for adipocytes differentiation, as well as miR-122, miR-370 (Iliopoulos et al., 2010), miR-335, miR-378/378*, and miR125a-5p all have a function in fatty acid and cholesterol metabolism regulation (Fernández-Hernando et al., 2011).

1.5.2.3 The relationship of miRNA and motility in male fertility:

Sperm motility is a critical factor in assessing male fertility. Several studies have reported differential miRNA expression between impaired and normal semen samples implying that miRNAs are key regulating players in spermatogenesis and production of a new, viable sperm in males (Wang et al., 2004; Reza et al., 2019). Some studies have established that some gene transcripts are regulated by miRNAs related to sperm structure, sperm morphology, motility, and metabolism. miRNAs studies were conducted on human sperm miRNAs with abnormal motility outcomes have found a positive relationship between the miRNAs expression level and semen quality which significantly affects reproduction patency (Ghorbian 2012; Abu-Halima et al., 2013). In addition, gene transcripts related to sperm motility are regulated by miRNAs. They have a role

during spermatogenesis, chromatin packaging, and early embryonic development, when delivered to the oocyte after fertilization, it can be passed down to the further generations (Wang et al., 2004; Jedrzejczak et al., 2007; Jodar et al., 2012; Kawano et al., 2012; Liu, Cheng et al., 2012; Abu-Halima et al., 2013).

miRNAs have different expression patterns between fertile and infertile men (Khazaie and Esfahani, 2014). Altered expression levels of sperm-specific miRNAs have been implicated with abnormal sperm parameters (Abu-Halima et al., 2013) and the propensity for transgenerational amplification of some conditions such as obesity and type-2 diabetes (Fullston et al., 2013). Marczylo 2012, reported that environmental changes targeted histone modification and miRNAs (for instance, altered the expression of hsa-mir-146b-5p, hsa-mir509-5p, hsa-mir-519d, and hsa-mir-652) profile in the sperm of infertile men as well as leading to alter next generation phenotype through impairment of male germ cells functions (Marczylo et al., 2012). Sperm function is influenced by miRNA expression changes. miR-122 is a specific sperm motility-related transcripts that have a role in male fertility via targeting TNP2, a testis-specific gene that involved in chromatin remodeling during spermatogenesis (Yu et al., 2005; Jodar et al., 2012; Lin et al., 2012). Hence, miR-122 inhibits the expression of proteins that have a significant impact on sperm development process (Liu et al., 2013). Sperm miRNAs are responsible for early embryonic development in mice (Liu et al., 2012). miR-34c was found in the sperm and it is important for cellular maturation, its inhibition causes detrimental development in mouse zygote (Choi et al., 2011) and expressed in human sperm by targeting DLL1 and NOTCH1 genes important for spermatogenesis (Krawetz et al., 2011). It is obvious that the lack of specific

miRNA can result in spermatogenesis impairment and infertility (Belleannée, 2015). miR-18 has a significant role in spermatogenesis by targeting Hsf2 gene (Björk et al., 2010).

miRNA function in spermatogenesis is still unclear. However, previous studies have compared normal fertile with impaired infertile cohorts (Figure 1.5), but to our understanding, there have not been any studies verifying miRNA expression in the same group of spermatozoa, i.e. actively motile and non-motile groups of spermatozoan according to the grading system of motility.

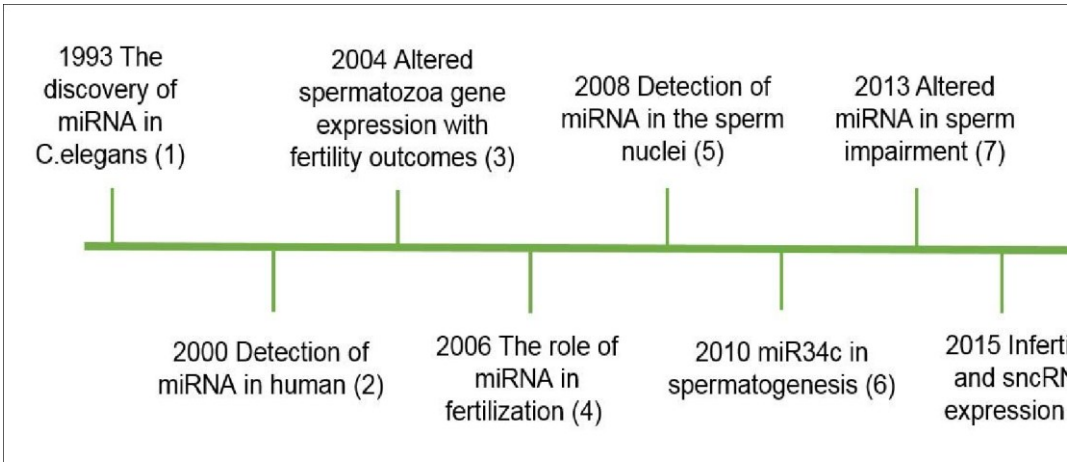

Figure 1.5 Historical miRNA discovery through a timeline since the first miRNA has been discovered in 1993.

Different studies of miRNAs in spermatozoa. 1 (Almeida et al., 2011), 2 (Wild and Roudebush, 2000), 3 (Garrido et al., 2004), 4 (Amanai et al., 2006), 5 (Yan et al., 2008), 6 (Bouhallier et al., 2010), and 7 (Abu-Halima et al., 2013).

1.5.2.4 miRNA and early embryonic development

miRNAs are important for normal human and animal development. Changes or loss of the miRNA expression can cause embryonic death through the posttranscriptional mediation of pluripotent cells (Berardi et al., 2012). Different miRNAs can be specific targets of different pathways and transcripts during spermatogenesis and early embryonic development. miR-122 and miR-34c are spermatids specific miRNAs and maintain cell development by targeting c-Kit and Tnp2 genes (Figure 1.6) (Smorag, 2013).

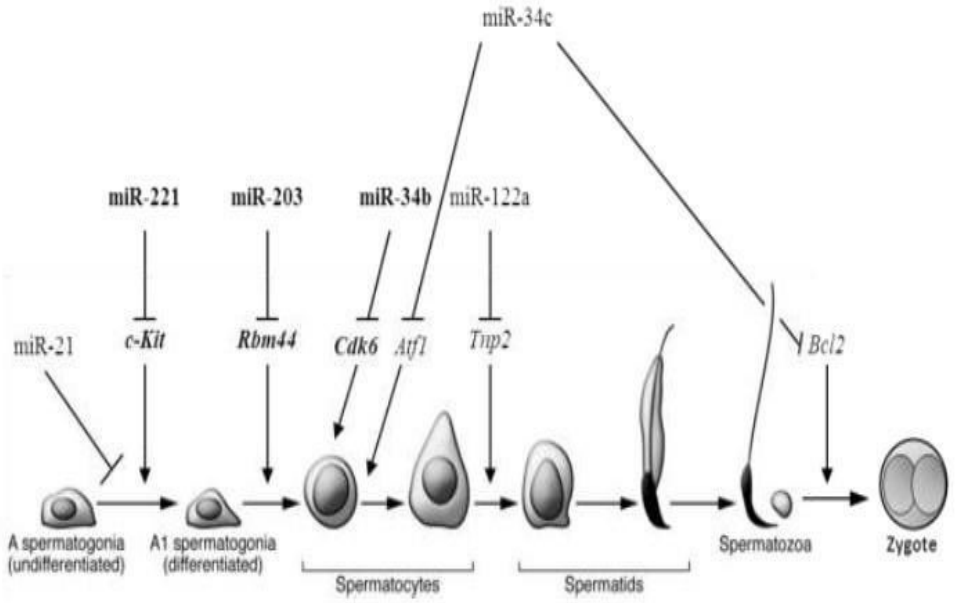

Figure 1.6 miRNA and spermatogenesis.
miRNA targets different genes during spermatogenesis and after fertilization (Smorag, 2013).

Let-7 was the first miRNA that have found related to the development of *C. elegans* larvae by suppression of lin-28 and controls the transition of the larval stage into an adult (Reinhart et al., 2000). Lin (2013) reported that miRNA is highly expressed in the human embryos and some specific miRNAs are

upregulated at various stages of development as well as in tumorigenesis, which was confirmed using the microarray technique (Lin et al., 2013).

miR-17-192 cluster also was downregulated in week 6 of human embryogenesis and have oncogenic features at the same time by targeting CDKN1A (p21) and RUNX1 which is very important in controlling cell cycle and apoptosis. miR-290-295 cluster in mouse similar to 371-373 cluster in human and miR-302 clusters have a vital role during embryogenesis, also important in the cell cycle of embryonic stem cells and pluripotency phase by manipulating the expression of some cell cycle gene inhibitors like Rb1, Rbl1 (Yuan et al., 2017), (Figure 1.6). they regulate embryonic stem cells by repressing mRNA embryonic stem cells regulators transcripts such as Nanog, Oct4, and Sox2 transcripts (Figure 1.7).

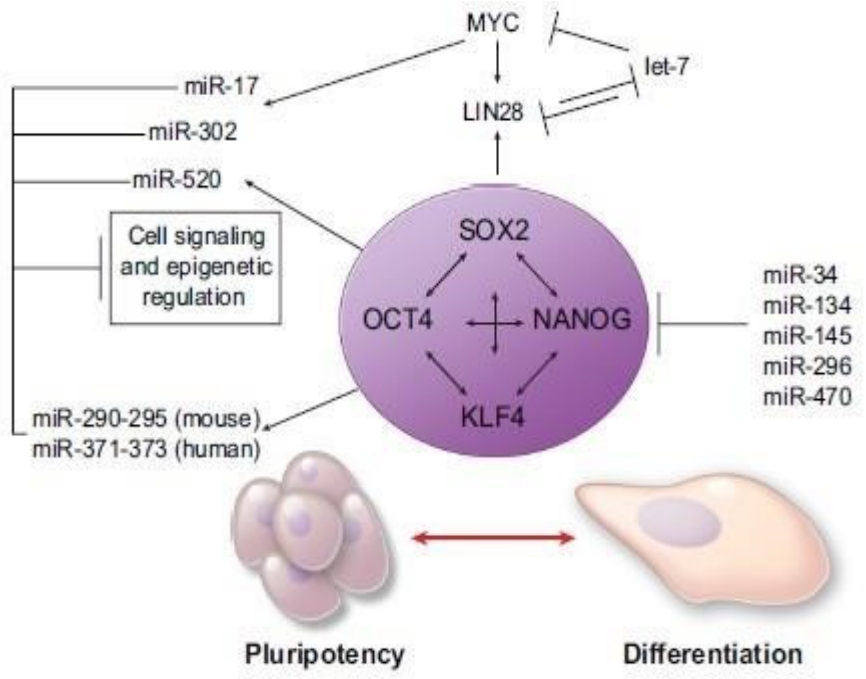

Figure 1.7 miRNAs and embryogenesis signaling pathway schematic diagram. The miRNAs target genes involved in the up-regulation of core pluripotency factors that have an impact in embryogenesis and pluripotency.Let-7 repress differentiation transcripts (lin-28 and MYUC) and lin-28 conversely repress let-7 to maintain embryonic stem cell development. miRNAs on the right are suppressing other transcript and involved in the process of development also by repressing Casp3 leads to apoptosis. Pointed arrows representing activation while blunted arrows for repression. (adapted from (Berardi et al., 2012).

1.5.2.5 Methods of miRNA quantification

To study the cellular performance of miRNAs, mature miRNAs profile is the main target in normal and disease condition of different organs (Wark et al., 2008). Because of the miRNA short nucleotides length and limited expression level in a confined cell at a particular stage, as well as large sequence similarities make it a difficult molecule to isolate. Numerous quantification techniques were identified to quantify the miRNA expression with some limitations of advantages for each method (Baker, 2010).

1.5.2.5.1 Quantitative real-time PCR (qRT-PCR)

The polymerase chain reaction (PCR) is one of quantification method that allows a specific known region of DNA amplification using oligonucleotide primer complementary to the known sequence of DNA template. The DNA polymerase then used to make an extension of the primers on single strand DNA (ssDNA) in the presence of dNTPs under specific conditions, after that heat denaturation of double strand DNA (dsDNA) and cooling annealing (primer binding) to synthesize new DNA strands. The data output of quantification of gene expression using RT-PCR by cDNA amplification and transcription from RNA samples is expressed as a fold change or a fold difference of expression levels (Newton and Graham,

1994). qRT-PCR needs normalization of cDNA with the same RNA input to ensure the actual output obtaining using fluorescent reporter molecules emitted following each PCR cycle (Bustin et al., 2005).

1.5.2.5.2 Microarray

The microarray is a potent high-throughput implement to profile a large number of miRNA in parallel. Around 1000 miRNAs simultaneously can be detected in one run using a flexible probe and fluorescent dye, in order to label the specific gene of interest then dsDNA was formed by hybridization. Finally, the genes and their expression level were detected (Liu et al., 2008; Yang et al., 2008). However, it has several limitations like requisite of a previous understanding of the sequence being investigated, also the cross-hybridization of similar sequences, besides the inability to identify low or high expression level genes (Shendure, 2008).

1.5.2.5.3 Illumina High Through-put Next-generation sequencing

High Throughput Next-Generation Sequencing (NGS) is an advanced technology that allowed DNA and RNA sequencing directly by the synthesis *in situ* and dNTP can be detected simultaneously by Illumina sequencer system on a flow cell cartridge at millions of specific positions to provide an inclusive understanding of gene nature and function (Corney, 2013). RNA sequencing also known as RNA-Seq is a procedure of transcriptome analysis including mRNA analysis using various methods to study the presence and quantity of RNA in biological samples and to investigate gene expression profiling of the organisms. Simply, RNA samples are converted into cDNA library fragments to obtain short reads between 200-500 bp to be sequenced on Next-Seq 500 (an Illumina Genome analyzer

with a high throughput sequencing platform), which then aligned to a reference genome to generate a base-resolution expression profile (Datta and Nettleton, 2014).

1.5.2.6 miRNA therapy

miRNA are now well approved as biological pathways and functions regulators, and participating in the disease development. Various research has tried to modify miRNA to renovate therapeutics to be offered in the markets especially cancer therapeutic. With the unique miRNA conserved sequence among species that made it easy to synthesize to produce anti-miRs that have a high affinity to bind to miRNAs target and causing degradation of over-expressed miRNA (Christopher et al., 2016). Inactivating the pathological miRNAs and correcting the imbalance in genetic pathways caused by miRNA dysregulation can be achieved via introducing artificial miRNA, which will lead to increase the miRNA amounts and functional inhibition will be initiated (Negrini et al., 2007).

1.6 Summary

miRNA emerges to participate in many cellular function and biology and needs further investigations. This chapter of current research will emphasize the potential RNAs specific for progressively motile sperm, non-progressively motile sperm, and immotile sperm and may lead to identifying novel pathways and biomarkers associated with male infertility.

1.7 Hypothesis

Expresisons of mRNA and subpopulations of sperm micro RNAs responsible for sperm function and fertility is of increasing interst both in terms of sperm biology and potential fertility biomarkers.

The main aim of this research is to explore the relative epxresion of RNA in the sperm contains a myriad of RNA that expressed differentially in term of sperm activity.

The second aim is to explore the relative expression of mRNA and miRNA from different populations of isolated sperm cells from humans and to investigate the relative expression of sperm-specific miRNAs in an obese mouse model.

To achieve this, two main objectives of our research were:

1. Isolate and sequence humam sperm mRNA from motile and immotile sperm, establish optimal sperm RNA isolation [mRNA dan miRNA] and amplificaiton methodology from sperm samples – and sequence using the Ilumina platform.
2. To investigate the impact of highfat diet and obesity on the differtntial expression and epigenetic regulation of miRNA populations – an obese mouse model was utilised.

Chapter 2: General Materials and Methods

2.1 Exploring the relative mRNA expression in motile and immotile human sperm

In order to explore the transcriptome content of motile and immotile human sperm, RNA sequencing (RNA-Seq) was performed using the Illumina Next-generation sequencing (NGS) platform (NextSeq 500 sequencer, Illumina, UK).

2.1.1 Procurement of semen

All sample participant consent and percurmment adhered to faculty ethics approval [SE111 229A Appendix 2]. Semen samples were obtained from recruited donors (aged 19-30 years) by masturbation after 2-5 days of abstinence. Donors were asked to fill a questionnaire detailing for health, lifestyle, and medications (Appendix 2). All participants have consented following faculty ethical approval at Manchester Metropolitan University (Appendix 2). Semen samples were produced on site in a designated, secure room were collected in sterile plastic containers (Sterilin, UK). Samples were divided into two portions for motile and immotile sperm from the same sample. Motility was assessed using the CASA system (Sperminator,® Procreative, UK).

2.1.2 Sperm preparation from human semen samples:

Isolating motile sperm from seminal plasma is a routine procedure in assisted reproduction technology (ART). These techniques are based on isolating viable, normal and motile sperm capable of fertilizing the oocyte. In order to separate different motility grades (Grade A - fast progressive motility, Grade B - slower progressive motility, Grade C- nonprogressive and Grade D immotile – *referred to as A, B, C, and D henceforth*). Conventional swim up methods was used to such isolation (Grunewald and Paasch 2012), (Figure 2.1).

Samples of interest were assessed regarding sperm concentration, motility and the presence of round cells in semen sample according to WHO semen analysis criteria (2010) using CASA system software (Sperminator®, Procreative, UK). To select motile and immotile sperm from non-sperm and remove contaminants, a 55:80% discontinuous density gradient was performed.

Samples were allowed to liquefy for 30 min at 37^{o}C before further analysis to enable sperm to acquire swimming ability. Semen was analysed for volume, sperm concentration, and motility according to WHO guidelines (2010). Sperm concentration and grade motility were then assessed on a pre-warmed (37◦C) stage using CASA software (Sperminator®, Procreative, UK). Samples with more than 1x10^{6} round cells/ml were excluded from the study.

Sperm preparation was done using two swim-up purification methods (WHO, 2010). Firstly, sperm was separated by a discontinuous density gradient, a separation method based on cellular density separation. Briefly, 1 ml of semen samples were gently layered on top of 2 ml 55:80% Supra Sperm^{TM} media

(SPM) (Origio, Denmark), then centrifuged at 300 xg for 20 min at room temperature, then sperm pellets were washed twice in sperm preparation medium™ (Origio, Denmark) at 300 xg for 10 min. The sperm was then suspended in 1 ml of SPM for motile sperm portion and counted. While immotile sperm (D) was isolated from the intermediate layers of the gradient (Figure 2.1). The immotile portion washed twice with 1ml PBS at 300 xg for 10 min to ensure that sperm are free from seminal plasma and any other decapacitated contaminants. Finally, the washed portion was suspended with 0.5 ml PBS.

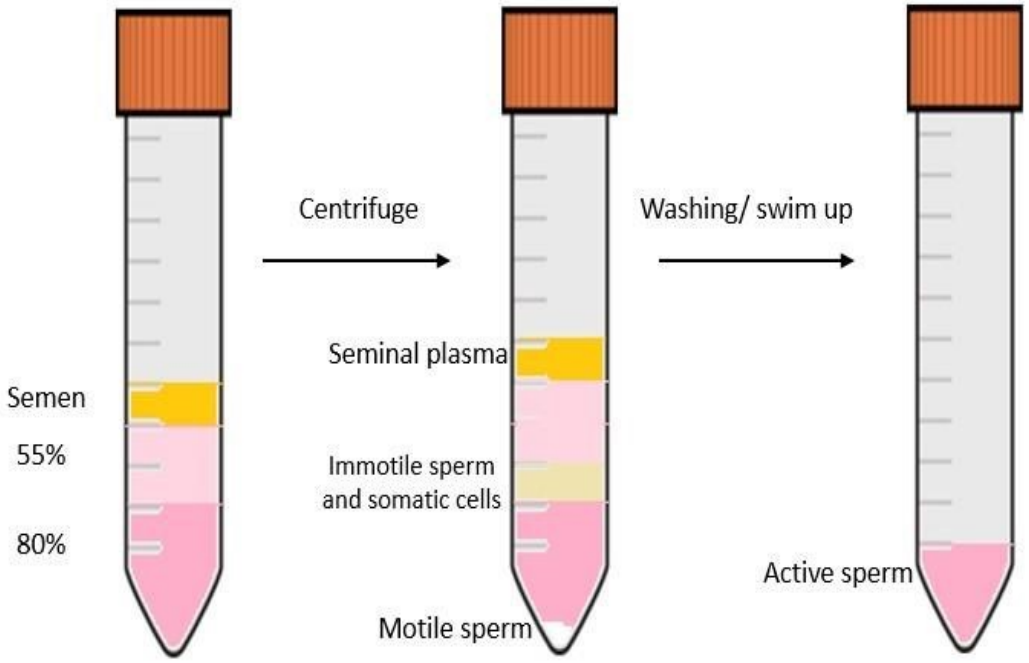

Figure 2.1 Schematic illustration of Discontinuous Density gradient method in vitro.

Secondly, the simple layer technique was done by gently placing 1 ml of semen sample underneath 2 ml of SPM (Origio, Denmark) at the bottom of a conical Falcon test tube, and placed in a 6.0% CO_2 gassed incubator at 37°C at an angle of 45º with a loose cap. The entire supernatant contains the active motile

sperm was isolated after 30-60 min and 0.5 ml of supernatant was taken for motile active sperm (A) after examined for no somatic cell contamination under X400 light microscope (Bongso et al., 1993). All samples were cryopreserved by adding Sperm freezing medium (FertiPro, Belgium) dropwise in a 1:1 volume to the samples and stored at -80^{o}C until further use.

2.1.3 Total RNA isolation

Isolation of RNA from sperm population is challenging as RNA can quickly degrade due to RNase activity present in various contaminants. Therefore, to avoid RNA degradation and to optimize the yield of RNA; all work was carried out on a bespoke RNAase-free workstation only. Furthermore, the sperm has a very little total RNA content (10-20 fg of RNA in haploid spermatozoon, 450 fg of RNA in a haploid spermatid while diploid somatic cells contain 10-20 pg) and even less of small RNAs, especially miRNA. Another point, the sperm nucleus is highly condensed that adds more difficulties to isolate its content (Das et al., 2010). For that reason, we chose to compare different kits.

Three different kits were tested to isolate RNA from the eight human semen samples contain 2550 m/ ml spermatozoa of different motility grades to find out the best one regarding RNA quantity and quality. The kits were:

1. Total RNA purification kit (Norgenbiotek, Canada).

2. Mini-miRNeasy kit (Qiagen, UK).

3. Trisure kit (TRIsure™, bioline, UK).

Samples were centrifuged at 300 xg for 10 min after thawing and washed twice with 1X PBS. Cells were counted using Neubauer improved counting cell chamber, under x200 microscope. 80 µl of ß-mercaptoethanol was added to an

adjusted 20-40 m/ml of sperm to lyse the cells with vortexing for 30 sec. Samples were incubated for 30 min on ice before proceeding with each kit.

2.1.3.1 Total RNA purification kit (Norgen biotek, Canada):

This protocol isolates total RNA including miRNA without phenol-chloroform contamination. The purification is based on a spin column chromatography using Norgen's proprietary resin as the separation matrix. The sperm cells were first lysed using the provided lysis solution and vortexed for 15 min. Ethanol (100%) was added and vortexed for 10 min. The lysate was added to the spin column provided with the kit and centrifuged for 1 min at high speed. Then, the column was washed with wash solution, centrifuged for 1 min and the flow through was discarded. Lastly, RNA was eluted into 30 µl of the provided elution buffer.

2.1.3.2 Mini miRNeasy kit (Qiagen, UK):

The RNA isolation was performed according to the manufacturer's instructions, Qiazol was added to the samples and vortexed to mix, then incubated at RT for 5 min. Chloroform was added to the tube containing the homogenate, vortexed for 15 sec and centrifuged at 12,000 xg for 15 min at 4°C. The upper colourless portion was transferred to a new collection tube. Ethanol (70%) was added and centrifuged at ≥8000 x*g* for 15 sec at RT. The flow-through was discarded and RWT buffer was added then centrifuged at ≥8000 xg for 15 sec, another wash buffer RPF was added, centrifuged at ≥8000 xg for 15 sec, and discarded the flow-through, this was repeated twice. This step is important to remove phenols, salts, and other contaminants during the centrifugation. Finally, RNA was eluted in 40 µl of RNase-free water.

2.1.3.3 Trisure RNA extraction method (TRIsure™, bioline):

RNA extracted following the manufacturer's protocol. 1 ml of Trisure added to the samples and incubated for 5 min at RT, followed by the addition of 200 µl of chloroform. Samples were shaken vigorously for 15 sec and incubated for 5 min at RT then centrifuged at 12,000 xg for 15 min at 4°C. Three phases appeared; the upper colourless portion that contains RNA was transferred into RNase free tubes; the pellet containing the DNA and other insoluble constituents like polysaccharides and membranes (Rauber, 2008). RNA was precipitated by adding 500 µl of cold isopropanol followed by sample incubation for 10 min at RT then centrifuged at 12,000 xg for 10 min at 4°C. The supernatant was removed and RNA wash was performed by adding 1 ml of 75% ethanol to the pellet and vortexed then centrifuged at 7500 xg for 5 min at 4°C. Finally, the pellet was air dried and dissolved in 25 µl DEPC-treated water.

All isolated RNA from tested kits were stored at –80°C until further use.

2.1.4 RNA library generation methods

mRNA library preparation was performed using Nextflex™ Rapid RNA-Seq kit (Illumina compatible/ Bioo Scientific, USA) paired with the Agencourt® AMPure® XP magnetic beads (Beckman Coulter, USA). To qualify gene expression levels, target 3' poly (A) mRNAs sequence selection method was approached via a magnetic beads-conjugated oligo (dt), and depletion of other sorts of RNA occurs at this point in order to enrich the RNA sequence of interest.

RNA library preparation consists of seven basic steps to prepare RNA for sequencing: RNA fragmentation, Reverse transcription, Adaptor ligation, Library

clean up, Amplification, and Library quantification, finally, Quality control and sequencing as illustrated in Figure 2.2.

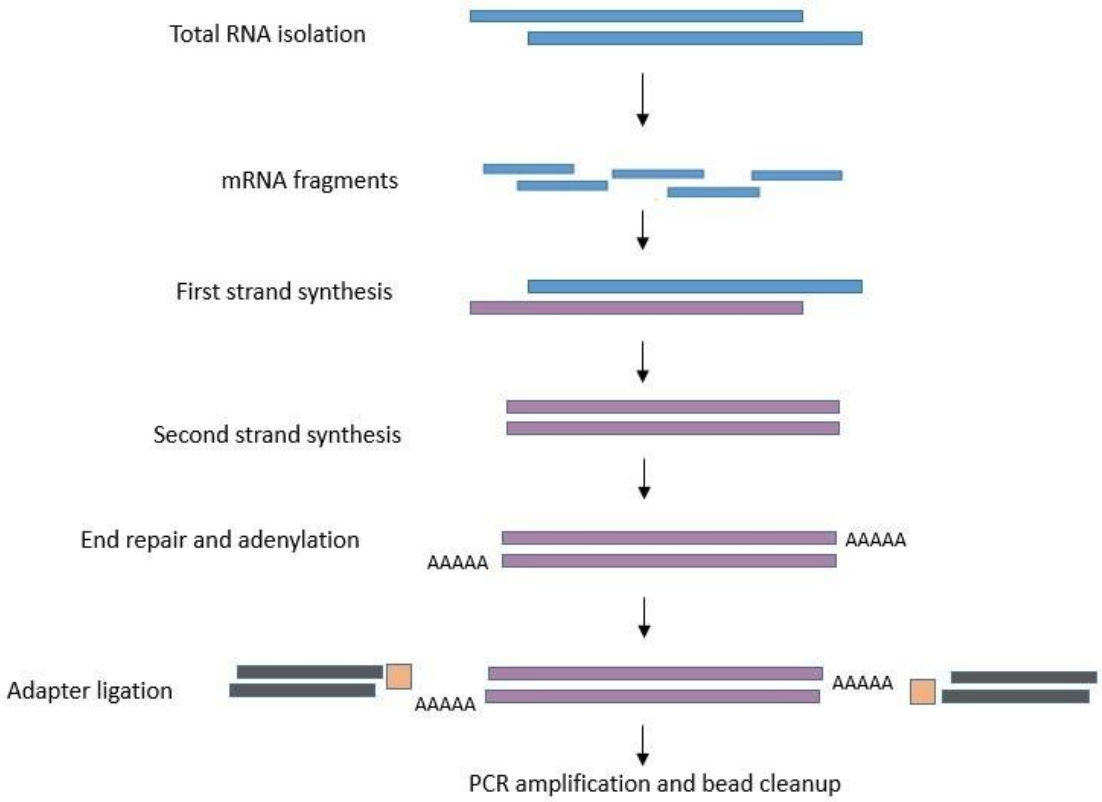

Figure 2.2 Basic flow chart of RNA sequencing preparation steps from sperm samples.

The RNA library preparation was done according to the manufacturer's instruction as the followings:

Step 1: RNA fragmentation

The first step of RNA fragmentation into smaller strands fragment of 200-500 bp length was done by adding 5 µl of Nextflex fragmentation buffer to 14 µl of RNA in the nuclease-free water on ice. The mixture then heated for 10 min at 95°C before proceeding to the second step of first strand synthesis.

Step 2: First strand synthesis

To convert mRNA into cDNA, reverse transcription was done for each reaction of the fragmented RNA. 1 µl of Nextflex™ first strand synthesis primer (5' AATGAT ACGGCGACCACCGAGATCTACAC) was added and incubated for 5 min at 65°C then placed on ice. Next, 4 µl of Nextflex first strand buffer mix and 1µl of Nextflex rapid reverse transcriptase were added to the samples and incubated on a thermocycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA) as shown in Table 2.1, after has been thoroughly mixed.

Table 2.1 Cycling conditions of the first strand

Time	Temperature°C
10 min	25
50 min	50
10 min	70

Step 3: Second strand synthesis

Second strand cDNA was generated by adding 25 µl of Nextflex second strand synthesis mix to the reaction tubes from step2, mixed well and incubated for 60 min at 16°C.

Step 4: First Bead Cleanup

Agencourt® AMPure® XP magnetic beads (Beckman Coulter, USA) was used for the clean-up step. It consists of first bead binding, beads regeneration, second bead binding and final elution as described in Figure 2.3. After this stage, the product can be stored safely at -20°C before proceeding to the next step of adenylation of the purified ssDNA product.

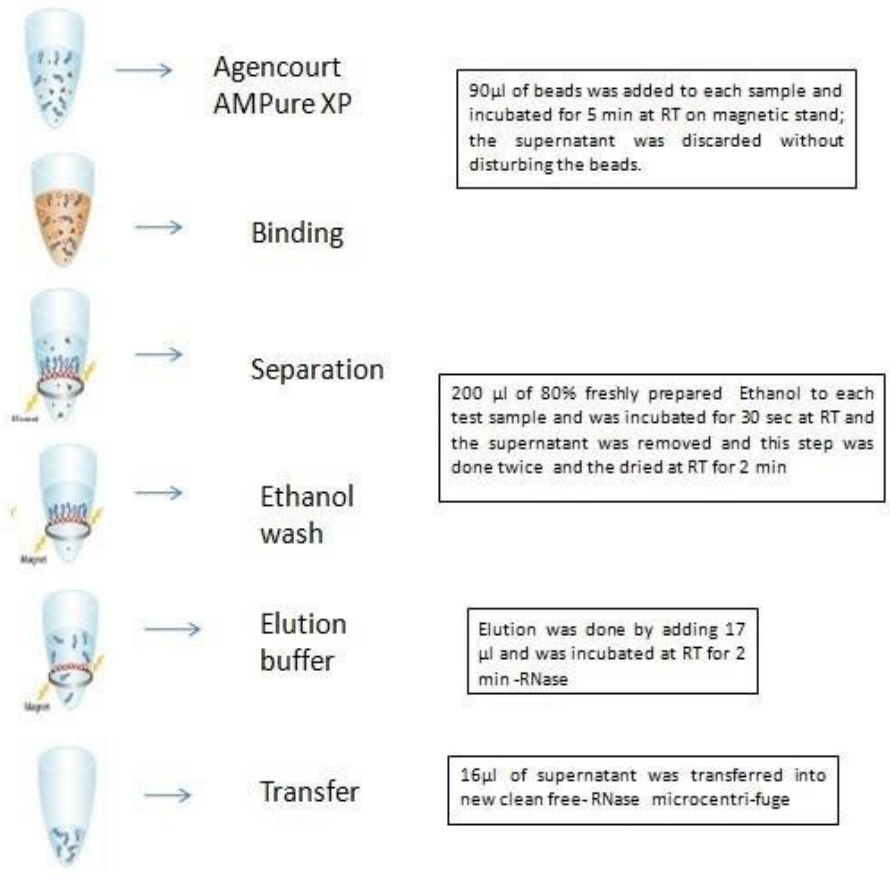

Figure 2.3 Brief description of beads clean-up step.

Step 5: Adenylation

For each sample of purified second strand synthesis product from step 4, 4.5 µl of Nextflex Adenylation mix was added on ice. The mixture was then incubated on a thermocycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA) for 30 min at 37°C.

Step 6: Adapter ligation

The 5’ and 3’ ends of cDNA fragments were repaired and adaptors were ligated (thus providing a unique label for different samples being pooled in a single sequencing reaction and allowing the hybridization of the sequences on a flow cell). 20 µl poly A beads were used as recommended for batch purification of pure intact mRNA upstream of RNA-Seq library preparation. Therefore, mRNA was expected to compromise 100 ng (~80 nm) as polyA is 1-5% of total RNA content. On ice, a mixture of 20.5 µl of adenylated DNA, 27.5 µl of Nextflex ligation mix and 2.0 µl Nextflex RNA-Seq barcodes or called indices of six random bases (random hexamer) to bind to a random position of the template (Table 2.2) were mixed thoroughly and incubated for 15 min at 22°C.

Table 2.2 Nextflex RNA-seq Indices

Barcode adaptor (n)	Sequences
Barcode adaptor1	CGATGT
Barcode adaptor2	TGACCA
Barcode adaptor3	ACAGTG
Barcode adaptor4	GCCAAT
Barcode adaptor5	CAGATC
Barcode adaptor6	CTTGTA

Step 7: Second Bead Clean-up

Two bead-binding steps were needed to ensure maximum removal of Ribosomal and non-messenger RNA contaminants where magnetic beads are incubated with total RNA in the presence of a binding buffer. Magnetic based separation is used to retain poly(A) mRNA while removing all other transcripts. Beads are subsequently washed and mRNA is eluted, releasing purified poly(A) mRNA as detailed in Figure 2.4.

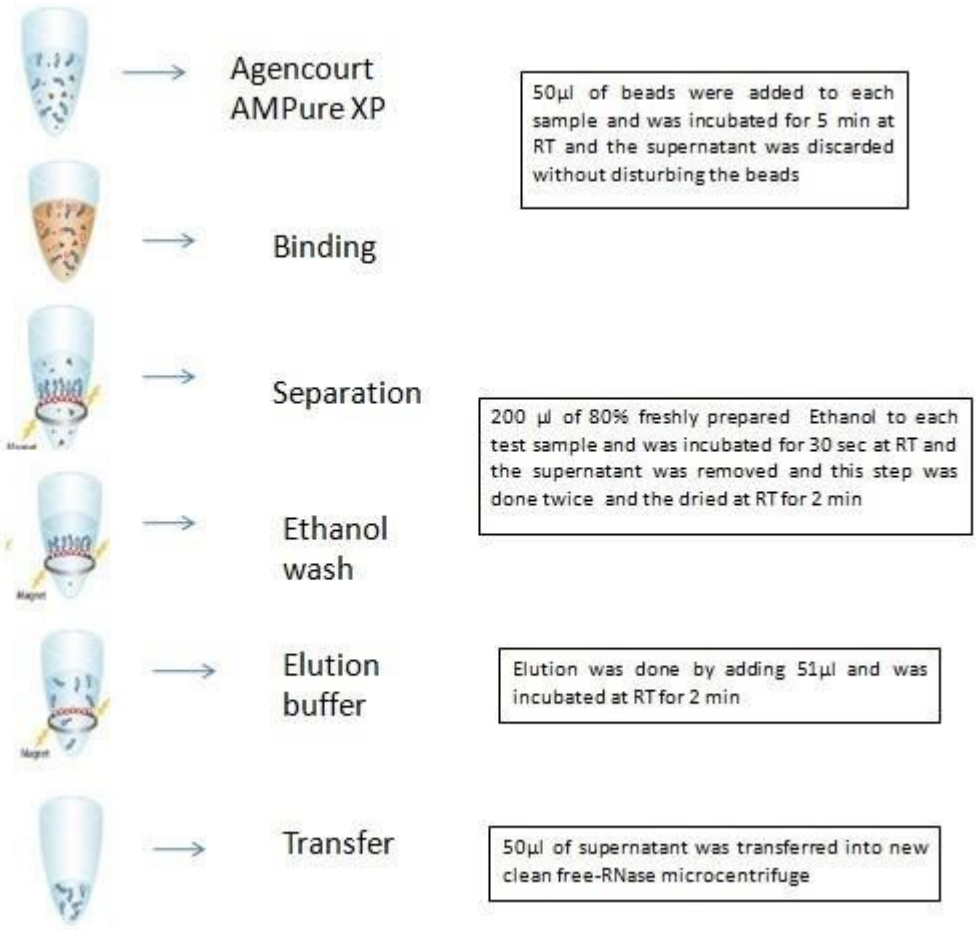

Figure 2.4 Brief description of second beads clean-up step

After that, 50 µl of AMPure XP beads was added to each sample and incubated for 5 min at RT. The mixture was then placed on a magnetic stand for 5 min at RT. The supernatant was removed and 80% freshly prepared 200 µl ethanol was added and incubated for 30 sec at RT, this was repeated twice. After that, the dried beads were re-suspended in 37 µl of resuspension buffer, mixed well and incubated for 2 min at RT. The suspended beads were placed on a magnetic stand for 5 min at RT. Lastly, the clear supernatant was transferred to a new microcentrifuge for a further step of PCR amplification.

Step 8: PCR amplification

PCR amplification was achieved in order to obtain enough material to be sequenced. In a nuclease-free microcentrifuge tube, a total of 36 µl of Adapter-ligated DNA, 12 µl of Nextflex PCR master mix, and 2 µl of Nextflex primer mix to bring a total volume of 50 µl, and then incubated according to Table 2.3:

Table 2.3 Cycling condition for real-time PCR

Steps	Time	Temperature°C	Cycle
PCR initial activation	2 min	98	
Denaturation	30 sec	98	
Annealing	30 sec	65	15 cycle
Extension	60 sec	72	
Final extension	4 min	72	

Step 9: Third Beads Clean-up

Samples were mixed with Ampure XP beads after removing the tube from the magnetic stand. Then were proceeded to the first bead binding, beads regeneration, second bead binding and final elution of 32 µl resuspension buffer which was incubated for 2 min, placed on a magnetic stand for 5 min at RT. Eventually, 30 µl of clear supernatant was transferred to a new fresh microcentrifuge tube before library validation (Figure 2.5).

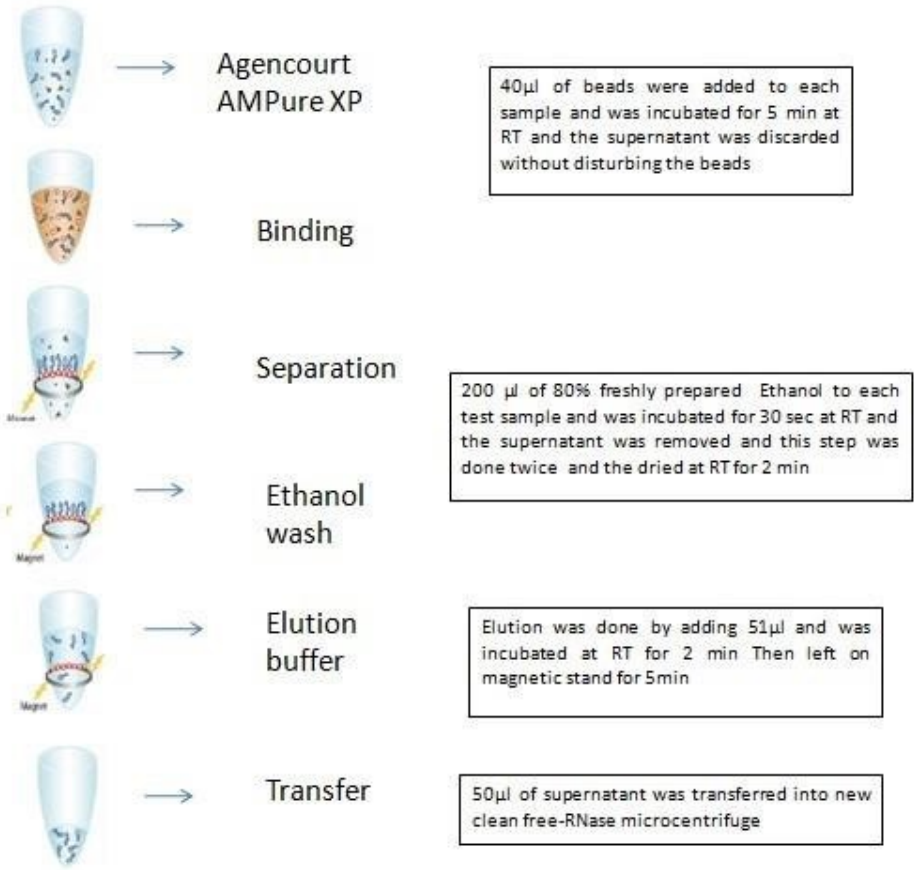

Figure 2.5 Brief description of third beads clean-up step

2.1.5 Library quantification

Non-multiplexed libraries were validated with a Bioanalyzer 2100 fluorimeter (Agilent technology, USA) tracing the size and quality of the libraries. Libraries were normalized using standard normalization method then pooled into an equimolar ratio (4 nM) and stored at -20°C before proceeding to cluster generation process and being ready for sequencing.

2.1.6 Cluster generation

The critical step of NGS is when the DNA is attached to a flow cell cartridge which contains 8 lanes but only a single or pooled libraries can be sequenced on it with the adaptors on both ends of sheared cDNA and performing bridge amplification to generate millions of strands from one molecule then annealing of sequencing primer will occur. To start, libraries were normalized using a standard normalization protocol then uploaded onto the reagent cartridge from a single reservoir, which transferred automatically to the flow cell to all lanes. Following the manufacturer's protocol cluster generation was done as the following steps:

2.1.7 Library denaturation and dilution

The pooled and diluted library of 4 nM final concentration (5 µl) was combined with 5 µl of freshly prepared 0.2 N of NaOH in a microcentrifuge tube, vortexed and centrifuged briefly then were incubated at RT for 5 min.

After that, 5 µl of 200 mMTris-HCl (pH7) was added, vortexed and centrifuged again. The denatured libraries were then diluted to 20 pM by adding pre-chilled HT1 and centrifuged briefly.

2.1.8 Loading concentration preparation

The final library volume was 1.3 ml at 1.8 pM was achieved by combining 117 µl of denatured library solution with 1183 µl of pre-chilled HT1, was mixed and centrifuged.

- **PhiX control preparation**

10 nM Phix (10 µl) was used as sequencing control and was combined with 15 µl RBS to achieve 4 nM PhiX which was mixed and centrifuged briefly.

- **PhiX denaturation**

The following were combined in a microcentrifuge tube to denature the diluted PhiX

4 nM PhiX (5 µl)

0.2 N NaOH, freshly diluted (5 µl)

The mixture was vortexed and centrifuged, then 5 µl 200 mMTris-HCl, PH 7.0 was added and mixed well.

- **Denatured PhiX dilution**

In order to prepare loading concentration of denatured PhiX, 985 µl of prechilled HT1 was added to the denatured PhiX to bring the total volume 1 ml at 20 pM, to dilute the denatured PhiX to 1.8 pM, the followings were mixed :

117 µl Denatured PhiX

1183 µl Pre-chilled HT1

The mixture was mixed and centrifuged at 280 x g for 1 min.

2.1.9 Library and PhiX control combination

13 µl of denatured and diluted PhiX was combined with 1287 µl of the denatured and diluted library from step 2.2.7 and was placed on ice for downstream workflow.

2.1.10 Library loading on the cartridge

1.3 ml was added to the Next Seq Mid output reagent cartridge V2 (Illumina, USA) of 150 cycles on the well number 10 (Figure 2.6)-, which then introduced to the Illumina genome analyser, the Next-Seq 500 (Illumina Genome analyser, UK).

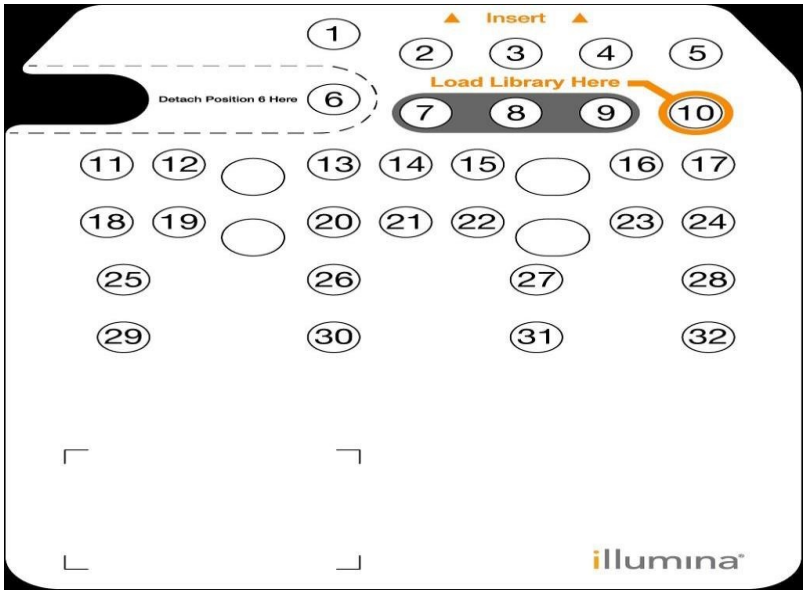

Figure 2.6 The Illumina reagent cartridge. All reagents required for sequencing and the library loading well number 10 of the cartridge.

2.1.11 Data analysis

Computational data analysis pipeline and bioinformatics study of selected mRNA sequences were done. The analysis was comprised of downstream quality control like trimming of adapter's sequencing and poor quality scores reads (Q<20) removal. Next, mapping reads sequences to a reference genome, counting the mapped reads, statistical analysis of differential expression, identification of novel transcripts, and finally pathway analysis. Three software packages have been developed for data analysis (Illumina base space software (http://basespace.illumina.com/): RNASeq Alignment using TopHat for reads alignment and mapping, Cufflinks for transcripts assembly and quantification and DESeq2 for differential expression.

2.1.11.1 RNA-Seq Experimental Design (Figure 2.7)

Paired-end 70bp reads have been generated from human sperm dscDNA using high through-put Illumina Next-seq 500 (Illumina Genome Analyser, UK), (Trapnell et al., 2012).

Our data was performed without technical or biological replicates to explore the differential gene expression (DGE) because of the cost of sequencing consumables. For sequencing multiple libraries in a single run, barcodes for each library were introduced to allow all libraries to be sequenced simultaneously over a single flow cell lane. The Illumina flow cell cartridge with four lanes and samples were spread equally over the four lanes. Next, demultiplexing of reads based upon barcode sequence was performed and analyzed the resulting short-read sequences accordingly.

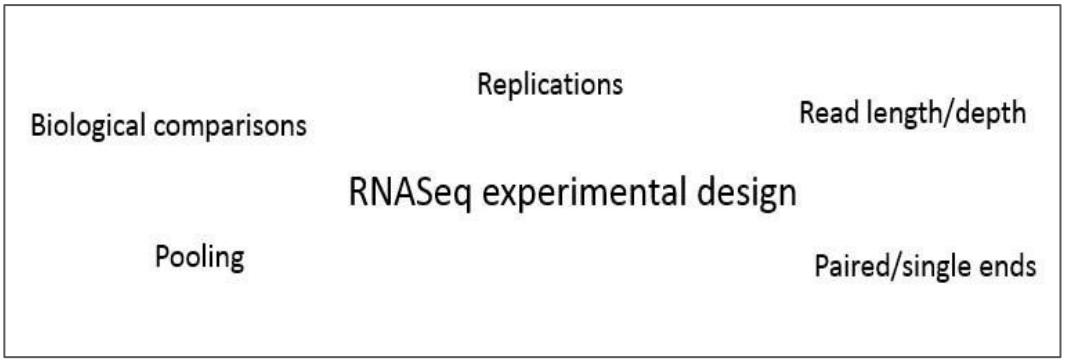

Figure 2.7 The experimental design of RNA sequencing.

As shown in Figure 2.8 Illumina libraries of ssDNA are bound to the surface of flow cell channels that contain a lawn of dense bound oligonucleotides complementary to the sequencing adapters, which have introduced during the library preparation. In Illumina sequencing by synthesis, all four dNTPs are fluorescently labelled and blocked with 3'-OH group. During each cycle of the sequencing procedure, the dNTPs are concurrently introduced into the flow cell cartridge. This allowed only one nucleotide incorporation per one cycle. Clusters then build on the flow cell via PCR bridge amplification were initiated by adding unlabelled nucleotides and polymerase enzyme to produce separate clones of 1 μm in diameter. The original strand is then washed away leaving only the strands that had been synthesized to the oligos attached to the flow cell, multiple copies of the same sequence of fragmented RNA was then formed through PCR amplification by synthesis and denaturation of the dsDNA. The first base is incorporated after adding sequencing reagents, and signals were detected in an imaging phase, then a new cycle added after deblocking (removal of 3'-OH group) and removal of previous fluorescent signal that can read as sequential

images of a cluster (Figure 2.8). The next step is to find the original sequence of the large sequence by alignment to a reference genome.

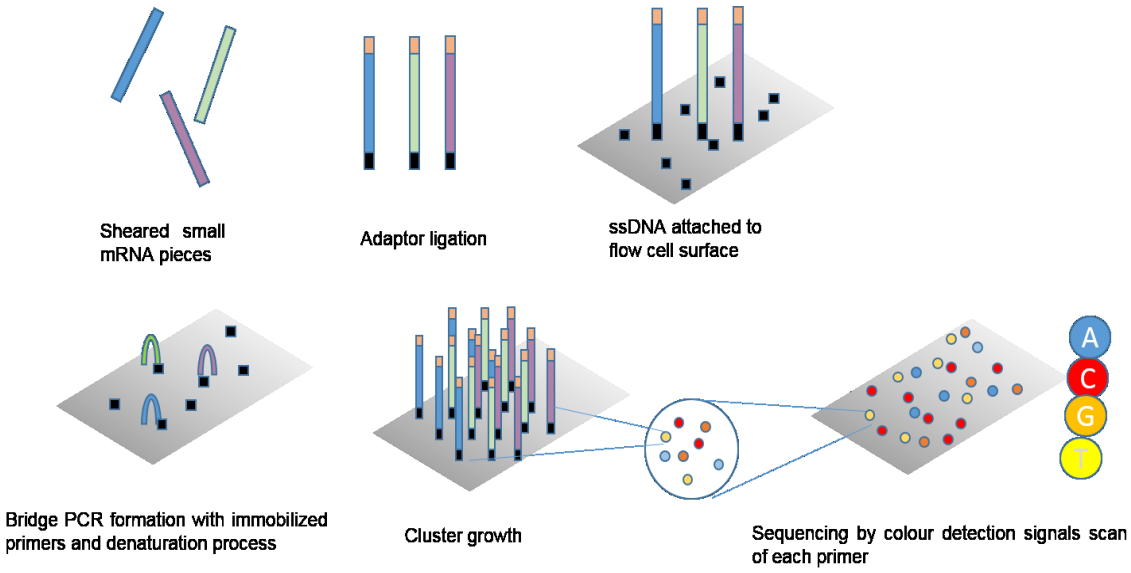

Figure 2.8 RNA-NGS experiment flows.

Cluster generation and read sequencing of mRNA where fragmented mRNA attached to the primers on the flow cell cartridge then are detected by fluorescent-labeled nucleotides.

2.1.11.2 Quality control

In order to evaluate and normalize the sequenced raw data for the length and the total number of reads, we conducted NGS-QC generator application of Galaxy platform (http://www.galaxyproject.org, htt://www.usegalaxy.org) to display the basic statistics and allow quick evaluation of the resulting sequence. BAM file format output was generated from FASTQ- format sequence files that include all reads sequenced from the NGS platform, and QC analysis including counting the number of reads presented and translated into base call quality. The GC content percentage per the followings: a base sequence quality score (a measure of

confidence of correct base calling), (Figure 2.9), per base sequence content (a representation of each nucleotide at each base position to visualize position/ sequence bias), per base N content (a plot of uncalled nucleotides (N's) at each base position), duplicated reads (typically a result of PCR over amplification during library preparation), and overrepresented sequence Kmer (Corney, 2013).

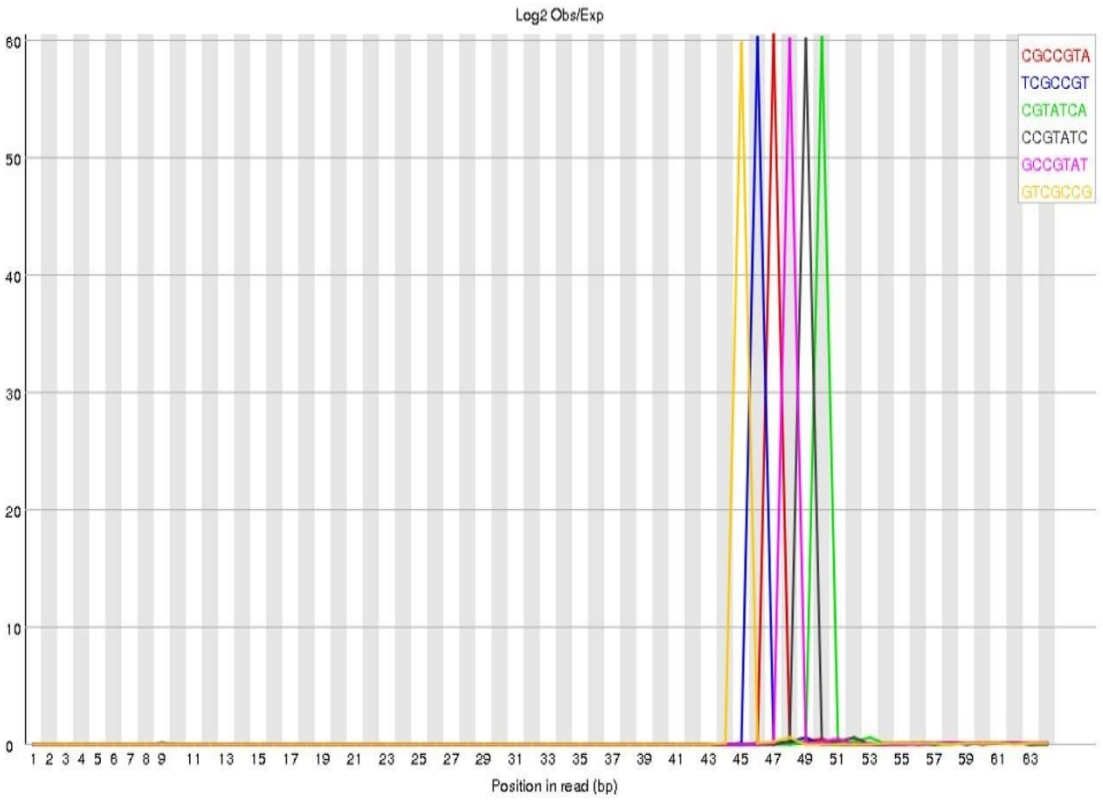

Figure 2.9 graph illustrating the Kmer content.

[Reads per Kilobase of transcripts per million mapped reads (PRKM)], is showing the abundance of mRNA using Galaxy software.

The RPKM metric reads normalize a gene's read count by both transcripts length and the total number of mapped reads in the sample (Kukurba and Montgomery, 2015).

The FASTQ file is a text-based format for sorting biological sequences and its corresponding quality scores. Files contain nucleotide sequencing read data and

associated per-base quality scores together with read identifier while BAM file is a tab-delimited text file containing read alignment data, flags to indicate the numbers of matches, mismatches, and presence of correct mate read (in the case of paired-end reads). BAM is a binary version of SAM-format file (a tab-delimited text format) which is a standard format generated from all mappers (Cock et al., 2009).

2.1.11.3 Reads Alignment

RNA-Seq Alignment software (FASTQ Generation | version: 1.1.0 engine) has been launched to align to the annotated reference genome, called Homo sapiens

(PAR-masked) /hg19 (RefSeq) or *Homo sapiens* (UCSC hg 19) via the aligner TopHat (version: 2.1.0). Bowtie2 read mapping algorithms (version: 2.2.6) based on BWT (Burrows-Wheeler index transform) was used for alignment and resolve spliced reads by splitting reads mapping (Trapnell et al., 2009). The reference genome provides the position from which the reads initiated. Mapping RNA-Seq reads with the STAR aligner and assigns aligned reads to RNA-Seq data, followed by differential expression quantification with DESeq2 software

(version: 1.6.3), which is an exon-based approach built on beta negative binomial (BN) distribution to model read counts across samples and to capture the overdispersion (Robinson et al., 2010). The main advantages of alignment are to generate reads, post alignment statistical summary, and to eliminate the contaminating reads. The aligners can align reads across intron-axon boundaries.

2.1.11.4 Gene quantification and differential gene expression testing

The analysis DESeq2 software package has been used to study the differentially expressed genes and their biological functions in the motile and immotile human sperm samples of our study, the number of reads is mapped to each gene to find the level of expression between different conditions in terms of their read counts, when transcripts reconstructed from short-read data. Cufflinks Assembly software (version: 2.2.1) has been used for reads counting that mapped to the full-length transcripts, as well as, for both gene assembly and quantification. The cufflinks are a graph based programme, the aligned reads were assembled into transcripts, isoforms, and genes simultaneously identifying transcriptional start sites (TSSs) where expression statistical significance can be recorded (Trapnell et al., 2010).

2.1.11.5 Transcripts pathways and functions

Networks of transcripts that were differentially expressed in motile and immotile sperm were identified via online resource GeneMania system (http://www.genemania.org) in order to identify some of the genetic architecture of human sperm and associated network function of the gene lists that have been identified.

2.1.12 Statistical analysis

The statistical significance for gene expression analysis was calculated through a Fisher's exact test, using a 2x2 contingency table, but instead of assuming that the probabilities follow a hypergeometric distribution, they follow a negative binomial distribution parametrized from the mean and the estimated dispersion

(Auer and Doerge, 2010). Bonferroni cut-off was used to define probability significance values. All other analysis was carried out using computational apps provided by Illumina Base space software analysis (http://basespace.illumina.com/).

2.2 Exploring miRNA in human sperm

2.2.1 Sperm preparation

Semen samples were collected from consenting donors (n= 12, mean age: 24.58 ± 0.86) under Faculty approved ethics. Samples were produced by masturbation after 2-5 days of sexual abstinence. Samples of interest were assessed according to WHO semen analysis criteria (2010) using CASA system software (Sperminator®, Procreative, UK). To select motile and immotile sperm from non-sperm contaminates, a 55: 80% discontinuous density gradient was performed. Also, sperm was gained by the simple layer method as discussed in details in section 2.1.1. Sperm samples were kept at -80°C until further analysis.

2.2.2 Total RNA isolation

Total RNA was extracted and purified from each sample using the Total RNA isolation kit (Norgen Biotek, Canada) as per section 2.1.2.1.

2.2.3 RNA integrity assessment:

The RNA quality and quantity were measured via the Nano-Drop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA), by assessing the ratio

of absorbance at 260/280 and 260/230 nm. Bioanalyzer 2100 Agilent (Agilent technology, Santa Clara, USA) was used to evaluate the RNA integrity and looking for the presence of small RNAs in sperm samples using Total RNA 6000 pico kit, Agilent small RNA analysis kit, RNA ladder and Agilent analysis software (Agilent technology, Santa Clara, CA, USA). Samples were then kept at -80°C until further use.

2.2.4 miRNA expression - qPCR for motile and immotile human sperm

Eighty-four sets of mature miRNAs- specific forward primers (miscript primer assay) were selected. The isolated total RNA was adjusted at 250 ng/ 5 µl of total RNA enriched with miRNA for further PCR Array analysis.

2.2.5 Reverse Transcription:

Isolated RNA was poly-adenylated and reverse transcribed into cDNA using oligo(dt) primer and miScript HiSpec buffer on ice prior to Preamplification process using miScript II RT Kit (Qiagen, UK), (Table 2.4). Following the manufacturer's protocol, a PCR thermal cycler used for the synthesis of the first strand cDNA, by incubating samples for 60 min at 37°C, then 5 min at 95°C to inactivate miScript Reverse Transcriptase and stored at -20°C until use.

Table 2.4 Reverse transcription reaction components mixture

Components	Volume/ reaction
5x miScript HiSpec Buffer	2 µl
10x miScript Nucleic Mix	1 µl
RNase-free water	4 µl
miScript Reverse Transcription Mix	1 µl
Template RNA	2 µl
Total volume	**10 µl**

2.2.6 cDNA Pre-amplification

In order to obtain a reliable amount of miRNA, the array was tested with sample kit provided by Qiagen with and without pre-amplification using the misprint PreAMP PCR Kit (Qiagen, UK), which performed before proceeding on miRNA quantification method.

Briefly, 10 µl of template cDNA from reverse transcription reaction was diluted in 40 µl, and then 5 µl of diluted cDNA was used in the reaction volume at RT for pre-amplification reaction mixed with another master mix as shown in Table 2.5. The mixture was mixed, briefly centrifuged, and then placed on ice.

Table 2.5 Pre-amplification reaction components

Components	Volume/ reaction
5x miScript PreAmp Buffer	5 µl
Hotstar-Taq DNA Polymerase	2 µl
miScriptPreAMP Primer Mix	5 µl
RNase-free water	7 µl
miScript PreAMP Universal Primer	1 µl
Template cDNA	5 µl
Total volume	**25 µl**

The 25 µl of preamplified cDNA was incubated at 95°C for 15 min to activate HotStar-Taq DNA polymerase, then 12 cycles of amplification (30 sec at 94°C, 3 min at 60°C) was done on a thermal cycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA), (Table 2.6).

Table 2.6 Cycling conditions for the Pre-amplification reaction

Step	Time	Temperature
PCR initial activation	15 min	95 °C
2-step cycling		
Denaturation	30 sec	94 °C
Annealing/ Extension	3 min	60 °C
Cycle number	12 Cycles	

After the run, the pre-amplified cDNA product was diluted in 475 µl RNase free water to obtain a 20-fold dilution factor to ensure an equal amount of PreAmplified cDNA, which will be used as a template for the next step of real-time PCR miRNA analysis.

2.2.7 Real-Time PCR for mature miRNA expression profiling:

Pathway-focused miScript miRNA PCR array assay (Custom miScript PCR Array system, Qiagen, UK) was chosen for mature miRNA profiling. The Array included all steps of RNA conversion into cDNA and subsequent RT-PCR detection of miRNA using miScript-Syber green PCR kit that contains QuantiTect Syber Green master mix, the miscript Universal primer was used to allow detection of miRNAs, the PreAMP PCR kit, and the 84 different miRNAs primers (Table 2.7). A custom 96-well format was used in order to profile the 84 different miRNAs that can be expressed in the sperm. A replicate of endogenous reference RNA including: Cel-miR-39-3p (C.elegans miR-39 miScript Primer assay), which is used as an alternative data normalizer, 5 different SNOR (snoRNA misript PCR controls defined as SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A), and the snRNA RNU6B (RNU6-2) were used for data normalization using the $\Delta\Delta C_T$ method of relative quantification using the miScript PCR system. miRTC primer assay (miRNA reverse transcription control) is for the assessment of reverse transcription (RT) performance. Finally, the PPC (positive PCR control), which is used for quality assessment of PCR performance.

Table 2.7 miRNome miScript miRNA PCR Array layout

miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID
hsa-let-7a-5p	hsa-let-7b-5p	hsa-let-7c-5p	hsa-let-7d-5p	hsa-let-7e-5p	hsa-let-7f-5p	hsa-let-7i-5p	hsa-let-7g-5p	hsa-miR-100-5p	hsa-miR-101-5p	hsa-miR-106a-5p	hsa-miR-125-5p
hsa-miR-126**-3p**	hsa-miR-128-3p	hsa-miR-130b-3p	hsa-miR-132-3p	hsa-miR-139-5p	hsa-miR-142-3p	hsa-miR-142-5p	hsa-miR-145-5p	hsa-miR-146a-5p	hsa-miR-146b-5p **	hsa-miR-147a-5p	hsa-miR-148a-3p
hsa-miR-150**-5p**	hsa-miR-155-5p	hsa-miR-15a-5p	hsa-miR-15a-3p	hsa-miR-15b-5p	hsa-miR-16-5p	hsa-miR-17-5p	hsa-miR-17-3p	hsa-miR-18a-5p	hsa-miR-181b-5p	hsa-miR-181c-5p	hsa-miR-181d-5p
hsa-miR-182-5p ***	hsa-miR-184	hsa-miR-191-5p	hsa-miR-195-5p	hsa-miR-199a-5p	hsa-miR-19a-3p	hsa-miR-19b-3p	hsa-miR-204-5p	hsa-miR-18a-5p	hsa-miR-20a-5p **	hsa-miR-20b-5p	hsa-miR-21-5p
hsa-miR-210**-3p**	hsa-miR-214-3p **	hsa-miR-221-3p	hsa-miR-222-3p	hsa-miR-223-3p	hsa-miR-23a-3p	hsa-miR-23b-3p	hsa-miR-24-3p	hsa-miR-25-3p	hsa-miR-26a-5p	hsa-miR-26b-5p	hsa-miR-27a-3p
hsa-miR-27b**-3p**	hsa-miR-28-5p	hsa-miR-29a-3p	hsa-miR-29b-3p	hsa-miR-29c-3p	hsa-miR-30a-5p	hsa-miR-30b-5p **	hsa-miR-30c-5p	hsa-miR-30d-5p	hsa-miR-30e-5p	hsa-miR-31-5p	hsa-miR-326
hsa-miR-331**-3p**	hsa-miR-335-5p	hsa-miR-342-3p	hsa-miR-346	hsa-miR-34a-5p **	hsa-miR-365b-3p **	hsa-miR-423-5p	hsa-miR-574-3p**	hsa-miR-92a-3p	hsa-miR-93-5p	hsa-miR-98-5p	hsa-miR-99a-5p
Cel-miR-39**-3p**	Cel-miR-39-3p	SNORD61	SNORD68	SNORD72	SNORD95	SNORD96A	RNU6-6B	miRTC	miRTC	PPC	PPC

Real-time qPCR array Master mix multiplied by the number of samples was prepared using the diluted preamplified cDNA as in Table 2.8, according to the manufacturer's protocol.

Table 2.8 Reaction mix for pathway-Focused miScript miRNA PCR Custom array (96-well)

Components	Volume / µl
2x Quanti Tect Syber Green PCR master mix	1375
10x miScript Universal primer	275
RNase-free water	1000
Template Preamplified cDNA	100
Total volume	**2750**

Finally, 25 µl of the reaction mixture was added to each well of the plate, the PCR plate was tightly sealed with optical adhesive film and was centrifuged for 1 min at 1000 xg at RT and the programme of the qPCR cycler was performed via Applied Biosystems Step one analysis (StepOne^{TM} Real-Time PCR System, Thermo Fisher Scientific), (Table 2.9).

Table 2.9 Cycling condition for real-time qPCR analysis

Step	Time	Temperature
PCR initial activation	15 min	95 °C
3-step cycling		
Denaturation	15 sec	94 °C
Annealing	30 sec	55 °C
Extension	30 sec	70 °C
Cycle number	40 Cycles	

Data analysis was carried out using the ΔΔCt method of relative quantification for miScript miRNA PCR Arrays available at http://pcrdataanalysis.saiosciences.com/ mina, (miscript® miRNA PCR Array, Qiagen, UK).

2.3 Illumina sequencing for miRNA for motile & immotile human sperm

In order to explore the miRNA expression from motile and immotile human sperm, High throughput NGS technology using Illumina sequencer platform (sequencing by synthesis) was adopted, a protocol used to selectively targets the small RNA molecules by size fractionation through gel electrophoresis, because they lack polyadenylation and has a short size (15-30 nt) length. The incorporation of dNTPs is being detected simultaneously on flow cells after preparation of RNA-Seq libraries contains sequencing adaptors complementary to an oligo(nt) positioned on a lawn of the flow cell. Once hybridization of sequences on the flow cell occurs, the activity of DNA polymerase extension commences and copies of cDNA are made when subjected to several rounds of PCR amplification.

2.3.1 Sample collection:

Sperm was isolated from 12 semen samples from MMU volunteers and sperm kinetics was examined using CASA (Sperminator®, Procreative, UK) to prove sample normozoospermia according to WHO 2010 guidelines, all samples that have round cells more than $1x10^6$/ml were excluded from the study in order to

avoid somatic cell contaminations. Samples were processed, sperm with 25-50 m/ml density was used to separate motile (A) sperm by simple layer swim-up method and the isolation of immotile (D) sperm from the same individual was done by density gradient swim-up method as previously mentioned in section 2.1.1, samples then kept in -80°C for downstream RNA extraction.

2.3.2 RNA isolation and validation of isolation methodology:

RNA isolation was done using Total RNA isolation kit (NorgenBiotek, Canada) according to manufacturer's protocol, after thawing the frozen samples, centrifugation with fresh sperm preparation medium (Origio, Denmark) at 500 xg for 10 minutes was performed twice, 80 µl of β-mercaptoethanol was added, then the same workflow steps from previously discussed in section 2.1.2.1 were followed.

Testing of RNA quantity and purity:

Obtaining a good yield and quality of RNA free of contaminating proteins, salts, and DNA that could hinder downstream analysis was another major goal of this study. Great care had to be taken when isolating the sperm RNA, as sperm contains a thousand times less RNA than other somatic cells (Marczylo et al., 2012). To establish the best method for RNA isolation from sperm, three different protocols were tested. Table 4.3 compares the results of RNA yields and quality from the different kits, Total RNA Purification Kit (Norgenbiotek, Canada) recorded higher values when compared with the miniRNeasy kit (Qiagen, UK) and Trisure (TRIsure™, bioline, UK).

Table 4.3 Comparisons of Total RNA isolated from human sperm using different procedures

RNA purification method	RNA concentration ng/µl	260/280	260/230
Trisure kit N=4	50.35±39.65 (5.4-70)	1.28±0.28	0.38±0.15
Total RNA purification kit N=12	57.94±65.64 (14 -242)	1.83±0.14	0.82±0.66
Mini MiRNeasy kit N=4	34.5±32.51 (2.3-70.5)	1.58±0.06	0.28±0.22

Data are mean ± SD, N=numbers of recruited men.

Sperm is known to have many strong disulfide bonds, making RNA extraction difficult (Gilbert et al., 2007). Therefore, β-mercaptoethanol was used during the lysis step in the kits to reduce disulfide bonds and maximize the RNA yield. Importantly, as sperm contains far less RNA than other cells (Marczylo et al., 2012), pre-amplification step using miScript PreAMP PCR Kit (Qiagen, UK) was included before the microRNA PCR analysis.

A total cell number of 30 million sperm was used for RNA extraction. The quantity of RNA determined using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) showed a range of yields from 50- 200 ng/µl from the different groups of study(Table 4.3). An absorbance ratio at 260/280 nm of 1.8-2.0 is considered to be acceptable for RNA purity and it is free from proteins, DNA, and organic contaminants. The 260/230 ratio was also measured; this should be higher than the 260/280 ratio, i.e. ~ 2 with lower values suggesting contaminations through organic factors.

The quality of the isolated RNA was also measured using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara- US). This platform is

based on the microfluidic system and allows the detection and visualization of low RNA concentrations as well as quantitative analysis of the detected RNAs. Normally, the RIN (RNA integrity number) value given by the Bioanalyzer is the cell integrity quality control value, as it confirms the presence of 18S rRNA and 28S rRNA. However, sperm does not contain rRNA (Johnson et al., 2011), so the RIN value was used to confirm that the samples were somatic cell-free.

In sum, testing methods for extraction showed that it was possible to produce RNA of high quality, quantity, and purity, particularly using the Total RNA Purification Kit (Norgenbiotek) together with ß-mercaptoethanol.

2.3.3 miRNA libraries preparation and sequencing:

Small RNA libraries were constructed using Illumina TrueSeq small RNA Library Preparation kit (Illumina, USA) to study the differences between motile and immotile sperm miRNA profile and to find some novel miRNAs in sperm then study their target genes and their relation to male infertility.

According to manufacturer's guidelines, the input of purified RNA was converted into cDNA after adapter ligation to attach to the 3' hydroxyl group on both ends of miRNA (most mature miRNAs have 3' hydroxyl and 5' phosphate group because of cellular pathways). The RNA 3' adapter is modified to target miRNA and other small RNAs that have 3' hydroxyl group which produced by Dicer enzymatic cleavage. Then, small RNA was isolated by denaturing PAGE gel in the 14-30 nt region, then purifying the gel slice, sample barcoding followed by an equal amount of barcoded samples were pooled to generate the library product as detailed below:

2.3.3.1 Adaptor ligation

In the first step of miRNA library preparation, adaptors were ligated to each end of RNA to attach to the 3' and 5' end of the miRNA and allow its amplification. In brief, on ice, 500 ng/µl of total RNA was ligated to 1 µl RA3' (5' TGGAATTCTCGGGTGCCAAGG) in 5 µl nuclease-free water (Table 2.10), then was placed on preheated PCR thermal cycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA) at 70 °C for 2 min.

Table 2.10 1st step of RNA ligation

Reagent	Volume (µl)
RNA3' adapter	1
RNA in nuclease free-water	5
Total volume	6

Then, in the presence of T4 RNA ligase2, Deletion Mutant (Epicentre, VWR, UK), the following mixture multiplied by the number of samples was made on ice (Table 2.11). this step allows adaptor ligation to the ends of the miRNA population of the samples.

Table 2.11 T4 RNA ligation reaction

Reagent	Volume (µl)
Ligation buffer (HML)	2
RNase inhibitor	1
T4 RNA Ligase 2, Deletion Mutant	1
Total volume	4

The above mixtures were mixed together thoroughly with the RA3' adaptor, in order to bring the volume to 10 µl. The mixture was then placed in a preheated PCR thermocycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA) at 28 °C for 1 hr. After that, 1 µl of STP solution was added and mixed well before has been incubated at 28 °C for 15 min.

The second step of ligation was performed by placing 1.1 x N µl of RNA5' adaptors (5' GUUCAGAGUUCUACAGUCCGACGAUC) into preheated PCR thermal cycler at 70 °C for 2 mins. A total of 1.1 x N µl of 10mM ATP was added to the RNA 5'' adaptor aliquot with N (where N is the number of samples being prepared) and were mixed very well, then 1.1 x N µl T4 RNA ligase was added and was mixed. 3 µl of the above mixture then added to the mix of RNA 5' adaptor that was prepared previously to have a total of 14 µl mixture. The latest mixture was put into preheated PCR thermal cycler at 28 °C for 1 hr and placed on ice until further use.

2.3.3.2 Reverse transcription

Reverse transcription followed by amplification was adopted to create cDNA molecules based on the previous step and enrich RNA fragments selectively with

adaptors on both ends, this step is critical for de novo transcripts detection. First, the dNTP concentration was diluted into 12.5 mM on ice.

Then, 6 µl of both adaptors of each sample was mixed with 1 µl of RNA RT primer and was placed on preheated thermal cycler at 70 °C then 6 µl of the adaptors of each sample was mixed with 1 µl of RNA RT primer at 70 °C for 2 mins and placed on ice.

The reverse transcription was prepared using the Superscript II Reverse transcriptase kit (Invitrogen, Life Technology, UK) according toTable 2.12

Table 2.12 Reverse transcription reagents

Reagent	Volume (µl)
5x First strand buffer	2
12.5 mM dNTP Mix	0.5
100 mM DTT	1
RNase inhibitor	1
Superscript II RTase	1
Total volume	**5.5**

2.3.3.3 PCR amplification

PCR was applied to amplify the libraries and create a double-stranded DNA from single-stranded miRNA transcripts by universal RT primer that binds to the Illumina adapters to create single-strand cDNA. After that, dsDNA has been amplified and an index tag is added using universal PCR primer and a 3' index specific PCR primer to be analysed in a single sequencing lane. PCR amplification was then conducted on cDNA using a common primer (F) and one of the 6-bp indices called "barcodes" sequences primers (R), this will allow all

libraries to be sequenced after pooling in the same sequencing reaction because the barcode can identify the reads from each sample (Blencowe et al., 2009). In addition, all samples are processed as parallel through RT-PCR amplification process, according to Table 2.13.

Table 2.13 PCR amplification reagents mixture

Reagent	Volume (µl)
Ultra-pure water	8.5
PCR mix (PML)	25
RNA PCR primer (RP1)	2
RNA PCR primer index (RP1X)	2
Total volume per sample	**37.5**

The reagents were mixed thoroughly on ice and then mixed with the 12.5 µl RT mixture from the previous reaction to make a total volume of 50 µl. Finally, the amplification was done in a thermal cycler following Table 2.14 steps.

Table 2.14 PCR amplification cycle

Temperature °C	Time	Number of cycles
98	30 sec	
98	10 sec	
60	30 sec	11 cycle
72	15 sec	
72	10 min	
4	hold	

2.3.3.4 Library validation

To validate the amplified libraries, high sensitivity DNA kit (Agilent Technologies, USA) was run to each sample before proceeding to the gel purification step and subsequent cluster generation. After that, samples were pooled by mixing 4 µl of each sample together to make a total volume of 48 µl which enables to run in a single run lane on a flow cell, this allows separating the amplified small RNA libraries from adaptors, adaptor dimers, non-small RNA library molecules, and other undesired products. Finally, samples are ready to be loaded onto the gel in the next step of purification.

2.3.4 Gel purification of the cDNA construct

To prepare the cDNA construct for cluster generation, a ready to use 6% Novex TBE page gel, 1.0 mm, 10 wells (Thermo Fisher Scientific, UK) were used to perform purification after samples have been pooled. The gel was loaded as shown in Figure 2.10 and positioned in a minicell electrophoresis unit.

Figure 2.10 Schematic figure illustrating the distribution of reagents on the gel.

H (High-resolution ladder), C (custom RNA ladder), and the pre-purified miRNAs in the middle are loaded on the 6% Novex TBE gel and run on the mini cell electrophoresis.

Lastly, the gel was run for 60 mins at 145 V with 1X Novex TBE running buffer (Invitrogen, UK) and 0.5 µg/ml of Midori Green (Nippon Genetics) was added to the gel for 2-3 mins, the resulted bands were viewed with UV transilluminator (Genetics, Taiwan).

After gel purification, bands should appear at the top of 160 bp CRL ladder and the end of 145 bp CRL band, which contains 22 nt bp of mature miRNA fragment with both adaptors in a total band length of 147 nt. The 147 nt bands were cut with razor blades and transferred individually into a gel breaker tube (0.5 ml tube with three holes that was made with a 21 gauge needle placed into a sterile, round bottom, nuclease-free, 2 ml microcentrifuge tube). Centrifugation at 20,000 x g for 2 mins at RT was done after 200 µl was added to the gel debris in the 2 ml tube. DNA elution was performed via overnight shaking at RT with 100 µl RNase free water on a thermo-shaker (TS-100; PEQLAB Ltd, Sarisbury Green, UK). The final eluate was transferred on top of 5 µm filter tubes (Merk, UK) and centrifuged at 600 x g for 10 sec and proceed to library validation.

2.3.5 Library quality control measurement:

Validation of the miRNA libraries was done with Bioanalyzer 2100 Agilent (Agilent Technology, USA) using the high sensitivity DNA kit (Agilent technology, USA) to estimate the libraries yield and purity.

2.3.6 Library sequencing

Unfortunately, the study could not develop the miRNA libraries size successfully, which should be 145bp and we could only get 113bp libraries length which mostly

of primer dimer rather than actual miRNAs as shown in Figure 2.11, for that reason the experiment was terminated here. This might be due to low miRNA contents in the sperm or RNA samples been degraded during processing samples.

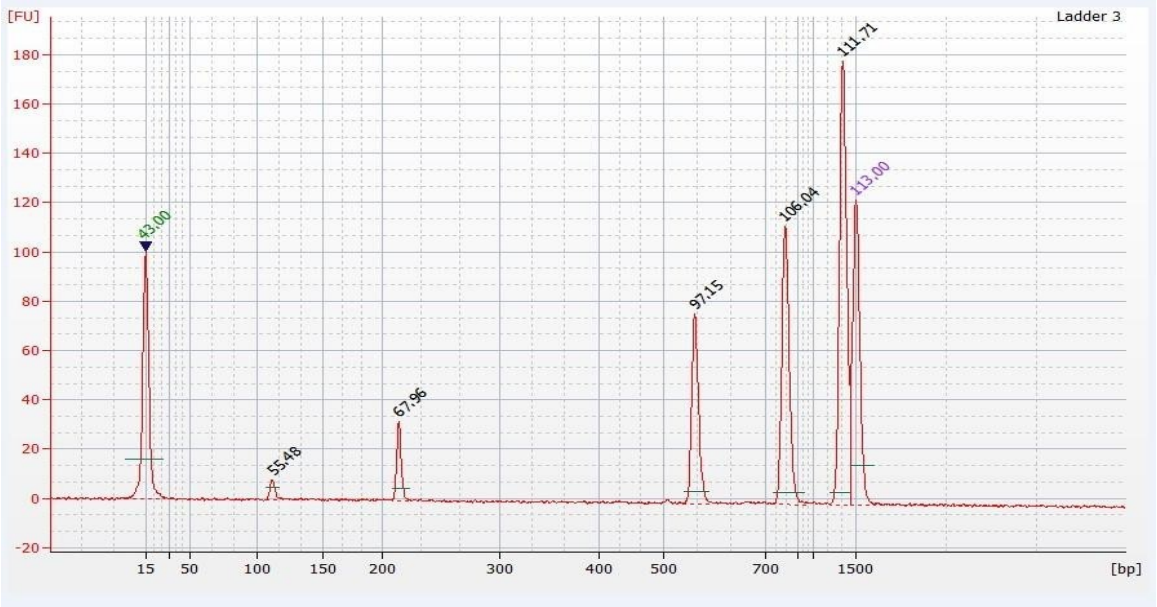

Figure 2.11 Electropherogram of pooled purified cDNA libraries from the human sperm,

Motile and immotile samples libraries appeared <113 bp length size at wavelength 55.48 using Agilent 2100 Bioanalyzer system.

Chapter 3: Exploring Relative expression of mRNA in motile and immotile Humam sperm

3.1 Background

Male infertility is in decline with a decrease in sperm count (oligospermia), (Levine et al., 2017) and motility (asthenozoospermia) (Mehra et al., 2017). This decline may be associated with environmental and lifestyle effects (Virtanen et al., 2017), which may influence spermatogenesis and sperm-specific transcripts that are vital to produce functional human sperm and early embryo development (Boerke et al, 2007). Several studies support the conclusion that sperma contain a complex repertoire of mRNAs, where these mRNAs are provide some insight into past events of spermatogenesis, and potentiallty offer avenues to explore potential fertility biomarkers. Sperm form differnet subpopulaitons - such as motile and immotile sperm – may contain a differential expression of transcripts.

Although mature sperm is considered transcriptionally quiescent (Betlach and Erickson 1976; Yang et al., 2009; Johnson et al., 2011), it has also been reported that there is a low but detectable level of transcription in mature sperm cells (Miteva et al., 1995; Martins and Krawetz, 2005). Other studies have demonstrated the existence of transcriptional and translational activities in human sperm, events are known during capacitation and acrosome reaction (Dierich, Sairam et al., 1998). Additionally, it has been suggested that mRNAs are residues of post-meiotic genes in early spermatogenesis events, which are activated again in mature sperm or after fertilization leading to the hypothesis that sperm is required for more than just providing paternal DNA to the oocyte during

fertilization and embryonic development (Nanassy and Carrell, 2008; Faucette, 2012). These studies may explain the presence and functional activities of mRNA in mature sperm. Thus, sperm-specific mRNAs may play important roles during the early stages of fertilization and contribute to paternal printing (Gekas et al., 2001).

Furthermore, analysing mRNA profiles in human sperm can be used as a diagnostic tool to evaluate male fertility, since they reflect spermatogenesis gene expression, and/or a prognosis value for fertilization and embryonic development, as sperm-specific RNAs are delivered to the oocytes.

The aim of this study was to explore the relative expression of mRNA in relation to sperm motility. Subpoplations of motile and immotile sperm were isolated and the relartive transcriptome investigated through NexSeg sequencing.

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3.1.1 Motile and immotile Sperm isolation

Swim up techniques were used to isolate motile and immotile sperm successfully without somatic cell contaminations from the eight participants as mentioned previously in section 2.1.1. Motile and immotile sperm were recovered from the same sample for each participant. Sperm concentration was adjusted to 25-50 m/ml for the next step of RNA isolation.

3.1.2 The motile and immotile Sperm RNA quantity and quality

Sperm contains a very small amount of 5-10 fg mRNA/sperm (Anderson, 2013). Using the Total RNA Purification kit (NorgenBiotek, UK), total RNA was isolated from eight normospermic semen samples contain 25-50 m/ ml sperm, two of the samples were excluded due to a technical error during processing the samples and only six samples were processed for RNA-seq technique. All semen samples were examined to be free of leucocytes and round or epithelial cells using a 400 X light microscope. The purity and specificity of the RNA were measured using the Nanodrop 2000c (Thermo Scientific, Wilmington, USA). The 260/280 and 260/230 UV/VIS ratio were measured to reflect whether the samples were free of protein, salt, and organic chemical- contamination.

3.1.3 mRNA quality

Total RNA adjusted to 500 ng from each sample was used to generate mRNA-specific cDNA libraries for the next-generation RNA sequencing on the Illumina NextSeq500 system (Illumina Genome Analyzer, UK). mRNA was isolated from the total RNA using the Nextflex ™ Rapid RNA-Seq kit (Illumina compatible, Bioo Scientific, UK). NEXTflex™ PolyA beads (Bioo Scientific Corporation, Catalog number: 512979, UK) were used to bind the mRNA. First and second strand synthesis was performed and a specific set of oligonucleotides with different sequences (NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1), New England Biolabs, UK) were used to allow specific individual adaptors to be added to each sample for multiplex Illumina library production. This “barcoding” enables the mRNA from different samples to be pooled and multiplex-sequenced at the same time.

The quality of the extracted mRNA was assessed using the bioanalyzer 2100 (Agilent technology, US). The final library peak was around 450 bp for 70 bp paired-end read libraries, this confirms the efficient single-read sequencing using Illumina sequencing technology without peaks of primer dimer (Figure 3.1).

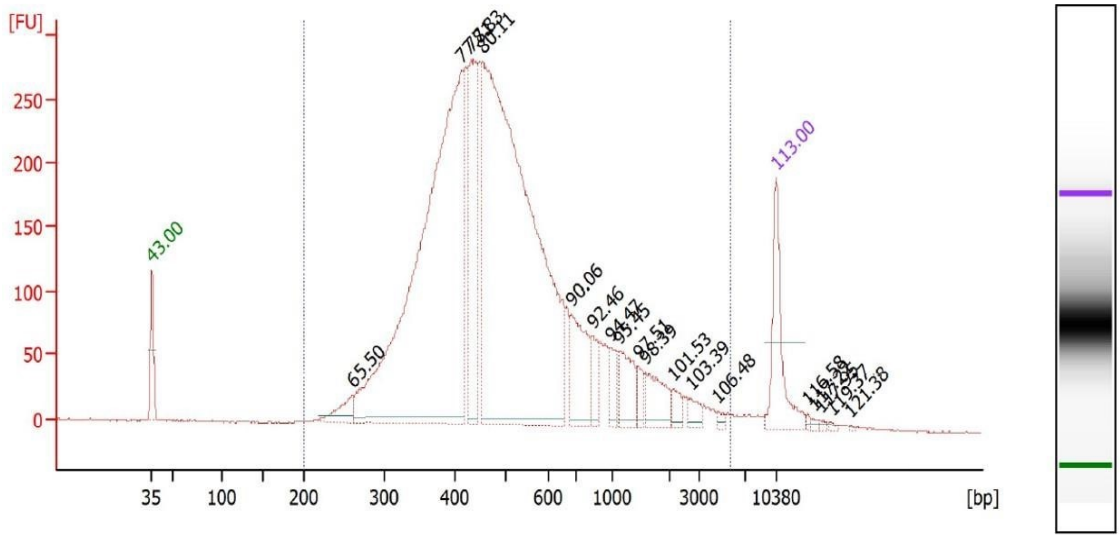

Figure 3.1 A representative bioanalyzer electropherogram of a human sperm mRNA library preparation.

A sample is shown alongside a gel-like image. The 35 bp and 10380 bp peaks corresponded to the size standard between the two markers with peaks of 43 and 113 bp for the lower and upper marker consequently. FU is the fluorescence units. At the right side, an agarose gel electrophoresis-like image of the good quality analysed mRNA is presented.

3.1.4 mRNA Illumina Sequencing Mapping and Analysis of motile and immotile human sperm

High throughput Illumina NGS platform of the spermatozoal mRNA was performed via Illumina NGS platform (NextSeq 500 sequencer, Illumina Genome analyser, UK). Reads x2 paired-end 70 bp sequencing with cycles of 6 multiplexed samples from spermatozoal ds-cDNA were generated for bioinformatics analysis. Sequence analysis was conducted with RNA-Seq alignment software version 1.1.0 from Illumina base space software (http://basespace.illumina.com/) to confirm the percentage of mapped reads.

Interestingly, motile sperm samples were generated higher sequence reads number ranged from 8,816554 -14,186,321 while immotile sperm was recorded

lower reads number ranged from 305,966 – 3,085,229 reads except from sample 2 where immotile sperm recorded higher reads count (12.485.32) than motile sperm (2.034.379) from the same sample, (Table 3.1).

Table 3.1 Summary of the total reads coverage data from motile and immotile sperm mRNA samples

Sample ID	Read length	Number of Reads	% Total Aligned	% Abundant	% unaligned	Median CV coverage uniformity	% Stranded
Motile1	70/70	14,186,32	57.43	2.81	42.57	1.15	50.19
Motile2	70/70	2,034,379	47.73	8.81	52.27	1.30	49.26
Motile3	70/70	8,816,554	37.83	17.13	62.17	1.43	49.55
Immotile1	70/70	305,966	31..49	24.29	68.51	0.69	52.20
Immotile2	70/70	12,485,32	51.74	6.58	48.26	1.27	49.99
Immotile3	70/70	3,085,229	48.44	6.88	51.56	1.42	50.94

Sequence reads were converted with FASTAQ generation software Version 1.1.0 before the library alignment step. The distribution of reads according to the regions of coding, non-coding or intergenic, UTR or intronic is shown in Figure 3.2. The majority of the reads distributed between intronic and intergenic regions around 40 and 50% respectively. These results exclude any bias when we performed trimming of the reads ends and other poor quality score reads to improve the quality of the read, so we choose only the sequence of interest and high quality reads more than 30% sores GC content.

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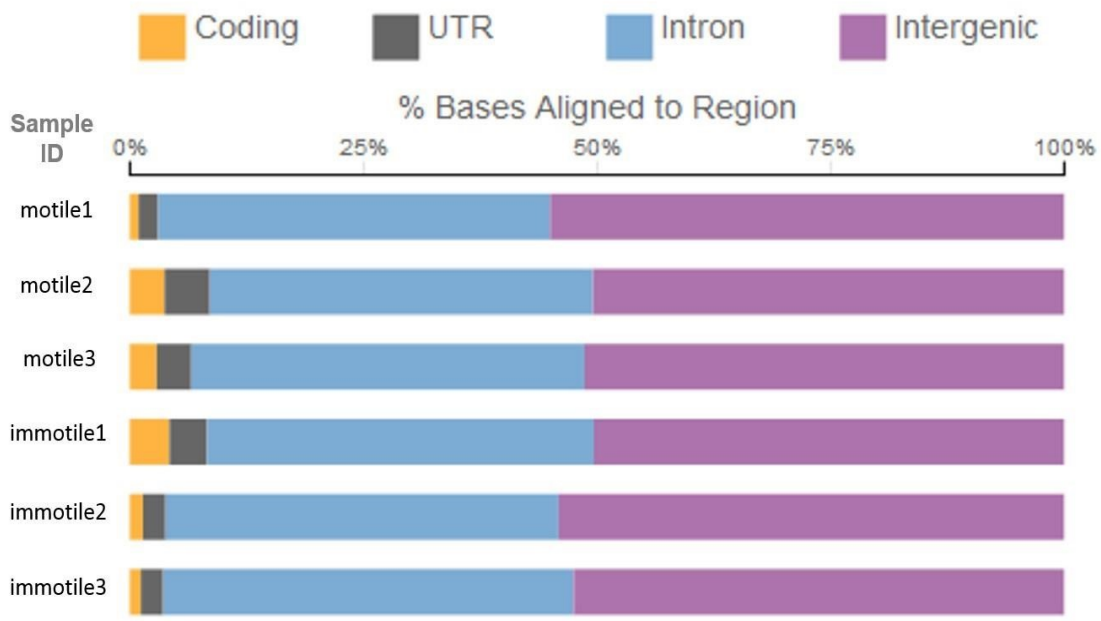

Figure 3.2 The distribution of alignments yields,

Percentages of sequenced or simulated read pairs by the number of bases, to the genomic regions including coding, UTR, intron, or intergenic/ non-targeted of the motile and immotile human sperm samples.

Galaxy software (https//galaxyproject.org) was used for quality assessment. Figure 3.3 shows the quality score of the sperm RNA sequencing output to be more than 30% with a GC% content of 39% and a total sequence of more than 350,000 reads. This indicates that FASTQ-format reads have a high-quality library and can proceed to the next step of analysis.

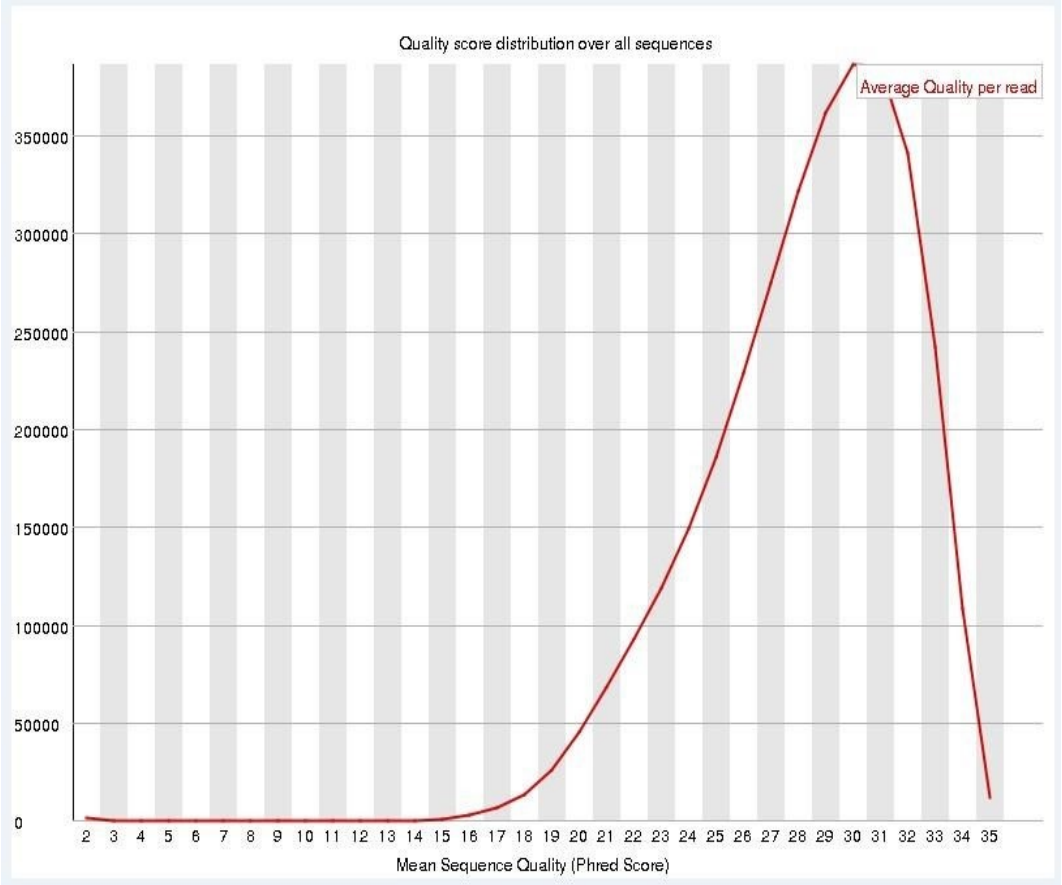

Figure 3.3 Per sequence quality scores using Galaxy software for quality control.

The illustration is showing a high-quality score per read of the mRNA multiplexed library of motile and immotile sperm samples.

3.1.5 Library alignment

RNA-Seq Alignment software version: 1.1.0 was conducted to align the FASTQformat reads into BAM-format against Homo sapiens (PAR-masked)/ hg19 (RefSeq) or Homo sapiens (UCSC hg 19) as a Reference genome and aligner of TopHat (Bowtie2) at the base space Illumina sequence hub (https://basespace.illumina.com/). After that, RNA-Seq reads were aligned with the STAR aligner and was assigned aligned reads to genes, followed by differential expression with DESeq2 software analysis (Figure 3.4).

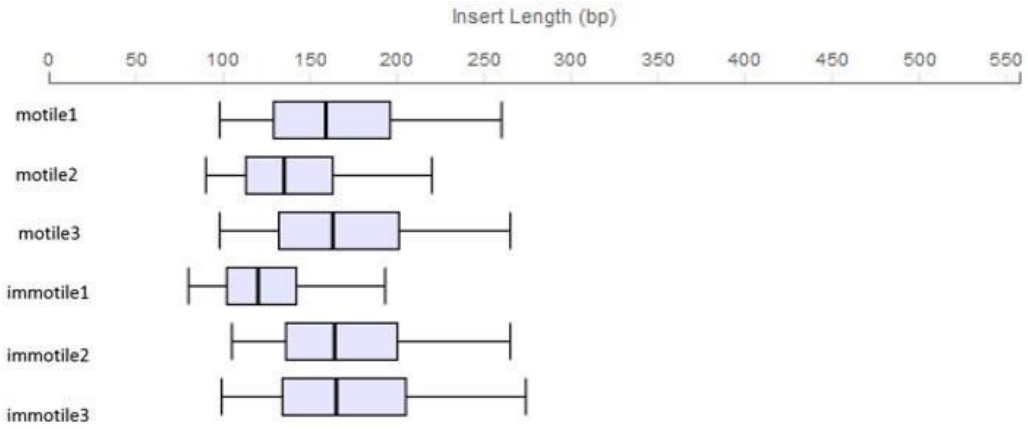

Figure 3.4 An insert length distribution boxplot,

The insert length distribution for the different sperm samples using DeSeq2 App is displayed.

3.1.6 Spermatozoal Transcripts profile

A total of 26,228 aligned transcripts were found in sperm mRNA from the motile and immotile groups. Those sequence alignments flagged as poor quality (using Galaxy software) were excluded from the results. Transcripts of 19 different genes were seen significantly ($P<0.05$) differentially expressed between the motile and immotile sperm samples, (Table 3.2). Interestingly, thirteen of the total 19 genes are coding for ribosomal RNA proteins were recognised, the majority specifically coding for either the large 60S (RPL) or small 40S (RPS) subunit ribosomal proteins gene family. The remaining transcripts coded for the Eukaryotic Translation Elongation Factors 1A1 and 1G (EEF1A1 and EEF1G respectively), Tumour Protein Translationally-controlled 1 (TPT1), Heat Shock Protein90kDa Alpha (Cytosolic), class A Member1 (HSP90A) and NK3 homeobox1 (NKX3-1).

Table 3.2 List of genes that are significantly different between the motile and immotile sperm groups.

Base means refers to the mean expression level of the identified genes; fold changes refers to the measurement of changing magnitude as FC=base mean of the motile/ base mean of immotile. An adjusted P value is given using the Bonferroni correction method

Gene Name	Base Mean	log2Fold Change	lfcSE	stat	P value	P adj	status
EEF1A1	112.15516	-1.98629861	0.662673	-2.9974	0.002723	0.03516	OK
EEF1G	15.0280264	-1.98129255	0.89644	-2.21018	0.027093	0.09204	OK
FTH1	14.8500056	-3.08653374	0.92376	-3.34127	0.000834	0.03035	OK
HSP90A	11.0341971	-1.64434698	0.821741	-2.00105	0.045387	0.13129	OK
NKX3-1	28.4326288	-2.71096618	0.956829	-2.83328	0.004607	0.04012	OK
RPL10	12.2745443	-2.08959362	0.946536	-2.20762	0.027271	0.09203	OK
RPL13	17.2673608	-2.09440441	1.004617	-2.08478	0.037089	0.11554	OK
RPL37	13.2437691	-1.92178536	0.918766	-2.0917	0.036465	0.11554	OK
RPL4	12.9069897	-1.64437411	0.880879	-1.86674	0.061937	0.17299	OK
RPL9	15.2353153	-2.33969927	0.962367	-2.43119	0.015049	0.07618	OK
RPLP0	12.7243196	-2.54134785	0.914422	-2.77918	0.00545	0.04012	OK
RPLP1	12.2316628	-2.69665265	0.993539	-2.71419	0.006644	0.04139	OK
RPS20	13.3281112	-2.64164032	1.048655	-2.51907	0.011766	0.06353	OK
RPS24	15.4260628	-2.95201735	0.968939	-3.04665	0.002314	0.03516	OK
RPS27	17.6073851	-2.89799967	0.999345	-2.8999	0.003733	0.03779	OK
RPS3A	14.8840981	-2.1662346	0.909973	-2.38055	0.017287	0.07790	OK
RPS4X	19.5065877	-2.89542136	1.064824	-2.71916	0.006545	0.04139	OK
RPS6	13.7425243	-2.09103976	0.886597	-2.3585	0.018349	0.07822	OK
TPT1	53.0915813	-2.70706791	0.913378	-2.9638	0.003039	0.03516	OK

3.1.7 DESeq2 App Analysis

The DESeq2 software was used to analyse the differential expression and differential post-translational processes such as translational elongation and termination, cytosolic ribosome, multi-organism metabolic process, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, ribosome and mRNA catabolic process, and many other processes. Results were represented as an MA plot of normalized mean counts of samples reads (A- values) against log2 fold change (M-values) of the motile versus immotile sperm (Figure 3.5).

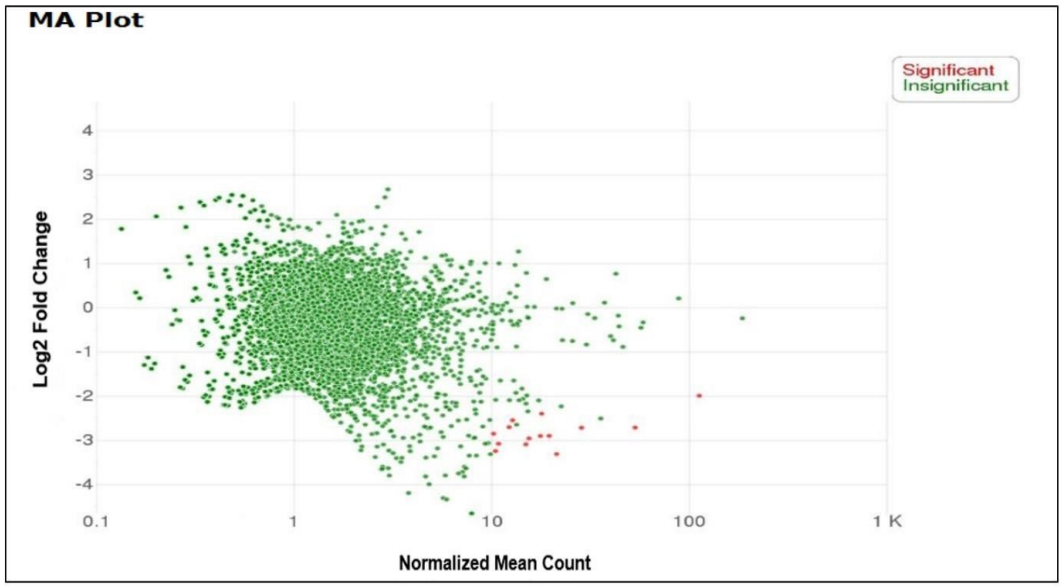

Figure 3.5 MA plot of the differentially expressed genes

Transcripts that are represented by red dots using DESeq2 package analysis. Log2 fold change values for motile vs immotile sperm samples are plotted against normalized mean count.

Expression heat map of the differentially expressed genes (genes highly expressed in the motile group compared to the immotile), from the samples, is showing the relationship between sample/genes (Figure 3.6). This analysis confirmed the previous outcomes of sperm transcripts contents of a wide range of Ribosomal mRNA content and confirms the significant expression differences

in motile A sperm when compared to immotile D sperm. The 19 expressed genes from 19,000 transcripts were included NKX3-1, RPS4X, RPLP0, EEF1A1, RPLP1, KLK2, RPS27, RPL5, RPL26, RPL3, FTH1, TPT1, and RPL7 were then further employed to Genemania analysis (Figure 3.8) to study their functions.

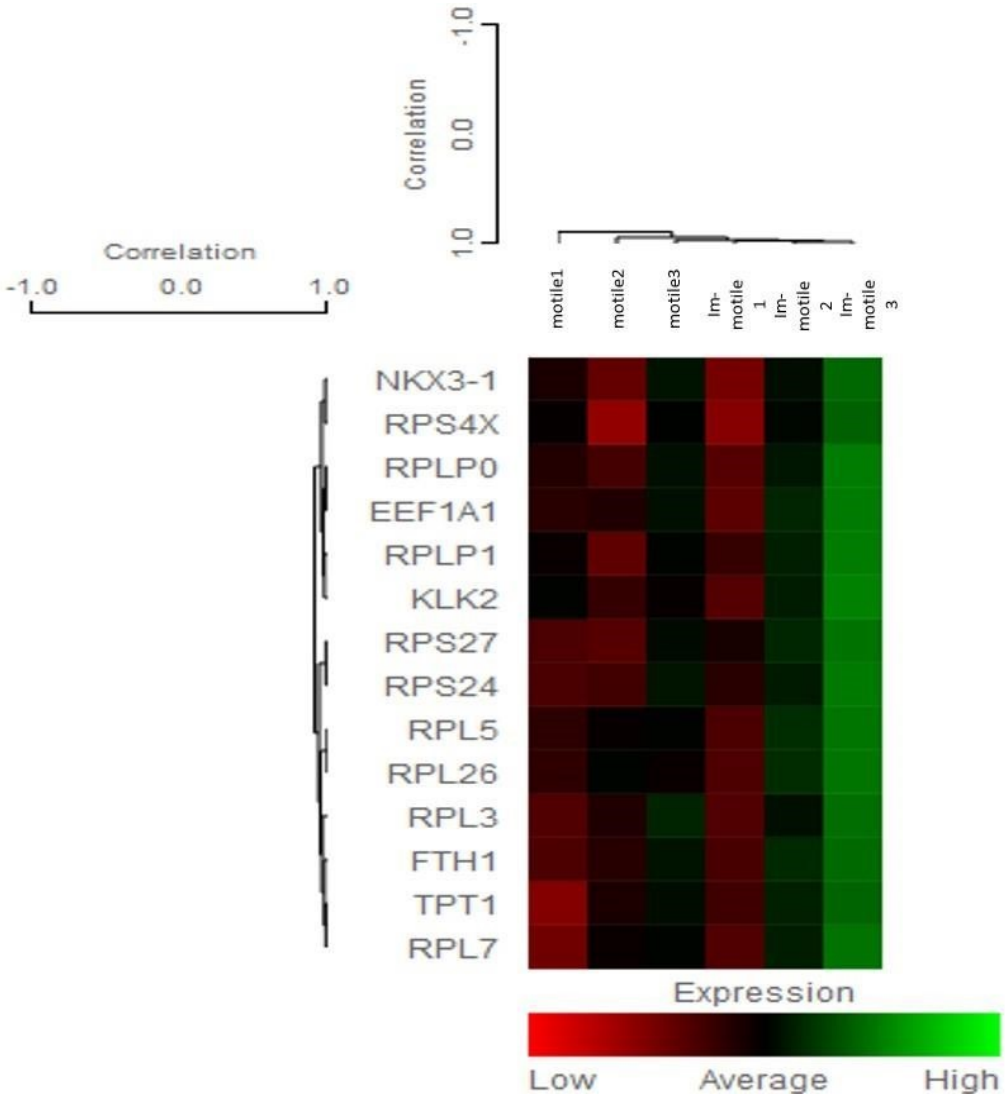

Figure 3.6 Comparison of expression of 14 selected genes based on the Illumina sequencing data using DeSeq2 analysis.

The heat map shows the expression of 14 regulated mRNA transcripts in the sperm. The highlighted key visualizes the colour coded of up and down-regulation.

The DeSeq2 analysis also provides a multidimensional scaling (MDS) in order to represent the (dis)similarities or distance among different samples, in order to measure the variance of dataset distribution according to the Principal component analysis (PCA) tool (Figure 3.7). A three-dimensional space containing PC1, PC2, and PC3 were plotted for samples, which demonstrates the greatest data separation according to PCA analysis tool as they were separated depending on the variation in gene expression among samples.

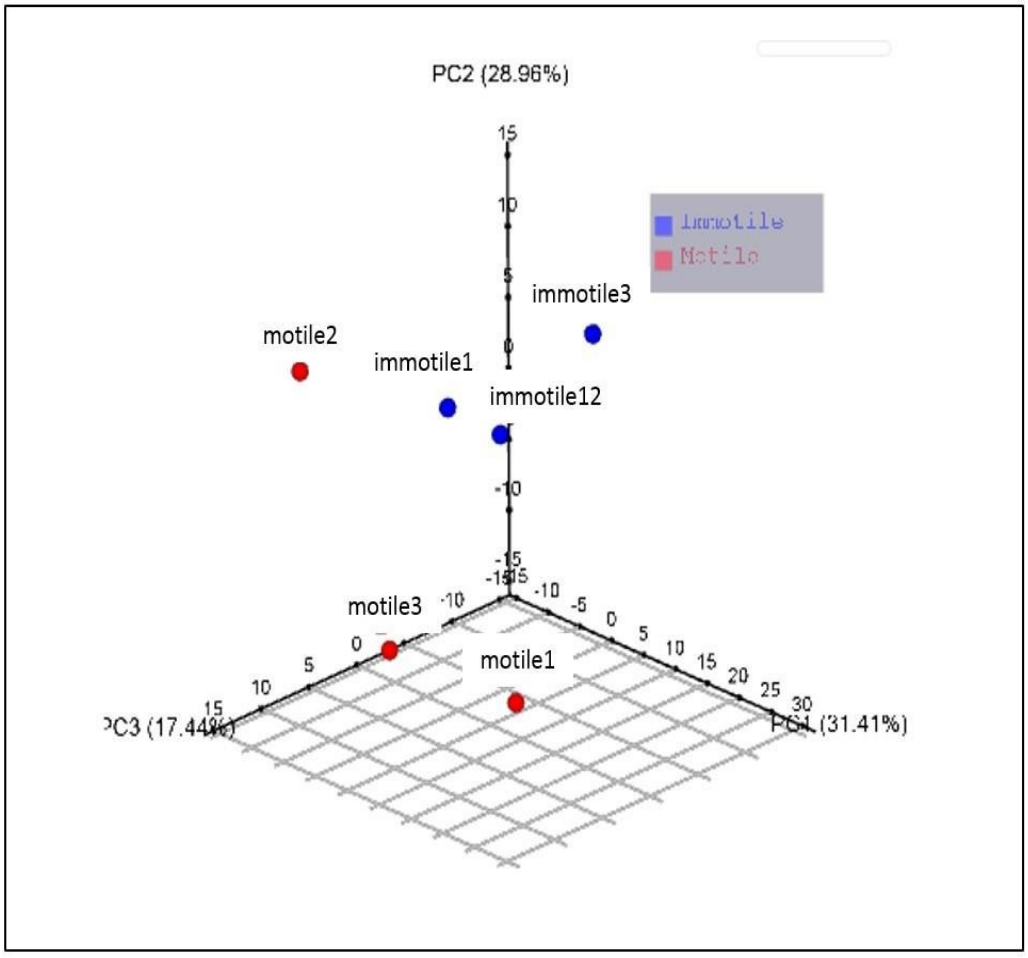

Figure 3.7 DESeq2 programme 3D analysis

The graph is displaying the cluster distribution of the motile (red) and immotile (blue) human sperm samples against the reference genome and the percent of variance explained by each principal component on each axis. The 3D PCA plot of RNA-Seq was generated using normalized and variance transcript expression data (DeSeq2).

3.1.8 Germania analysis

Further analysis was performed using the online Genmania software (http://www.genemania.org), in order to search the interaction of pathways and co-expression between significant transcripts that were recognized from the high-throughput sequencing analysis with other adjacent genes. Predictive functional relationships between genes were also indicated in the analysis of the transcripts from sequencing data (Figure 3.8). The results identified a network of Ribosomal Protein L (RPL) gene family that was more upregulated in motile sperm compared to immotile sperm. These were grouped into five classifications: co-expression, pathway, genetic interaction, physical interactions and predicted (Von Mering et al., 2002; Wang et al., 2015).

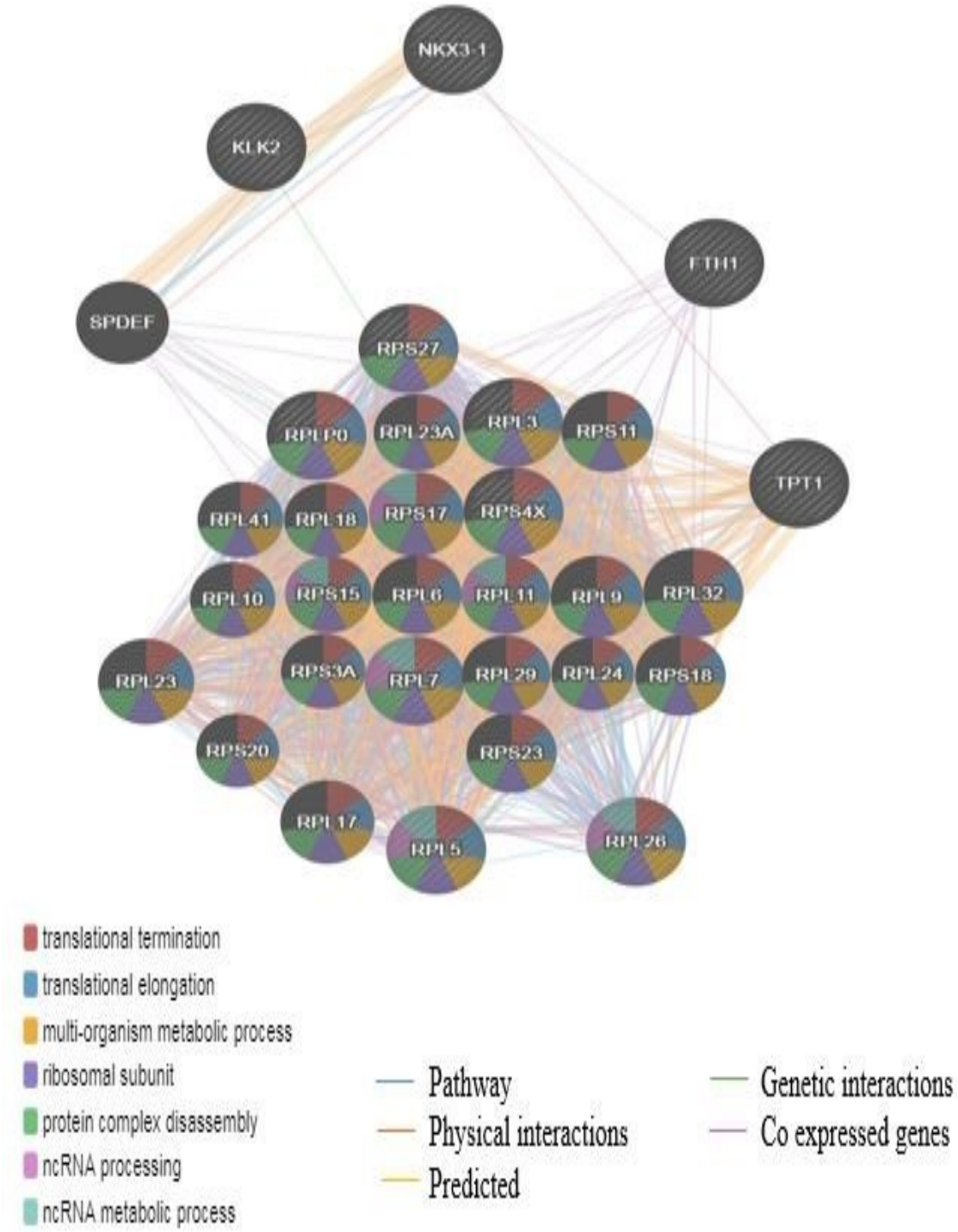

Figure 3.8 A gene regulatory network of signaling pathways for transcripts

Genes that are significantly different between motile and immotile sperm showing functionally related pathways and genetic interactions. Image from GeneMania (http://www.genemania.org).

Another transcript, glandular kallikrein protein family (KLKs), which was shown to be linked to RPS27 in motile sperm. KLK is expressed in the prostate gland, and responsible for semen liquefaction and is essential for sperm motility (Sotiropoulou et al., 2009; Li et al., 2017). Kallikreins are a subgroup of serine proteases that cluster on chromosome19. Members of this gene family are involved in a diverse array of biological functions. The protein encoded by this gene is a highly active trypsin-like serine protease that selectively cleaves at arginine residues (Fuhrman-Luck et al., 2014). By submitting KLK gene in Target scan software (http://www.targetscan.org), it appeared that KLK2 has multiple target sites for miRNAs like hsa-let7a-5p, has-let7b-5p, hsa-let7e-5p, hsa-let7f-5p, hsa-miR-124-3p, hsa-miR-30a-3p and has-miR-214-3p. This suggests a possible role of miRNA in the regulation of KLK transcript post-transcriptional expression in the human sperm. The other expressed transcripts were also submitted through Targetscan software to show the miRNA targets and were listed in Table 3.3.

Table 3.3 mRNA transcripts and their miRNA targets from Target scan

No.	Gene symbol	miRNA targets
1	NKX3-1	has-miR-142-5p, has-miR-26-5p, has-miR-129-5p, has-miR-302-3p/ 372-3p/ 373-3p/ 520-3p, has-miR-155-5p
2	RPS4X	has-miR-125a/b-5p, has-miR-23a/b/c-5p, has-miR-130a-5p
3	RPLP0	Poorly conserved
4	EEF1A1	33-5pa/b, 143a-5p, 133a/b-3p
5	RPLP26	miR-216a/b-5p
6	RPL27	miR-143-3p, miR-141-3p, miR200a-3p, miR365a/b-3p
7	RPLP1	miR-129-5p, miR-216-5p, miR-141-3p, miR-200a-3p, miR148a-3p, miR-152-3p, miR-148b-3p, miR-199a-5p, miR-181a-5p, miR-150-5p, miR-205-5p,miR-182-5p, miR-22-3p,
8	TPT1	miR-520c/b/d-3p, miR-302a/b/d, miR-372-3p, miR-150-5p
9	KLK2	miR-33a/b, miR-26a/b, miR-143, miR-9, miR-135a/b, miR216b, miR-15a/b, miR-16, miR-21-5p, miR-590, miR-212,miR-132
10	FTH1	miR-142-3p, miR-365a/b-3p, miR-590-5p, miR-21-5p

3.2 Discussion

The current study was carried out to explore the potential transcript markers of motile and immotile human sperm; human semen samples were divided into two portions for motile and immotile sperm from the same sample. Motility was assessed using the CASA system (Sperminator,® Procreative, UK).

The reported decline in male fertility is a concern, with sperm counts decreased by ~60% over the past 40 years (Levine et al., 2017). In the UK, the most common reasons for IVF treatment cycles being carried out were male infertility, with 37% of recorded reasons (HFEA.gov). Routine semen analysis is the first step of male infertility examination, which includes cell count, vitality, morphology, and motility (WHO, 2010). However, almost 50% of patients are diagnosed with idiopathic infertility, where genetic factors are likely contributors (Association and Medicine, 2006; De Jonge and Barratt, 2006; Lipshultz and Lamb, 2007; Yanagimachi, 2017).

At fertilization, spermatozoa provide the paternal genomic DNA, as well as a set of RNAs and proteins that have distinct roles in development. However, the role of spermatozoal RNAs was originally questioned based on the assumption that since transcription ceases in the round spermatid stage, where the cytoplasm is lost and thus with it, the components necessary for translational activity and any remaining male haploid RNA would be insignificant (Ramakrishna and Surani, 2018). It is now appreciated that spermatozoa contain a suite of unique RNAs that are delivered to the oocyte upon fertilization, which play important functional roles in many different processes including genome recognition, early embryonic development, and epigenetic transgenerational inherence. Furthermore, the

potential of spermatozoal RNAs to be used as a prognostic of live birth has been shown (Jodar et al., 2015).

Of increasing interest is the identification of molecular markers for sperm function and fertility potential, and gene expression profiling of spermatozoa has been proposed as a novel non-invasive tool to evaluate male fertility and testicular function (Bettegowda and Wilkinson, 2010; Dere et al., 2016).

Two swim-up techniques have been chosen in order to sort out motile sperm (A) and immotile sperm (D) from the same sample, simple swim up to isolate the motile sperm and density gradient for immotile sperm after ensuring the samples are normal and no more 1% of round cells in the semen samples. Both methods were proficient and reproducible to liberate of other seminal plasma and cellular debris in the ejaculate. This technique was previously used by other studies

(Weng et al., 2002; May-Panloup et al., 2003; Mengual et al., 2003; Lambard et al., 2004).

RNA isolation from the sperm is a challenging procedure due to the heterogeneous population of cells present in the ejaculate and the low yield of RNA per spermatozoon. Isolation of sperm RNA in other studies demonstrated a low yield, most research used low sperm number to extract the RNA (Lambard et al., 2004; Abu-Halima et al., 2013). In the present study- high sperm concentrations (20-50 m/ml) was required to enhance the sperm RNA recovery, with a yield of 50 ng/µl of total RNA. In previous studies, human sperm can yield about 15 ng of RNA per one million sperm (Miller et al., 2005; Lalancette et al., 2008). The reason that mammalian sperm has low RNA content (10-400 fg/ sperm) comparing to other somatic cells, as well as, disulfide bonds in the sperm

plasma membrane and between protamine and minimal cytoplasmic space (Krawetz, 2005). The head of the sperm also has very dense chromatin due to its replacement by protamine during spermatogenesis, which makes the nucleic acids highly condensed in the nucleus of mature sperm and difficult to extract the nucleic acid in contrast to mammalian somatic cells (Jodar et al., 2015). Some studies have used pooled semen samples in the interest to increase the RNA yield (Mao et al., 2014; Bansal et al., 2015). However, we found that using a single sample can give the approached result of sperm RNA content.

To achieve good RNA yield, ß-mercaptoethanol (ß –ME) was added to the sperm samples to lyses cells (Mengual et al., 2003). Sperm has strong sulfide bond making the lysis procedure demanding. ß-ME is a reducing agent that can denature the RNases activity via inhibition of bisulfite bonds of the sperm and prevent RNA degradation with a combination of denaturing power of the guanidinium isothiocyanate (GITC) in the lysis buffer of Norgen kit. In this study – it was demonstrated that RNA integrity could not confirm by Bioanalyzer. This may owe to the fact that RNA contributes only a small overall proportion and the absence of 18SrRNA and 28SrRNA in sperm that are normally used to calculate the RIN value means that this value is not available to estimate the RNA integrity (Kawano et al., 2012).

To explore the relative expression of mRNA in human sperm from motile and immotile populations – it was imperative to isolate good quality and yield. This study demonstrated the ability to separate motile from immotile sperm and obtain a good RNA quality in an adequate amount for downstream analysis.

High Throughput Next-generation sequencing (NGS) was applied to allow DNA and RNA sequencing directly by the synthesis *in situ* and dNTP that can be detected simultaneously by Illumina system at millions of fixed positions on a flow cell cartridge (Figure 2.8). RNA sequencing (also identified as RNA-Seq) is a procedure of diverse populations' transcriptome analysis including, mRNA analysis. Various software methods have been used to renovate the genomewide existence and number of transcriptomes in biological samples and to investigate the gene expression profiling of the organisms, hence, providing a comprehensive tool to study the key functions of genes (Auer and Doerge, 2010; Kukurba and Montgomery, 2015). RNA-seq analysis has much considerations and cautions should be taken into account to avoid bias sources through the experiment comprising the RNA isolation and library sequencing then data analysis to detect the candidate transcripts that linked to sperm function regulation.

This study revealed that different mRNAs were expressed in motile sperm comparing to immotile sperm from MMU recruited participants, providing data from previous studies that found the presence of mRNA in the sperm and involvement in sperm quality and embryonic development (Ostermeier et al., 2004; Martins and Krawetz, 2005; Nanassy and Carrell, 2008).

Sperm fractionation and RNA extraction have been mentioned previously and mRNA library from sperm has prepared via the Nextflex^{TM} Rapid RNA-Seq kit (Illumina compatible, Bioo Scientific, UK) based on magnetic beads isolation and polyA enrichment method with a gel-free technique. The computational analysis results confirmed high quality reads score (>30) of FASTQ-format file that was produced from BAM format files without any discrepancies. TopHat and Cufflinks

are highly performed apps due to its high revealing rate for multi-intron alignment for transcripts restoration. The alignment sequences of this study use the cis alignment (cDNA libraries of alignment sequences) and trans alignment (cDNA from homologous genes) to generate the whole genome sequence in order to identify the protein-coding transcripts within the genome of interest (Steijger et al., 2013). High throughput RNA-Seq found a challenging quantification process to identify the transcripts with low expression level and needs a more in-depth computational approach. Therefore, this study focused on the highly expressed transcripts for further analysis. The alignment was used because the sequences were mapped to a reference genome, the absence of introns refers to the transcribed RNA being sequenced that contains exons only (Zhao and Hamilton, 2007).

Overall, this study revealed that several diverse mRNA transcripts are differentially expressed between immotile and motile human spermatozoa. Transcripts such as, ribosomal transcripts (RPLs and RPSs) family group, HSP90A, EEF1A, and NKX3-1, were the most abundant genes in the human sperm, which have been related to each other functions when applied to Genemania analysis (http://www.genemania.org), these transcripts are found vital in apoptosis and regulation of physiological processes for reproduction and spermatogenesis (Anderson, 2013). HSPs are transcripts that involved in apoptosis and cytoprotection by inhibition actions, it can be linked to molecular markers of spermatogenesis and male fertility (Purandhar et al., 2014). HSP90 expression level increases in spermatogenic cells at meiotic prophase in mice also expressed during sperm capacitation (Du et al., 2019).

Differences between samples were also noted, which may be due to a large variation between individual's samples. However, due to the limitations of the small sample size - these variations might overcome by increasing sample size.

Although the introns are missing in the reads sequences from mature mRNA transcripts of the eukaryotic genome, which add another challenge when mapping the reads with intronic gaps, the mammalian introns account extensive lengths. Thus, the fixed length K-mers matches to the genome use small local regions for mapping (Kim et al., 2013). Differences in the level of expression suggesting that the sperm transcripts contents are varied according to its status and affected by sperm activity.

In spite of the absence of rRNA in sperm, the significant role of diverse types of RNA has been on focus to prove in term of its contribution to pregnancy outcomes and the health of the progenies. Also serves as a diagnostic tool for male fertility parallel to non-coding RNAs activities in reproduction. Diverse functions of mRNA transcripts in sperm including nuclear condensation (Prm1/2), capacitation (eNOS), cmyc and nNOS have found related to sperm motility (Lambard et al., 2004). Most of these transcripts have been hypothesised that they are remnant transcripts deposited throughout sperm transition period from diploid to haploid spermatids. The process of replacing histones protein by transition proteins (Tnp-1 and Tnp2) and protamines (Prm-1 and Prm-2) resulting in chromatin condensation and stability in the nucleus leaving the sperm transcriptionally silent in consequence (Zhao et al., 2004; Balhorn, 2007; Sillaste et al., 2017).

This study found that most of the ribosomal (RPLs and RPSs) genes exert a significant function in sperm activity. This has been confirmed by a study in 2012 by Bonache et al., when they found some of these transcripts (RPL23A, RPL4,

RPS27A, RPS3, RPS8, RPLI 0A, and RPS6) have a significant role in sperm from fertile and non-fertile male with pregnancy loss and success rate could be predicted according to the sperm content of these transcripts (Bonache et al., 2012). Ribosomal transcripts in the sperm have known to be translated *de novo* by mitochondrial type ribosomes (Zhao et al., 2009). To date, no previous study has elucidated them with the motile and immotile sperm.

HSP90A also expressed in our analysis. In previous studies, there was reported a wide expression in mammalian sperm, testes, and epididymis (Lambard et al., 2003; Lachance et al., 2010), where they reported to be residues of stored mRNA from earlier spermatogenesis and during the sperm-egg fusion process. Abnormal expression of HSP transcript can lead to aberrant sperm quality such as concentration, motility, and sperm morphology due to the fact that this transcript is upregulated in stress conditions as a protection mechanism (Beere, 2004).

To conclude, the sperm transcriptome has yet to be fully elucidated. This study attempted to identify sperm-specific mRNA in sperm from motile and immotile populations with the aim to provide potential avenues for diagnostic andrology.

Further analysis and a more in-depth bioinformatic approach are required to fully appreciate the sperm transcriptome in motile and immotile sperm.

Strenghts and Limitations

Next generation sequencing offers as powerful tool to explore expression data in any cells type. This approach has tremendous potential to reveal both functional and diagnostic data in human sperm. The main limitation is quality RNA isolation

from sperm and library preparation, additionally participant recruitment was also a limitaiton to this study.

Chapter 4: Exploring the relative expression of miRNA in Subpopulations of Human Sperm

4.1 Results

Dysregulation of sperm-specific miRNAs has been shown to be one contributing factor in male infertility. Lian et al., (2009) demonstrated 154 differentially down-regulated and 19 up-regulated miRNAs were found between a non-obstructive azoospermic group and a control group. (Lian et al., 2009). A more recent study has demonstrated that 50 miRNAs were up-regulated and 27 miRNAs were down-regulated in asthenozoospermic males. In oligo-asthenozoospermic males, 42 miRNAs were up-regulated and 44 miRNAs down-regulated when compared with normozoospermic males (Wang et al., 2011). They showed miRNAs that exhibited the highest fold changes were miR-34b, miR-122, and miR-1973 in samples from asthenozoospermic men and miR-34b, miR-34b, miR-15b, miR-34c-5p, miR-122, miR-449a, miR-1973, miR-16, and miR-19a in samples from oligo-asthenozoospermic men. These data revealed a comprehensive number of miRNAs that were differentially expressed in asthenozoospermic and oligo-asthenozoospermic men compared with normozoospermic men. Human sperm contains a unique family of miRNA, which is responsible for normal spermatogenesis and sperm function and their expression profile may serve as a sensitive, selective and non-invasive diagnostic tests for male infertility (Al-Gazi and Carroll, 2015).

The aim of this study was to explore the relative expression of sperm-specific miRNA in human sperm from motile and immotile sperm from a panel of sperm-specific miRNAs.

4.1.1 Study population:

Semen samples were procured from 12 consenting normospermic donors (18-30 years) and were processed as per section 2.1.1. Participants fulfilled WHO criteria (WHO 10th ed.) for semen parameters such as concentration, and motility (Table 4.1). Volunteers were asked to complete a questionnaire (Appendix 1) about their general health, lifestyle and any medications being taken.

Table 4.1 Semen parameter outcomes. A, B, C, and D are refers to sperm motility grades

Volume (mL)	Conc. m/ml	A	B	C	D	Velocity Micron/ s
3.57± 0.34	76.50± 2.46	36.00± 5.09	20.42± 2.16	13.61± 1.13	31.39± 4.38	24.56± 6.93

Data are mean of study parameters ± SEM

4.1.2 Identification and qualification of sperm motility

One of the main aims for this part of the study involved the isolation of sperm of different motility grades (grades A, B, C, and D) from the same semen sample. To achieve this, the swim up technique was performed. Briefly, grade A was obtained from simple layer techniques following 30- 60 mins of incubation with sperm preparation medium and using density gradient (55%-80%, vol. /vol.) technique. Immotile grade D sperm was extracted from the intermediate layer of the gradient after confirmation that there were no somatic cell contaminations

using microscopic examination (Lambard et al., 2003). Variations between individual samples affected semen sample sperm sorting. Low motile sperm, B and C samples were quite difficult to separate, due to some motility changes during sperm preparation procedure, which resulted in some of the cells becoming either motile A or immotile D. This further resulted in low cell numbers of B and C after preparation. For this reason, the study did not include sperm B and C grades for the microRNA analysis.

Table 4.2 illustrates the percentages of the means ± standard errors of the means (SEM) of the semen parameters after sperm preparation. The results showed that sperm isolated from motile (A) sperm contained the highest concentration in million/ml (66.58±12.31) and higher velocity (45.96±26.96) micron/sec. Sperm (B) showed a higher percentage of (Bs) (28.14±16.00) compared to other (B) in another group, but (A) recorded a higher percentage (48.64±17.9) in the same B group. Sperm (C) percentage was 14.26±2.94 and sperm (D) was a higher percentage (35.53±14.4) compared to A, B, and C in the same group. D sperm has shown a higher percentage (60.28±16.95) and a lower velocity (0.50±7.06 micron/sec) compared with the other groups.

Table 4.2 Semen parameters outcomes after preparation of sperm in vitro

Group s	N	Conc. m/ml	A %	B %	C %	D %	Velocity Micron/sec
Group A	1	29.16± 9.92	66.58± 12.3	13.83± 8.82	3.65± 2.28	15.89± 4.8	45.96± 26.96
Group B	3	21.86± 41.18	48.64± 17.9	28.14± 16.0	7.92± 3.36	15.3± 3.17	44.38± 31.42
Group C	3	17.17± 14.83	24.00± 9.61	14.26± 2.94	14.26± 11.20	35.53± 14.4	43.733± 14.6
Group D	12	19.16± 11.35	13.97± 10.07	10.91± 5.31	14.85± 7.33	60.28± 16.9	0.50± 7.06

Data are mean ± SD, N= the number of samples

All analyses were assessed using the CASA system (Sperminator®, Procreative, UK) prior to the sperm preparation by swim-up technique and a video frame capture of sperm tracks motion analysis (kinematics) were obtained (Figure 4.1) that confirms the presence of motile sperm in vast proportion.

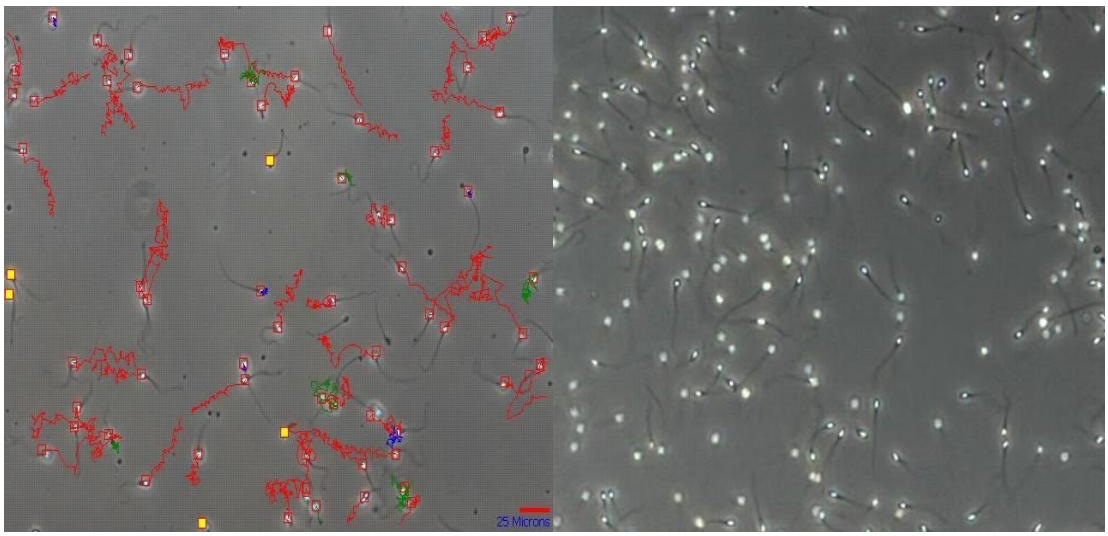

Figure 4.1 The videomicrography captured by in Sperminator® CASA system

The images are showing the original sample on the right where it is free of round cell contamination and the motile sperm after sperm preparation by simple layer method.

Figure 4.2 demonstrates the sensitivity and specificity of the sperm-separation showing the percentages of group D (Figure 4.1a) and group A (Figure 4.1b) in the separately prepared samples using CASA. It is clear that this is an effective method for isolating sperm as over 80% A or D sperm cells were successfully obtained from the samples of the study.

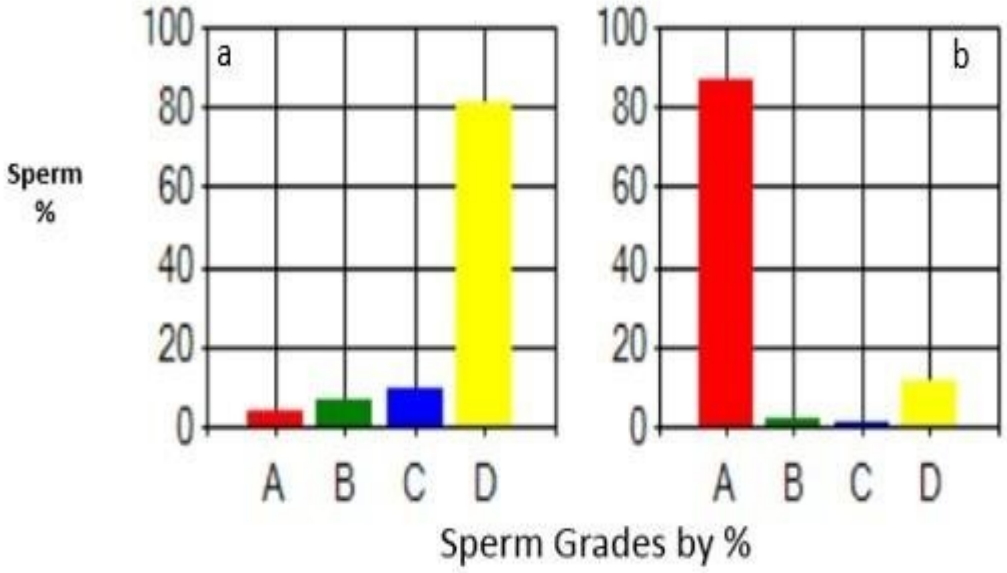

Figure 4.2 The images illustrating the percentages of each motility grade

A (red), B (green), C (blue) and D (yellow) in sperm that has purified by swim-up for D (a) and A (b) as appeared in Sperminator® CASA report.

4.1.3 miRNA expression profiles analysis of human sperm with different motilities

Running the miScript miRNA PCR array custom plates (Qiagen, UK) for motile (A) and immotile (D) sperm with and without Pre-amplification analysis using miScript PreAMP PCR kit (Qiagen, UK). Data from the PreAmp kit were more acceptable regarding the amplification profiles of the cDNAs. After preAMP, it was found that sufficient amount of miRNA, the preAMP cDNA, and the reverse transcription products were not affected by the quality or amount of the original RNA samples, which can affect the reverse transcription reaction and the miRNA expression profile.

The miRNA expression data were presented as normalized threshold cycle (Ct) values ($2^{-\Delta Ct}$), related to RNU6-6P reference gene and all miRNA Ct values

examined against the positive PCR control (PPC) gave values of 19±2 suggesting the samples contained cDNA of high abundance. Data analysis was performed via online software (https://www.qiagen.com/dk/shop/genes-and-pathways/data-analysis-center-overview-page/).

The Ct values of the reverse transcription miRTC (Ct^{miRTC}) values of our samples were less than 7 and showed no RT reaction inhibition, this was calculated by the following equation;

$$\Delta Ct = AVG\ Ct^{miRTC}) - AVG\ Ct^{ppc}$$

For the miRNA significant expression level ($p < 0.05$), the fold changes were calculated as log base 2-fold change ≥ 1 or ≤ -1. Data analysis was performed via http:// pcrdataanalysis. saiosciences.com/mirna online software.

Firstly, results from the analysis of miRNAs between the motility group of motile and immotile sperm revealed that all the 84 candidate miRNAs were tested were present within both motile and immotile sperm. Importantly, however, some of the miRNAs were expressed differently between the two extremes of motility.

The PCR data analysis revealed that among the 84 mature miRNAs identified in human sperm, eight miRNAs were down-regulated (fold change less than 1) significantly $P<0.05$ in immotile sperm (has-19a-3p, hsa-28-5p, has-miR-2233p, hsa-miR-27a-3p, hsa-miR-34a-5p, hsa-miR-106b-5p, hsa-miR-195-5p, hsamiR-191-5p). has-miR-19a-3p revealed the highest significant ($P=0.000019$) downregulation, in immotile sperm compared to motile sperm.

In addition, twenty of the miRNA species were up regulated (fold change greater than 1) in the same group of immotile sperm. These specifically included (hsalet7a-5p, hsa-let7b-5p, hsa-let7f-5p, hsa-let7g-5p, hsa-miR-150-5p, hsa-

miR-17-5p, hsa-mir21-5p, hsa-mir18a-5p, hsa-mir23b-3p, hsa-mir29b-3p, hsa-mir29c3p, hsa-mir30b-5p, hsa-mir30e-5p) compared to motile sperm. The microRNAs hsa-miR-222-3p and hsa-miR-326 showed the highest significant (P=0.000044 and 0.000047 respectively) upregulation (Figure 4.2 and Table 4.3).

The other mature miRNAs , hsa-miR-101-3P, hsa-miR-126-3p, hsa-miR-139-5p, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-154-5p, hsa-miR-146a-5p, hsa-miR155-5p, hsa-miR-181a-5p, hsa-miR-182-5p, hsa-miR-204-5p, hsa-miR-20b-5p showed no significant expression difference between the motile and immotile sperm.

Table 4.4 Mature miRNA expressed significantly (P<0.05) in human sperm

Mature ID	Fold Regulation	p-value	Mature miRNA sequence	Chromosome location
hsa-let-7a-5p	9.4759	0.01216	UGAGGUAGUAGGUUGUAUAGUU	9
hsa-let-7b-5p	6.3484	0.012181	UGAGGUAGUAGGUUGUGUGGUU	22
hsa-let-7f-5p	8.5267	0.010771	UGAGGUAGUAGAUUGUAUAGUU	9
hsa-let-7g-5p	9.6825	0.009698	UGAGGUAGUAGUUUGUACAGUU	3
hsa-miR-150-5p	10.8343	0.000309	UCUCCCAACCCUUGUACCAGUG	19
hsa-miR-17-5p	36.1945	0.232031	CAAAGUGCUUACAGUGCAGGUAG	13
hsa-miR-18a-5p	39.5785	0.000497	CAAGGUGCAUCUAGUGCAGAUAG	13
hsa-miR-21-5p	6.2609	0.000804	UAGCUUAUCAGACUGAUGUUGA	17
hsa-miR-221-3p	11.5157	0.000601	AGCUACAUUGUCUGCUGGGUUU	X
hsa-miR-222-3p	8.5087	0.000044	AGCUACAUCUGGCUACUGGGU	X
hsa-miR-23b-3p	5.0626	0.010292	AUCACAUUGCCAGGGAUUACC	9
hsa-miR-29b-3p	8.0132	0.0034	UAGCACCAUUUGAAAUCAGUGUU	7
hsa-miR-29c-3p	4.0141	0.014316	UAGCACCAUUUGAAAUCGGUUA	1
hsa-miR-30b-5p	5.0918	0.018172	UGUAAACAUCCUACACUCAGCU	8
hsa-miR-30d-5p	4.746	0.040947	UGUAAACAUCCCCGACUGGAAG	8
hsa-miR-30e-5p	8.154	0.0225	UGUAAACAUCCUUGACUGGAAG	1
hsa-miR-326	5.0486	0.000047	CCUCUGGGCCCUUCCUCCAG	11
hsa-miR-574-3p	106.0918	0.000066	CACGCUCAUGCACACACCCACA	4
hsa-miR-92a-3p	14.5077	0.000105	UAUUGCACUUGUCCCGGCCUGU	13
hsa-miR-93-5p	4.6391	0.096511	CAAAGUGCUGUUCGUGCAGGUAG	7
hsa-miR-106b-5p	-4.3938	0.005379	UAAAGUGCUGACAGUGCAGAU	7
hsa-miR-191-5p	-4.8533	0.01764	CAACGGAAUCCCAAAAGCAGCUG	3
hsa-miR-195-5p	-23.8299	0.006456	UAGCAGCACAGAAAUAUUGGC	17
hsa-miR-19a-3p	-37.0229	0.000019	UGUGCAAAUCUAUGCAAAACUGA	13
hsa-miR-223-3p	-58.3326	0.001021	UGUCAGUUUGUCAAAUACCCCA	X

hsa-miR-27a-3p	-7.0139	0.003014	UUCACAGUGGCUAAGUUCCGC	19
hsa-miR-28-5p	-35.4105	0.000117	AAGGAGCUCACAGUCUAUUGAG	3
hsa-miR-34a-5p	-30.66	0.005267	UGGCAGUGUCUUAGCUGGUUGU	1

The overall qRT-PCR results were confirmed by the Volcano plot, 8 miRNAs were down-regulated while 22 up-regulated and the rest show non-significant changes (Figure 4.3).

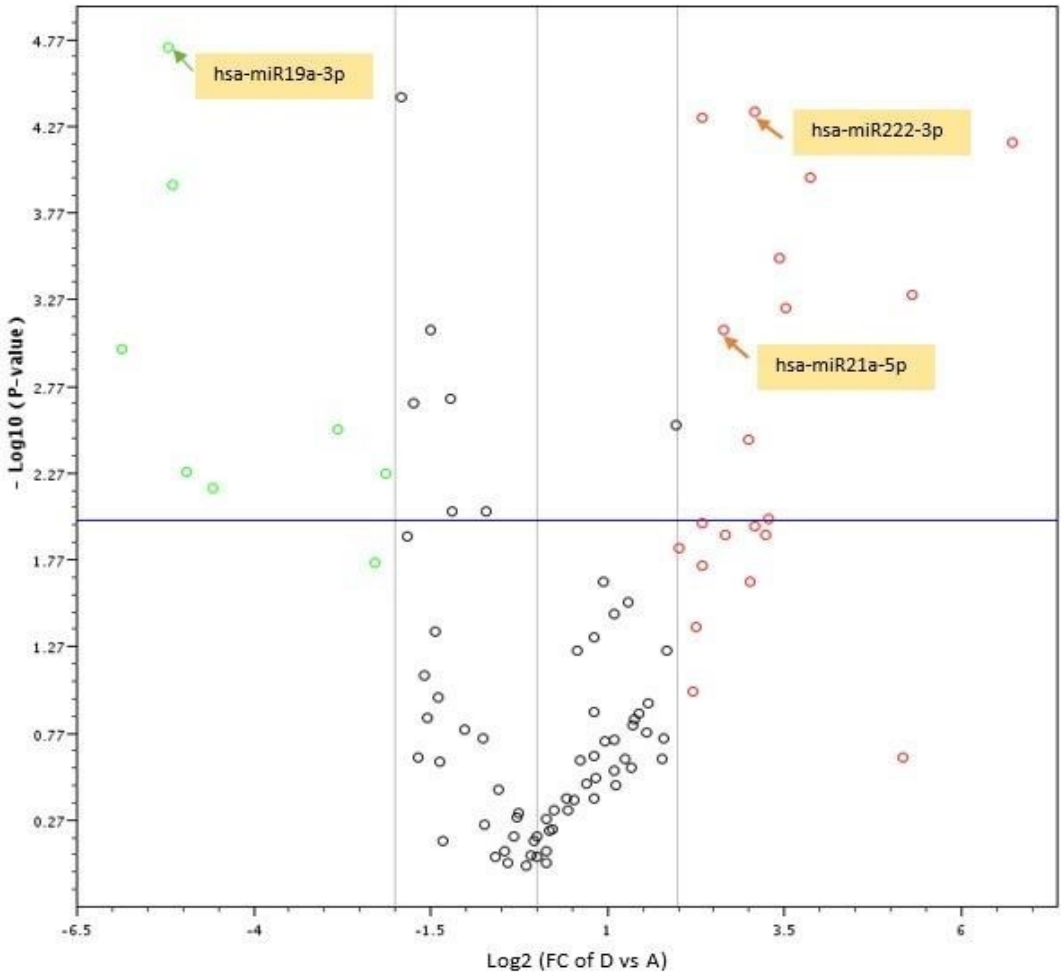

Figure 4.3 Volcano plot of miRNA differential expression in immotile sperm and motile sperm.

Red circles represent miRNAs that are upregulated. The green circles represent miRNAs that are downregulated. Black circles denote miRNAs that show no differences in regulation between motile and immotile human sperm (p-value 0.05).

The hierarchical clustering diagram measurements show up (red) and down (green) regulation relationship of these microRNAs in immotile sperm in comparison with the motile sperm, which appeared as a heat map analysis (Figure 4.4). Most of these miRNAs have been associated with reproductive system malfunctions, embryonic development, and cellular differentiation (Judson et al., 2009).

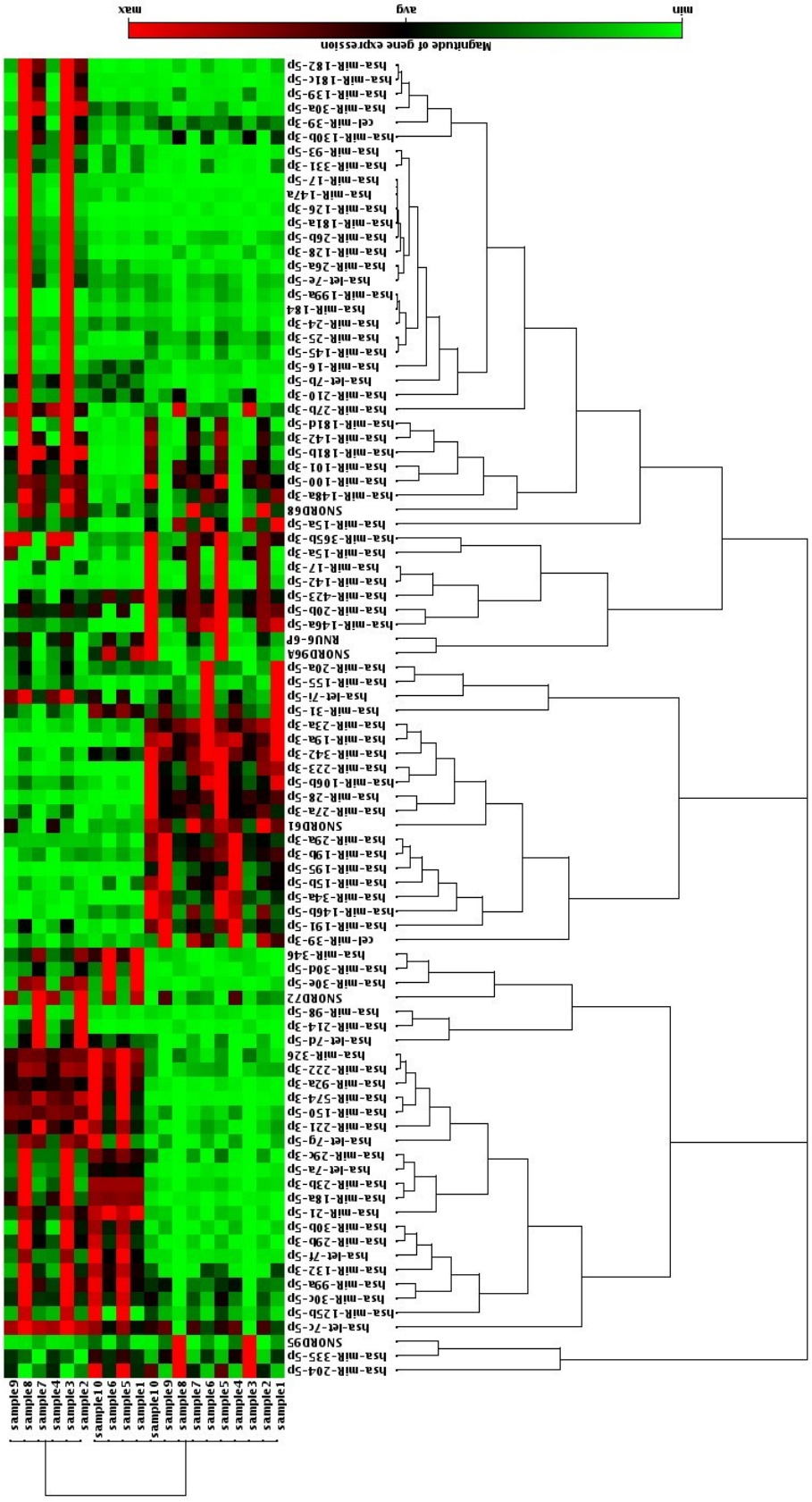

Figure 4.4 Hierarchical clustering analysis diagram of the PCR array.

The level of clustering of 84 miRNA expressed as relative signal intensities in immotile D (N=10) and motile A (N=10) human sperm from normalized high expression level of miRNAs in red colour bars to the low expression level in green colour bars, while dark colours are showing average expression. The miRNAs and the samples are shown in rows and columns respectively.

4.2 Discussion

4.2.1 Sperm miRNA Expression and motility

Since the first discovery in *C. elegans,* miRNAs have been established to play an integral part in organizing cellular functions and diseases post-transcriptionally by regulating target genes and suppressing transcription (Friedman et al., 2009; Johnson et al., 2011). miRNAs processed from hairpin loops in an ssDNA via RISC and DICER pathways and appeared that its expression is highly featured by tissue-specific and developmental stage (Ludwig et al., 2016). In reproduction, miRNAs can regulate gametogenesis and play a role in early embryonic development (Bettegowda and Wilkinson, 2010).

This study investigated the miRNA expression in sperm and has found that there is miRNA differential expression relating to sperm motility. To establish efficient miRNA expression data, we performed a qPCR array on the isolated RNA for a high-throughput sperm-specific miRNA expression profiling. RT-qPCR was used to quantify the mature miRNAs using the miscript primer as a forward primer and a universal primer as a reverse one, as well as, RNU6B primer assay as a control. These primers along with the oligo dts will ensure that mature miRNAs do not detect any genomic DNA.

Despite the notion that sperm is transcriptionally inert because of rRNA (18S and 28S), which are responsible for preventing the translational event, is depleted with cytoplasmic droplet during or post spermiogenesis (Kierszenbaum and Tres, 1975; Jodar et al., 2015). Many studies now demonstrate that sperm contains a profile of miRNAs that contribute to sperm development and function (Engel et al., 2003; De Gannes, 2014). Gene expression is controlled post-transcriptionally

in sperm either during pachytene spermatocyte or post meiosis stage before nuclear silencing, then most of the genes are stored for later spermatogenesis transcripts translation (McIver et al., 2011). Although the sperm miRNAs content (about 3% of total RNA) is lower than in oocyte, it appears that any abnormal sperm miRNA expression could be transferred after fertilization and cause aberrant embryonic development (Johnson et al., 2011; Bohacek and Mansuy, 2015; Pratap et al., 2017).

Pre-Amplification for cDNA samples was done in order to improve the miRNA input. Pre-Amp reaction contains primers and controls to selectively pre-amplify the miRNAs of interest by the PCR array to obtain an adequate amount of cDNA for further analysis and provide unbiased miRNA target amplifications, to ensure that there is enough target for quantification for the subsequent RT-PCR. Mature miRNA is not polyadenylated unlike mRNA, it contains 3' universal tag at one end and 5' degenerated anchor at the other end for that reason, using the polyA tails were used in reverse transcription reaction to allow the amplification of miRNAs in PCR reaction (Cirera et al., 2011).

In contrast to previous studies using the microarray and TaqMan arrays to investigate the miRNA contents and expression in sperm (Wang, 2009; Abu-

Halima et al., 2013; Nixon et al., 2015; Fullston et al., 2016), the current study used qRT-PCR for its simplicity and reliability. However, it is restricted to a set number of miRNAs. Despite this limitation, it is still widely used to validate miRNAs in a range of cell types. In this study, a known set of 84 sperm-specific miRNAs was explored via qRT-PCR analysis in motile and immotile sperm. The relative expression showed significant differential expressions of 18 miRNAs that were up-regulated ($P < 0.05$; fold regulation >2) in immotile sperm compared to

motile sperm. Furthermore, eight miRNAs were significantly down-regulated (P< 0.05; fold regulation <2) in immotile sperm compared to motile sperm. Most of the differentially expressed miRNAs are related to spermatogenesis, which was noted in a study by SalasHuetos et al., (2014) in fertile and infertile individuals and there are different miRNAs related to different physiological and pathological circumstances (SalasHuetos et al., 2014). miRNAs are known to be expressed during different stages of spermatogenesis, for example, let-7b with miR-145 is known to be expressed in pachytene spermatocyte, but not in spermatids and they have different roles and expression depending on the type of cell type they are expressed (Sangiao-Alvarellos et al., 2015).

Among upregulated miRNAs expression > +1 fold change in immotile sperm, let7 family in sperm of the current results was approved previously as negative regulators of lin28 transcripts and targets HMG2 mRNAs, the wide expression of let-7 miRNAs have been implicated in the control of androgen signaling and the repression of cell proliferation and oncogenic pathways (Nixon et al., 2015). Whereas miR-150 involved in embryogenesis through targeting c-Myb proto-oncogene (Lin et al., 2008).

Upregulation of miR-17/92 cluster at chromosome 13 in sperm has been associated with spermatogenesis abnormalities and knockdown of the miR106/25 cluster during renewal spermatogonium differentiation (Tong et al., 2012) by inhibiting PTEN signaling pathway important for sperm activation and development in low sperm motility (Capra et al., 2017).

miR-18a upregulation has been demonstrated to be involved in spermatogenesis through HSF2 target inhibition (Björk et al., 2010). miR-21 is also known to

regulate spermatogonium renewal and is a dynamic miRNAs in regulating various pathways, cellular differentiation, and development (Niu et al., 2011).

Other miRNAs that significantly ($p < 0.000044$ and $p < 0.000601$) up-regulated in this study are miR-222 and miR-221 respectively, they are known to be expressed in human malignancy and regulate cell function and development via P27 / 57 pathways and enhance apoptosis by targeting BCl2 via PUMA/ BAX transcript inhibition (Zhang et al., 2010). miR-29 is found to be related to cell motility via the PTEN pathway (Yan et al., 2007).

Some of the miRNAs are downregulated (fold changes< +1) in immotile sperm when compared with motile sperm, which agrees with some other previous studies when its inhibition reflect the cell stress condition through specific signaling pathway cascades and regulates genes that are activated or inhibited to cause cellular function modifications. Eight miRNAs showed significant ($p<0.05$) downregulation in the immotile sperm. miR-191 is known to be associated with sperm morphological changes in testis, also has found downregulated in teratozoospermia by targeting BNC2 transcript (Grinchuk et al., 2009; McIver et al., 2011).

miR-27a is inhibiting CRISP2 mRNA, motility fine-tune transcript and could be related to low sperm motility and are critical for pregnancy successful rate (Zhou et al., 2017).

Down-regulation of miR-34a in our study was confirmed in previous studies to inhibit BCl2 and induce apoptosis (Yan et al., 2007). miR-34b/c and 449 are present in sperm but not in oocyte and approved that injection of those miRNA prior ICSI treatments enhance success rates. A mouse knockout of these

miRNAs leads to semen abnormalities postulating that they are critical for spermatogenesis and early embryonic development (Yuan et al., 2015).

miR-34c has a role in early cleavage and belongs to a family member of miR34b/c and miR-449a/b/c, which have the same seed region and share the same target genes (He et al., 2007). miR-34c is also known to be involved in the inhibition of second meiotic spermatogenesis by targeting tgif2 mRNA (Damestoy et al., 2005) or participate in early-stage sperm development by inhibiting NOCH2 transcript (Kostereva and Hofmann, 2008).

One possible pathway using Genemania analysis (Figure 4.5), which is crucial for spermatogenesis and induce apoptosis is when up or down-regulation of miRNA will target PTEN pathway via suppressing AKT pathway and STAT it could lead to male infertility by increasing ROS level and modification of mitochondrial ATP levels that end up with sperm apoptosis (Lachance et al., 2013).

Profiling sperm-specific miRNAs are important in understanding how they may regulate spermatogenesis and contribute to the early embryonic development and offspring health (Llancette et al., 2008). Some studies have shown that exogenously added miRNAs can markedly affect fetal development and impact subsequent development (Grandjean et al., 2009).

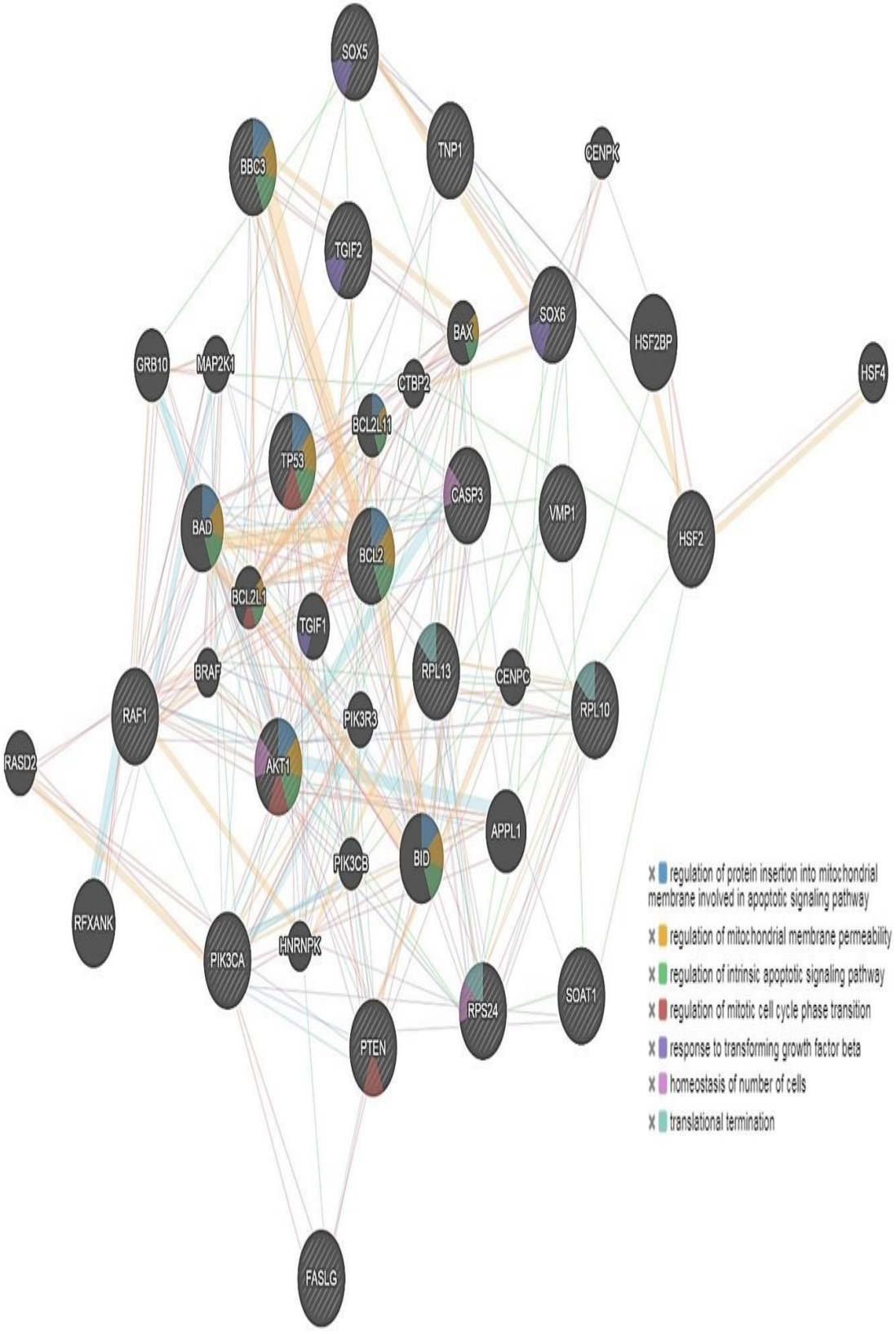

Figure 4.5 Illustration of different 20 related genes in sperm with 39 total genes of 364 total links, the colours are indicating gene functions from Genemania (http://www.Genemania.org).

4.3 Conclusion

The data presented here demonstrate the differential miRNA profiles in motile and immotile human sperm, suggesting that changes in sperm transcriptomes are related to sperm motility. It remains to determine whether the presence of these miRNAs in mature sperm may simply reflect earlier spermatogenesis pathways affecting sperm motility.

However, this study found some miRNA expression from early spermatogenesis are present in mature sperm that may indicate other roles for these miRNAs in later sperm development or in a subsequent process, such as fertilization and pregnancy outcomes.

Part of this study was to explore novel miRNA in motile and immotile human sperm using the Illumina platform for RNA sequencing (see section 2.3 for methodology) that could provide potential biomarkers of sperm function and infertility. However, due to repeated technical issues and time constraints, this part of the study had to be abandoned. The main technical bottleneck was the gel purification for library preparation, where sufficient and quality cDNA could not be obtained.

Chapter 5:

Investigating the relative expression and the epigenetic control of sperm-specific microRNAs in high-fat diet mice and age-matched controls.

5.1 Introduction

Obesity has reached epidemic proportions globally and is predicted to rise in both developed and developing countries worldwide. Recent studies have reported that in the UK, about 27% of adults are obese, another 36% are overweight, and the trend is continuously rising with the advance time as shown in Figure 5.1 (Baker, 2017).

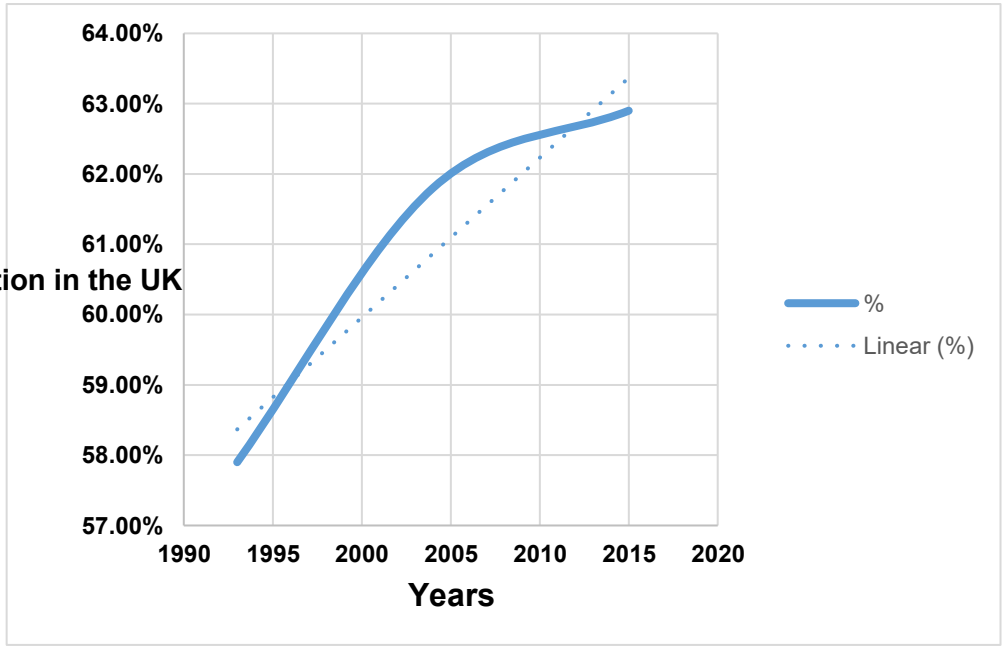

Figure 5.1 The progression of obesity trend in 1993, 2004 and 2015

Increasing the level of the obese population with time in the UK according to the UN Food and Agriculture Organization.

Obesity can be measured according to body mass index (BMI), which is based on the weight and height of the individual and is defined as the body mass divided by the square of the height (BMI = Kg/m^2), (de Castro Barbosa et al., 2016). Normal and abnormal ranges are presented in Table 5.1.

Table 5.1 obesity category according to BMI measurements

Category	BMI (Kg/m^2)
Normal	18.5-24.9
Overweight	25.0-29.9
Obese	30.0-34.9
Morbidity obese	40.0+

The primary reason for increased body weight is the high-calories food consumption that leads to the imbalance between energy intake and expenditure. Consequently, fat accumulation in particular parts of the body causes serious human health risks such as cardiovascular diseases, type II diabetes, and cancer, which results in increased morbidity and mortality (Pi-Sunyer, 2009). Furthermore, disorders of endocrine homeostasis can contribute to decreased fecundity (Jensen et al., 2004). Both the size and number of fat cells (adipocytes) increase and most pre-adipocytes proliferate into mature adipocytes, which have the ability to store a large amount of fat (A McGregor and S Choi, 2011).

Obesity leads to changes in the distribution of adipocytes, their differentiation, and apoptosis via excess fatty acid synthesis (Spalding et al., 2008). This is concomitant to the dysregulation of adiponectin and resistin, which is involved in the development of type2 diabetes, increased oxidative stress, and increase the level of adipokines (Al-Suhaimi and Scehzad, 2013). On the molecular level,

obesity can lead to epigenetic modification, which alters the gene expression level of some genes like PPAR family, a member of genes that regulate adipogenesis and obesity, and metabolism of tissues by increasing insulin resistance via different pathways like Akt, PDK-1, P53, and P85. Hence, the dysregulation of lipogenesis and lipolysis occurs (Samuelsson et al., 2008; Alfaradhi and Ozanne, 2011).

Epigenetic mechanisms have also been known to have an impact on obesity via processes such as DNA methylation, histone modification, and the action of noncoding small RNAs (Palmer et al., 2014). It has also been demonstrated that some epigenetic alterations are passed to further generations (transgenerational epigenetics), which can impact on the offspring health (de Castro Barbosa et al., 2016). Furthermore, mouse studies have shown that gametes from obese parents can negatively impact preimplantation embryo development, morphology, and metabolism (Finger et al., 2015; Wu et al., 2015). The known mechanism that can alter the gene expression accompanied with obesity, is due to DNA damage that occurs in the mutant cells because of oxidative stress and cell inflammation due to genome instability caused by obesity (Włodarczyk and Grażyna, 2019).

5.1.1 Male obesity and Infertility

According to the UN Food and Agriculture Organization, one in four British men are obese and it has been projected to be more than half the population obese by 2050.

Male reproductive potential is coincident with obesity, not only by impairing sperm function but in particular, alters the physical and molecular composition of germ cells in the testes and ultimately affects the maturity of sperm and its capacity (Shukla et al., 2014; Palmer et al., 2012). Obesity is also correlated with reductions in normal semen parameters such as sperm concentration and motility. In addition, obesity is correlated with increased sperm DNA damage (Dupont et al., 2013) and changes in reproductive hormones (Dulloo and Montani, 2012). Several mechanisms have been attributed to the obesity effects on sperm functions and male subfertility, such as the excessive conversion of androgens into estrogens. Changes resulting in sexual hormone imbalance and hypogonadism; adipokines produced by adipose tissue, which induce inflammation and oxidative stress in the male reproductive tract, thus impairing testicular and epididymal tissues (Aggerholm et al., 2008; Huang et al, 2016). However, more studies are needed to be elucidated to understand the obesity effects on male reproduction.

Sperm is known to be susceptible to oxidative stress that leads to sperm DNA damage and impairment to the sperm function (Aitken et al., 2014). Excessive oxidative stress is one of the potential mechanisms leading to poor sperm quality in obese males and studies have shown that oxidative stress in semen and testis were correlated to the increase in BMI and sperm DNA damage (Bakos et al. 2011).

Obesity can also induce insulin and glucose disturbances and result in sperm dysfunction. Hyperglycemia or insulin insufficiency decreased the level of GLUT9 in sperm and affect the sperm motility and fertilization rates (Kim and Moley, 2008).

Studies have demonstrated increased pregnancy loss rate in obese couples undergoing ART, which is attributed to reduced fertilization rate after IVF including sperm binding ability and blastocyst development (Bakos et al., 2011). However, other studies found no relation between BMI and IVF outcomes (Jungheim et al., 2013). Moreover, some reports have shown a decrease in the pregnancy rate and blastocysts development during ART of obese or overweight male partner (Bakos et al., 2011; Hammoud et al., 2008).

Furthermore, recent studies indicate that epigenetic changes may be a consequence of increased adiposity. A major effort to identify epigenetic determinants of obesity revealed that sperm DNA methylation and non-coding RNA modification are associated with BMI modifications and proposed to inherit metabolic comorbidities (Ornellas et al., 2017). Obesity causes sperm miRNA changes and germ cell hypomethylation of the genetic material (Fullston et al., 2013).

5.1.2 miRNA regulation and obesity:

The identified list of miRNAs is enormous; most of them are sharing similar functions. Target genes express themselves in different organs to be involved in cellular differentiation and proliferation, growth, apoptosis and in most common diseases. miRNA genes are located in intronic, non-coding exonic or intergenic regions (Schmittgen et al., 2008). The binding sites of miRNAs for RNA the polymerase and transcription factors were located in the core, while a proximal promoter consists of regulatory binding sites for transcription factors and CpG

islands for methylation a distal promoter for secondary regulatory factors (Berardi et al., 2012).

miRNA is one of an essential epigenetic mechanism post-transcriptionally that participate crucially in most of the biological and metabolic processes associated with obesity like, insulin and glucose homeostasis, cholesterol and lipid metabolism, that could be initiated by targeting mRNA transcripts that leads to molecular cascade changes which, ultimate with metabolic and reproductive disorders (Curry, 2010). Some of the miRNAs regulate fat accumulation through the action as fat cell maturation accelerator (proadipogenic factor) or inhibitor (antiadipogenic factor) by targeting different genes and pathways.

The function of miRNAs in adipose tissue is found to be regulation of differentiation through stimulation or inhibition of adipocytes and to regulate specific metabolic and endocrine functions. the miR-14 role has been found in adipogenesis from the genetic screening of Drosophila. miR-14 deletion leads to fat cell mass enlargement and apoptosis (Xu et al., 2003). Another miRNA, miR-278 has a significant expression difference in adipose tissue and regulates body metabolic rate through insulin signal transduction pathway in mammals and flies by control the level of circulating glucose and fat distribution. Mutant miR-278 cause obesity in Drosophila by changing glucose homeostasis and the production of insulin (Teleman and Cohen, 2006).

The human adipose tissue has multiple miRNAs content; however, few of them are expressed and may be changed in obese and type2 diabetes mellitus individuals, or differentially expressed miRNAs in various adipose depots. Obesity is accompanying with low-grade inflammation that is regulated by signal transduction systems, in which miRNAs, either directly or indirectly (by regulatory

transcription factors components), affect the inflammatory protein expression and secretion. Moreover, to their signalling multiple effects, miRNA and transcription elements can network to augment the influence of the inflammation. In spite of additional miRNA, signal links in human adipose tissue are not known yet; similar regulatory circuits have been designated in brown adipose tissue in mice. miRNA can also be secreted from fat cells into the circulation and serve as markers of disturbed adipose tissue might offer tangible targets for treating metabolic disorders (Arner and Kulyté, 2015).

The role of miRNA in obesity and type 2 diabetes in the pancreas, liver, and adipose tissue has been well reviewed, and attempts have been made to diagnosis the chronic diseases and therapeutic targets via miRNAs (Zhao et al., 2009), however, its role in sperm still not well understood.

miRNAs expression level has been approved changes in spermatozoa of a male with high BMI. Inhibition of miRNAs in the male pronucleus of fertilized oocytes leads to the production of a phenotypic variant of progeny that depends on miRNAs ratios. That means sperm RNAs are vital for the embryonic development, survival and progeny phenotypes (Shukla et al., 2014).

Various studies have been applied on HFD mice to induce obesity in order to study paternal or maternal phenotypic alteration that can perturb sperm function and transmitted via sperm transcriptome to further generations (Nishimura et al., 2007; McPherson et al., 2014; Navya and Yajurvedi, 2017).

In mice, experimental high-fat diet (HFD) can revise the molecular patterns or gene expression of the testes and sperm specifically miRNA-21 abundance by changing IGF-IR/Akt/P13k pathway (Esau et al., 2004; Fullston et al., 2013).

Phenotypic alteration of obesity can be transferred to the next generation via miRNAs and evidence showed that diet-induced paternal obesity has been shown to alter sperm miRNA content (Leibel et al., 2006). For instance, miR-133b and its target Igf-1R (insulin growth factor 1 receptor) are key regulators of proliferation and differentiation, Igf-1R dysfunction can cause early embryonic death in mice (Bedzhov et al., 2012). miR-133b has found to be overexpressed in HFD sperm, repressing Igf-1R and contributes to embryonic development failure (Mitchell et al., 2011). miR-196 has another role in regulating spermatogenesis and male infertility (Madison-Villar and Michalak, 2011). miR-196a-5p is a vital regulator during early embryonic development and infertility, it has found to be upregulated in HFD sperm and testes in mice by targeting HOX-A, B and C in mammals (Iimura and Pourquié, 2007) and repress NOBOX mRNA, a maternal transcript critical for EED (Wang and Yao, 2014). miRNA involved in lipid metabolism and glucose homeostasis, miR-375 in mice is significant in developing normal endocrine pancreas in mice, and mice lacking miR-375 (375KO) were hyperglycemic, increase the number of α-pancreatic cells and increase glucagon blood level, which in turns leads to increase gluconeogenesis, then insulin resistance will develop leading to severe diabetes (Poy et al., 2009). Obesity is induced by miR-143 expression differences in adipose tissue of high-fat diet mice by targeting Fgf7 gene (fibroblast growth factor 7) (Peng et al., 2014), and through ERK5 gene in human pre-adipocytes (Esau et al., 2004).

Chartoumpekis et al., (2012) reported the difference in the expression of several miRNAs accompanied obesity development, miR-342-3p, miR-142-3p, miR-1425p, miR-21, miR-146a, miR-146b, miR-379 were up-regulated, while miR-122, miR-133b, miR-1, miR-30a, miR-192, and miR-203 were downregulated

(Chartoumpekis et al., 2012). miR-205 and miR-340 expression have been changed in the testis and sperm in males fed on HFD, miR-205 has a principal role in cell cycle regulation and apoptosis (Madison-Villar and Michalak, 2011). the miR17/92 cluster also contributed to adipocyte differentiation acceleration by targeting Rb2 and p130 genes vital for adipogenesis (Wang et al., 2008) also targeting mTOR pathway signaling, which is important for sperm activity (Xie et al., 2016).

Evidence that inheriting obesity after HFD exposure has proved that testes transcriptome and miRNA profile of sperm in male mice and the causes of the miRNA dysregulation in germ cells of the testes, which contain multiple kinds of both germ and somatic cells. Additionally, paternal obesity in mice can cause perturbation of pathways signalling enriched for metabolic disease, cell death, production of ROS, DNA replication, NF-κB signalling, p53 signalling, recombination and repair, lipid metabolism, spermatogenesis and embryonic development (Fullston et al., 2013).

Furthermore, HFD mice produce phenotypic sub-fertile males that are heritable further two subsequent generations of offspring (Fullston et al., 2012). Thus, sperm miRNA that is downregulated by an HFD modulate or are predicted to modulate genomic integrity, epigenetic state, and embryonic development and provide a possible mechanism for paternal transmission of obesity and impaired metabolic health to the next generation by altering the epigenetic signature of the sperm (Fullston et al., 2013).

5.1.3 miR-21 genomic structure and regulation

miR-21 (Figure 5.2) is one of the highly significant miRNAs that has been identified at chromosome 17q23.1 in human (on chromosome 11qc in mice) overlaps with the eleventh intron of the 3'UTR TMEM49 (transmembrane protein9) gene or called VmP1 (vacuole membrane protein) (Ribas et al., 2012). It gets its own promoter transcription sites called VMP1and pri-miR-21, and is highly conserved among species, also identified as an oncomir and upregulated in response to malignancy by targeting BCL2 protein and promotes apoptosis (Yang et al., 2015). The expression of miR-21 has found RNA polymeraseII dependent on pri-miRNA precursor that are both capped and polyadenylated (Cai et al., 2004). It is also highly expressed in hepatocytes associated with insulin resistance and related to cellular differentiation, metabolism, and apoptosis (Calo et al., 2016; Palmer et al., 2014).

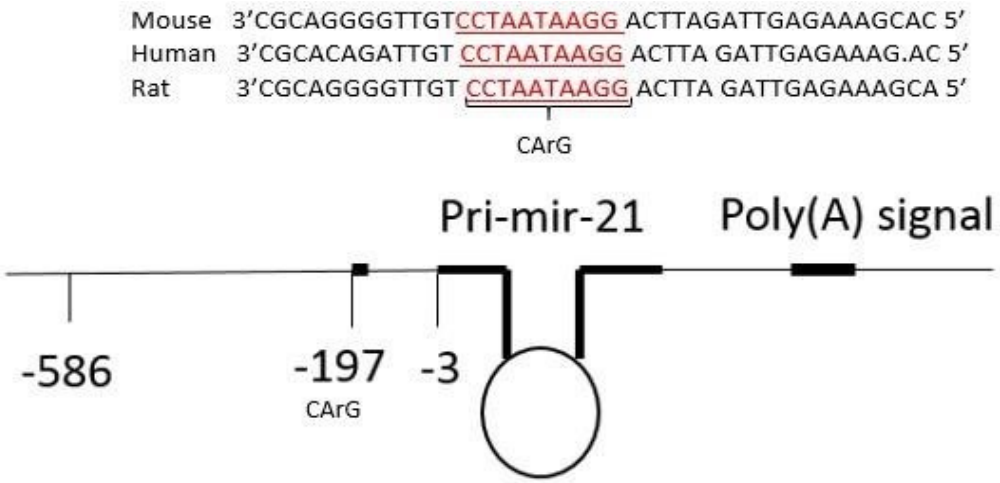

Figure 5.2 miR-21 conservation site

Different species of mice, human, and rats miR-21, the conserved seed area are shaded, and the promoter at -197 base pairs upstream of pri-miRNA-21 (stem-loop structure) is presented.

miR-21 is crucial for metabolism by regulating glucose and lipid uptake by targeting various mRNA including apoptosis regulators of BCl2 and the adipocyte differentiation inhibition pathway called, TGF-β (Kim et al., 2009), tumour suppressors PDCD4 and PTEN pathways. Downregulation of miR-21 can inhibit cancer proliferation via PTEN pathway (Meng et al., 2007). miR-21 has been found to be the best hit in the detection of miRNA regulation in cancer and cardiac diseases (Zhang et al., 2011).

Decreased expression of insulin signaling pathways such as GLUT4 (glucose transporter) is one of the major factors of insulin resistance and metabolic disorder that are regulated by miRNA-21 (Cai et al., 2004; Chakraborty et al., 2014). While, miR-21 overexpression was known to enhance insulin-induced phosphorylation in GLUT4 and IR adipocytes to control glucose motion across cell membranes (Ling et al., 2012).

5.1.4 DNA methylation

Epigenetics is simply known as the modification of non-genetic marks on DNA without changing the DNA sequence because of environmental changes, therefore, regulating gene expression and cellular development and differentiation (Chaligné and Heard, 2014). DNA methylation occurs in eukaryotic DNA by adding the methyl group in the 5th position of cytosine that followed by guanine base known as CpG dinucleotides to form 5-methylcytosine

(5-mC) in the presence of DNA methyltransferases (DNMTs) enzymes like DNMT1, DNMT3b, and DNMT3a (Figure 5.3). Cytosine methylation can also occur at non- CpG locations like CpC, CpT, and CpA sites, but are restricted to some type of cells such as oocyte and pluripotent stem cells to participate in epigenetic regulation (Maunakea et al., 2010; Jang et al., 2017). This usually results in gene silencing via active demethylation from 5hmC and converted into thymine driven by enzymatic deamination and leads to gene expression inhibition (dos Santos et al., 2015).

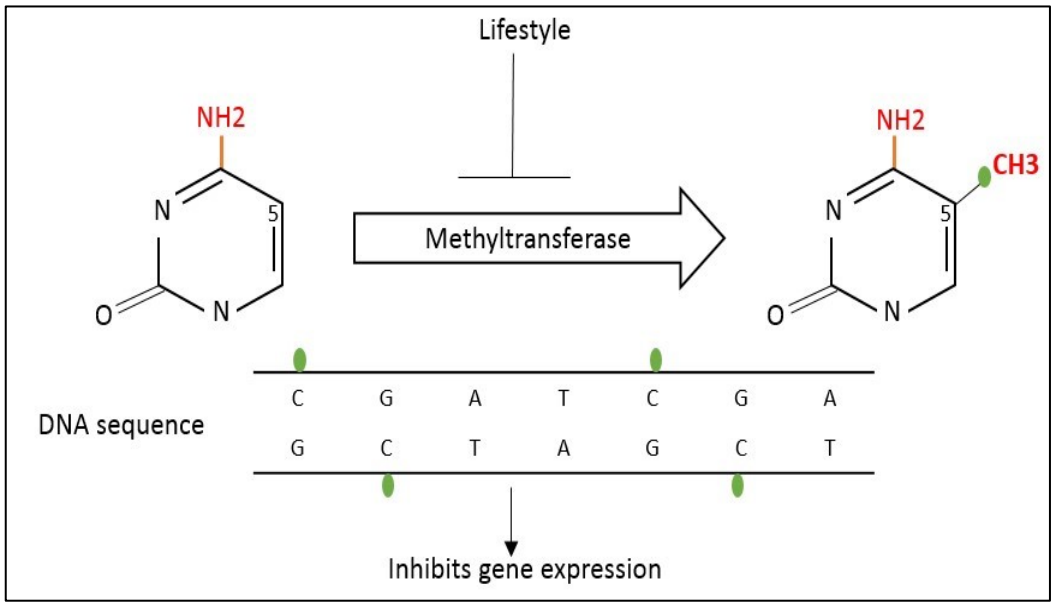

Figure 5.3 Methylation of cytosine as part of the main key of the epigenetic mechanism.

DNA epigenetic marks occur on CpG sites by adding methyl group (green circles) to the Cytosine base at position 5 of the DNA at promoter regions to form 5-methylcytosine in the presence of methyltransferases.

Most genes in vertebrates contain more than 50% of CpG rich regions defined as CpG islands. Exogenic factors can imply a potent impact on the methylation status of an individual, which originally came from gametes (Norouzitallab et al., 2018). Many types of research were conducted on animals and proved that paternal lifestyle can be transmitted via the epigenetic landscape to the next generation and causes metabolic disorders and infertility (Donkin et al., 2018). Experimentally, 5mC can be detected by treating DNA with bisulfite, the unmethylated cytosine will converted into uracil and then into thymine via PCR amplification while methylated one will resist that conversion and remained intact as cytosine (Soubry et al., 2016). Over a period, all methylated cytosine, turn into thymine because of spontaneous deamination process unless there is another reason for keeping them as cytosine (Table 5.2).

Table 5.2 DNA Sequences after bisulfite treatment and PCR amplification

	Original sequence	After bisulfite treatment	After PCR amplification
Unmethylated DNA	C-A-A-T-C-G-T-C-G	U-A-A-T-U-G-T-U-G	T-A-A-T-T-G-T-T-G
Methylated DNA	C-A-A-T-C-G-T-C-G	C-A-A-T-C-G-T-C-G	C-A-A-T-C-G-T-C-G

Simply, DNA methylation is the addition of a methyl group from the DNA sequence and any disturbance may lead to embryogenesis interruption also incorporated in other diseases and cancer development. Active genes at

transcription start site of a specific gene usually unmethylated and controlled by transcriptional factors (Lin and Zhang, 2014).

Here we hypothesise that epigenetic processes such miRNA expression differences and DNA methylation can be a non-invasive diagnostic method of male infertility due to high- fat diet.

5.1.5 Aims

The aim of this study is to identify the relative expression of sperm-specific miRNA in an obese mouse model and explore the epigenetic regulation of this expression.

To achieve this, objectives are to:

- Extract sperm from mice fed on a high-fat diet [and controls]
- Isolate total sperm RNA and miRNA for array analysis
- Explore any epigenetic regulation of miRNA expression through pyrosequencing.

5.2 Materials and methods

5.2.1 Study Design

C57BL/6 mice (n = 12) were fed on rodent high-fat diet (HDF) with 60% kcal% fat and age-matched controls (AMC) were fed on rodent diet with 10% kcal% fat (n = 12) for 24-28 weeks postweaning, with water *ad libitum* [all animals were supplied by Dr A. Greenstien, Univeristy of Manchester, as carcasses surplus to

use]. Mice were sacrificed by cervical dislocation by an experienced technician following home licence procedures [Project licence number: PPL40_3558].

5.2.2 Mouse sperm collection

Both testes were removed and the epididymis was dissected after being washed with PBS to avoid blood contamination, carefully separated from adipose and overlying connective tissues and immediately expressed into PBS and kept on ice for downstream extraction of sperm, total RNA, DNA, and protein. The epididymis was then transferred into sperm preparation media (SPM^{TM}; Origio, Denmark) and sperm was recovered by a simple layer technique. Simply, the epididymis was placed into a 15 ml Falcon tubes containing 1 ml of SPM, multiple incisions with razor blades and micro scissor were applied in the epididymis wall. Samples were kept for at least one hour in 6% CO2-5% O_2 at 37◦C incubator to allow actively motile sperm swim-up freely into the medium. The sperm was then collected from the top layer and washed twice with PBS to remove contaminating cells, and the epididymis with other contaminated tissues was left in the bottom of the tube to be discarded (Huang et al., 2007; Anderson et al., 2015). Finally, sperm pellet was mixed with 50 µl PBS and counted using Neubauer chamber counter as per WHO references guidelines 2010 for human sperm examination (WHO 5th edition), and kept in Eppendorf tubes at -80◦C after mix it with sperm freeze medium 1:1 dropwise until further RNA, DNA and protein processing.

5.2.3 Total RNA extraction

Total RNA isolation from purified mouse sperm was carried out as per section 2.2 using the Norgen biotek kits. Briefly, the cell membrane that contains a high

amount of disulfide bonds in the sperm head was broken down by adding β-ME as a strong antioxidant after two steps of wash with 1% dPBS. The cells were vigorously shaken for 30 seconds and allowed to stand for 30 minutes on ice to release the nucleic acids and keep the RNA content intact and efficient, then proceed to RNA extraction using Total RNA Isolation (miRNA enriched) kit, (Norgen biotek, UK), (refer to section 2.1.2.1). Purity and concentration of RNA samples were assessed by the NanoDrop 1000 spectrophotometer (ThermoScientific, Wilmington, USA). Only those samples that contain a concentration between 250- 500 ng and a 260/280 ratio of 1.8 - 2.0, a reflection of protein-free RNA, were only considered for further analysis.

5.2.4 Reverse transcription and Pre-Amplification

Approximately 125-250 µg of RNA samples were converted into single strand cDNA using miScript II RT kit (Qiagen, UK), followed by Pre-Amplification reaction using miScript Pre-AmP kit following the same procedure mentioned in section 2.2.7.

5.2.5 Quantitative Real-Time PCR for mature miRNA profiling:

qRT- amplification was conducted to measure the relative gene expression of different 84 miRNAs that were chosen depending on previous studies (Krwatz et al., 2011; Nixon et al., 2015) via miScript miRNA PCR custom plate array kit (Qiagen, UK), for 24 male mice (12 from each group), (Table 5.3).

Table 5.3 Different 84 miRNAs included in the custom PCR array plate

miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID	miRNA ID
let-7a5p	let-7b5p	let-7c -5p	let-7e5p	let-7i3p	miR-1a-3p	miR-10b-5p	miR101 b-3p	miR106 a-5p	miR-122-5p	miR-124-3p	miR-127-3p
miR129-2-3p	miR130 a-3p	miR133 a-3p	miR133 b-3p	miR-141-3p	miR142 a-3p	miR142 a-5p	miR-143-3p	miR145 a-5p	miR146 a-5p	miR146 b-5p	miR148 a-3p
miR-15a-5p	miR-155-5p	miR16-1-3p	miR16-2-3p	miR-17-5p	miR-17-3p	miR181 b-5p	miR-18a-5p	miR-19a-3p	miR-192-5p	miR193 a-3p	miR196 b-5p
miR-20a-5p	miR-20b-5p	miR200 b-3p	miR-203-3p	miR-204-5p	miR208 b -3p	miR-214-3p	miR-21a-5p	miR-22-3p	miR-222-3p	miR-223-3p	miR-24-3p
miR-27a-3p	miR-29a-3p	miR-300-3p	miR-30a-5p	miR-30e-5p	miR-320-3p	miR-34a-5p	miR-34b-5p	miR-34c-5p	miR-340-5p	miR-342-3p	miR-375-3p
miR378 a-5p	miR-379-5p	miR-409-5p	miR465 a-5p	miR465 b-5p	miR-470-5p	miR471-5p **	miR-499-5p	miR-539-5p	miR-582-5p	miR743 a-5p	miR-871-5p
miR-880-3p	miR883 a-3p	miR376 b-5p	miR-468-5p	miR-93-5p	miR-99a-5p	miR19b-3p5p	miR-183-5p	miR-468-5p	miR465 a-5p	miR465 b-5p	miR465c -5p
CelmiR39-3p	CelmiR39-3p	SNORD61	SNORD68	SNORD68	SNORD72	SNORD95	RNU66P	miRTC	miRTC	PPC	PPC

5.2.5.1 miR-21 expression

We were next interested in exploring any epigenetic regulation of the expression of miR21-a, which was found to be within the promoter region of VmP1 gene.

Bonferroni correction was applied to find the miR-21 expression in the sperm of HFD mice and study the DNA methylation epigenetic of sperm in HFD and AMC mice model.

5.2.5.2 Spermatozoa DNA isolation:

DNA from the same sperm collected in section 5.2.2, 20-30 x 10^6 purified sperm was isolated following the protocol described by Weyrich (Weyrich, 2012). Briefly, previously frozen isolated sperm was thawed at room temperature, then washed twice with 1 ml of 1% dPBS, and sperm was counted using the Neubauer

chamber. Ethanol 70% (500 µl) was added and the samples were centrifuged for 5 min at 13000 rpm after that the supernatant was removed. The pellet was then incubated overnight in lysis buffer containing 1M Tris-HCl (Sigma, UK) pH 8.0, 3 M NaCl, 0.5 M EDTA (Lonza, Belgium) and 2.5 µl of 20% SDS. 2.5 µl Triton-X100 (0.5%), 21 µl DDT (1M) as a strong antioxidant, and 40 µl proteinase K (10 mg/ml) (Sigma, UK) also added for complete cells lysis. Samples were incubated at 50°C overnight in a thermos-shaker (TS-100; PEQLAB Ltd, Sarisbury Green, UK), then were centrifuged for 10 min at 15000 xg. The supernatant containing the DNA was carefully transferred into a new Eppendorf tube. DNA precipitation was performed by adding 10 µl βME, 1/10 volume of 3M NaOAc and 2 volume of ice-cold 100% ethanol, mixed and left at 20°C overnight. The mixture was centrifuged for 20 min at 15000 rpm and the supernatant was carefully removed and subsequently cleaned twice with 500 µl 75% ethanol, spun for 10 min at 12000 rpm. The pellet was dissolved in 100 µl Nuclease-free H2O and kept overnight at 4°C. DNA yield and quality were measured using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and was considered pure if the ratio of 260/280 was 1.8-2.0; samples were kept in -20°C for the next step of bisulfite conversion.

5.2.5.3 Bisulfite Pyrosequencing assay design

The sequencing primers for the methylation assay in the regions of interest of mouse, VmP1 including promoter for miR-21 was chosen based on a previous study (Zhang et al., 2011), which demonstrated to contain CpG regions for DNA methylation. One of the PCR primers was biotin labeled to isolate a single-stranded DNA of the target product that serves as a template in the sequencing

reaction. The sequence was retrieved from the mouse genome assembly GRCm 38/mm10 on the UCSC genome browser (Genome Bioinformatics Groups of UC Santa Cruz, 2015). We looked at the presence of CpG islands specifically close to promoters or regulatory regions. Sequences were introduced into EMBOSS CpG predictor software (Genome Bioinformatics Groups of US Santa Cruz) with a minimum CpG length of 100 bp in order to restrict the secondary structure formation in an ssDNA, which in turn inhibit the sequencing reaction or increase the background signals by 3' end extension. PCR and sequencing primers of the proximal promoter region were designed as a custom oligonucleotide via the PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany), and purchased through Life Technologies (UK) (Table 5.4).

Table 5.4 Pyrosequencing custom oligonucleotide for mouse VmP1 gene

Gene	Chromosome location	Amplicon length bp	Sequence to analyse (5'- 3')	CpGs
VmP1 Exon 11	Chr11:86,583, 86586,683,822	148	AAGGATGA**CG**CAGGGGTTGTCCTAATAAG GACTTAGATTGAGAAAGCACCTCCCACCCA CCCATCCCCCTGAGAAGA**CG**	2

5.2.5.4 Bisulfite modification of DNA of interest

A total of 500 ng of sperm genomic DNA in nuclease-free water was sodium bisulfite- treated to sulphonate unmethylated cytosine nucleotides into uracil with the EpiTec Fast Bisulfite Conversion kit (Qiagen, UK) following the manufacturer's instructions. DNA was added to each reaction tubes containing 80 µl of bisulfite buffer and 15 µl of DNA protect buffer to a final volume of 140 µl. The reaction was carried out in a thermal cycler (Sure Cycler 8800; Agilent

Technologies Inc., Santa Clara, USA) under conditions detailed in Table 5.5.

Table 5.5 Thermal cycler steps of Bisulfite conversion reaction

Step	Temperature C◦	Time (min)
Denaturation	95	5
Incubation	60	10
Denaturation	95	5
Incubation	60	10
Incubation	20	Hold

Following PCR reaction, the samples were transferred into 1.5 mL microcentrifuge tubes and 310 µl of BL buffer was added to each of the bisulfite DNA and vortexed. Next, 250µl of 100% ethanol was added and mixed further. The mixture was then purified using the supplied spin columns. The flowthrough was discarded, followed by a washing step with 500 µl BW buffer before 15 min incubation at room temperature in buffer BD for desulphonation step. Samples were centrifuged for 1 min at 12000 rpm and the flow-through was again discarded. After that two washes in 500 µl BW buffer with 1 min centrifugation at 12000 rpm, followed by 250 µl ethanol 100% wash step, the mixture was spun at 12000 rpm and incubated for 5 min at 60◦C Consequently. Samples were then incubated for 1 min after eluted in 15 µl of prewarmed elution buffer. ssDNA concentration was determined spectrophotocally at 260/280 nm and was stored at -20◦C before used for PCR amplification using the PyroMark PCR kit (Qiagen, UK).

5.2.5.5 PCR amplification of bisulfite DNA

PCR amplification of the region of interest was carried out by the PyroMark PCR kit (Qiagen, UK). In order to generate a biotin-labeled amplicon on one of the two strands of the primer designed to target the bisulfite DNA, a master mix was prepared on ice according to the reaction mix in Table 5.6. Amplification was checked along with control of no template by agarose gel electrophoresis. Samples were kept at -20◦C until sequencing.

Table 5.6 Reaction Composition for PyroMark PCR

Components	Volume µl	Final concentration
PyroMark PCR Master Mix	12.5	1x
Coralload concentrate	2.5	1x
Forward Primer: GGGGGAGGTGTTTTTTTAATGT	1	0.3 µM
Reverse Primer: **Biotin-CTACCCTCCCTCTCTCTAAC**	1	0.3µM
NF-H2O	5	-
DNA	3	5 ng/µl

The cycling conditions using the thermal cycler (SureCycler8800; Agilent Technologies Inc., Santa Clara, USA) directly applied to the above master mix as shown in Table 5.7. All samples then were analysed by Pyrosequencing using the PyroMark Q24 system (Qiagen, UK).

Table 2.7 Cycling protocol of PyroMark PCR

Steps	Time	Temperature ◦C	Cycle number
Initial activation	**15 min**	**95**	**1**
Denaturation	**30s**	**94**	
Annealing	**30s**	**56**	**45**
Extension	**30s**	**72**	
Fiunal extension	**10 min**	**72**	**1**
Hold	**-**	**2**	**-**

emitting a visible light, which is detected by the pyrosequencer and appears as a peak of the resulting data output of pyrogram trace (Figure 5.4). The production of light is directly proportional to the number of nucleotides incorporated and the

level of methylation is automatically counted by the PyroMark software and presented in the pyrogram as methylation percentage.

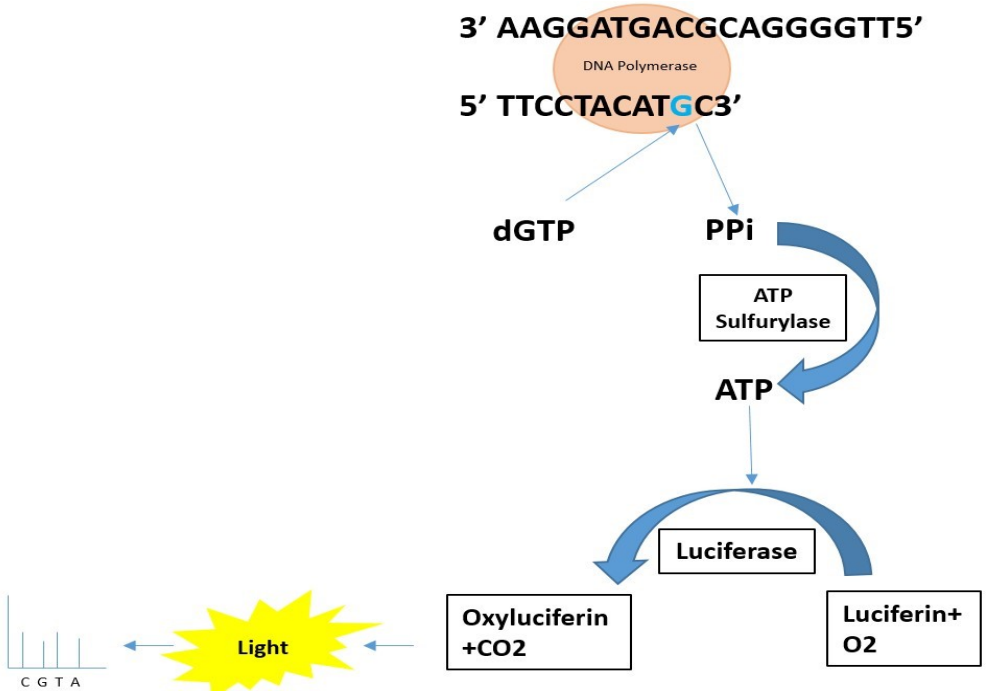

Figure 5.4 enzymatic cascade involved in pyrosequencing process.
Ppi is the pyrophosphate released after incorporation of dNTP (biotin-labeled) in a primer-directed polymerase extension. The quantity of light ejected after ATP reacted to produce oxyluciferin is proportional to the number of nucleotides assimilated and will appear as the peak for each nucleotide integrated.

Sequencing was done by binding the ssDNA to Streptavidin-coated Sepharose High-Performance beads (GE Healthcare Biosciences, UK). A master mix was made up of 1 µl of the beads into 40 µl binding buffer and 29 µl nuclease-free H2O (per sample volume). Then, 70 µl master mix was transferred to a 24 –well PCR strip and 10 µl of the PCR product was added bringing the total volume to

80 µl, and was shaken on a thermos-shaker (TS-100; PEQLAB Ltd, Saribury Green, UK) at maximum speed for 10 min at room temperature to maintain dispersion of the beads.

After that, 0.3 µM of the sequencing primer was prepared in annealing buffer (Qiagen, UK) and 25 µl of diluted sequencing primer was dispensed into each well of a 24-well Q24 PyroMark reaction plate (Qiagen, UK).

The PyroMark Q24 workstation was then run: using a vacuum tool, DNA-bound beads were sequentially washed into 70% ethanol (Sigma, UK) for 5 sec, denaturing buffer (Qiagen, UK) for 5 sec, wash buffer for 5 sec then on the sequencing primer plate for 10 sec. The plate was immediately transferred to a heat block set to 80◦C and incubated for 2 min. Following this, the plate was secured into the Pyrosequencerafter has been cooled for 5 min at room temperature.

A new assay was generated on the PyroMark Q24 software for each specific site and the sequence to analyse was input to produce a nucleotide dispensation order. The required assay was selected for each run per each sample in the plate, which gave the volumes of enzyme and substrate mixtures, and dNTPs to load into the cartridge (Qiagen, UK) based on the length of the assay sequence. The cartridge was loaded and secured in place in the Pyrosequencer. Finally, the sequencing experiment was run and the Pyrosequencing output was in the form of a pyrogram and results were analysed using the PyroMark Q24 software.

PyroMark Q24 software gave an accurate reading for each of the CpG sites analysed based on how correct the nucleotides in the surrounding sequence has

been sequenced: red (failed), yellow (check) and blue (passed). Red reads were repeated or excluded from the analysis.

5.2.5.6 Statistical analysis

Data analysis of gene (miRNA) expression profile of the study cohorts was done through miRNA PCR Array data analysis online software (Qiagen, UK) (http://pcrdataanalysis. sabiosciences.com/mirna). A *t-test* was applied to study the significant difference in miRNA expression profile between motile HFD and AMC groups of the study. Before analysis, all data were tested by Shapiro-Wilk statistic for normality distribution. Values of $P < 0.05$ were considered significant. Bonferroni correction analysis was used to find the most significant miRNAs.

IBM SPSS statistical software, independent *t-test* was performed for each variable of this study comparing means and standard deviation of the mean for methylation data observations at 95% CI.

5.3 Results

5.3.1 miRNA expression qRT-PCR

To confirm the relative expression of sperm-specific miRNAs in HFD and AMC mice, a qRT-PCR (Applied Biosystem) was carried out on the isolated RNA. A set of 84 spermatozoal miRNAs were selected using custom miScript miRNA PCR array and Pre-Amplification protocol from Qiagen-UK.

Relative expression levels were normalized to a ıel of six housekeeping genes (cel-miR-39-3p, SNORD 61, 68, 72, 95, 96A). miRTC was a gene for the reverse transcription reaction control and PPC as a positive PCR control. The other controls were used for data normalization. The analysis demonstrated that 29 of them were overexpressed (fold changes greater than 1) or significantly upregulated ($p < 0.05$) in the sperm of HFD mice, while three miRNAs were downregulated (fold changes less than 1), but only miR-883a-3p was significant ($p < 0.05$) as listed in Table 5.8, and highlighted in the scatter plot (Figure 5.5). The remaining 54 miRNAs showed no significant differences in the expression of miRNA in the sperm of HFD male mice when compared to controls.

Table 5.8 Fold change and P values of miRNA miScript PCR Array in HFD comparing with AMC,

All miRNAs are highly expressed significantly, only miR-883a-3p was downregulated. The underlined sequences are representing the miRNA seed regions

miRNA	Fold Regulation	P value	Mature miRNA Sequence	location
let7a-5p	70.2859	0.005627	UGAGGUAGUAGGUUGUAUAGUU	ch13
let7b-5p	54.3505	0.022252	UGAGGUAGUAGGUUGUGUGGUU	ch15
let7c-5p	63.9943	0.005654	UGAGGUAGUAGGUUGUAUGGUU	ch16
let7e-5p	9.9594	0.027242	UGAGGUAGGAGGUUGUAUAGUU	ch17
miR-10b-5p	180.004	0.00995	UACCCUGUAGAACCGAAUUUGUG	ch2
miR-145a-5p	146.8859	0.032656	GUCCAGUUUUCCCAGGAAUCCCU	ch18
miR-148a-3p	291.5883	0.004335	UCAGUGCACUACAGAACUUUGU	ch6
miR-17-5p	196.3796	0.040346	CAAAGUGCUUACAGUGCAGGUAG	ch14
miR-19a-3p	146.7403	0.021939	UGUGCAAAUCUAUGCAAAACUGA	ch14
miR-20a-5p	206.3355	0.018187	UAAAGUGCUUAUAGUGCAGGUA	ch14
miR-20b-5p	176.3626	0.04279	CAAAGUGCUCAUAGUGCAGGUAG	chX

miR-200b-3p	21.262	0.003506	UAAUACUGCCUGGUAAUGAUGA	ch4
miR-204-5p	122.5673	0.020412	UUCCCUUUGUCAUCCUAUGCCU	ch19
miR-214-3p	39.8525	0.009967	ACAGCAGGCACAGACAGGCAGU	ch1
* **miR-21a-5p**	61.3726	0.000643	UAGCUUAUCAGACUGAUGUUGA	ch11
miR-22-3p	32.5092	0.039342	AAGCUGCCAGUUGAAGAACUGU	ch11
miR-222-3p	55.6404	0.04257	AGCUACAUCUGGCUACUGGGU	chX
miR-223-3p	60.5419	0.005702	UGUCAGUUUGUCAAAUACCCCA	chX
miR-24-3p	111.0388	0.016333	UGGCUCAGUUCAGCAGGAACAG	ch13
miR-27a-3p	86.0089	0.026101	UUCACAGUGGCUAAGUUCCGC	ch8
miR-29a-3p	169.679	0.043498	UAGCACCAUCUGAAAUCGGUUA	ch6
miR-30e-5p	95.771	0.024435	UGUAAACAUCCUUGACUGGAAG	ch4
miR-320-3p	31.0873	0.00703	AAAAGCUGGGUUGAGAGGGCGA	ch14
miR-34a-5p	99.449	0.03182	UGGCAGUGUCUUAGCUGGUUGU	ch4
miR-34c-5p	80.3869	0.020111	AGGCAGUGUAGUUAGCUGAUUG	ch9
miR-342-3p	23.2586	0.017736	UCUCACACAGAAAUCGCACCCGU	ch12
miR-375-3p	47.665	0.010424	UUUGUUCGUUCGGCUCGCGUGA	ch1
miR-465b-5p	23.6548	0.015052	UGCAAUGCCCUAUUUAGAA	chX
miR-183-5p	92.3576	0.004631	UAUGGCACUGGUAGAAUUCACU	ch6
miR-883a-3p	-4.233	0.045197	UAACUGCAACAGCUCUCAGUAU	chX

***miR21 is highly significant upregulated via the Bonferroni correction method (p<0.0006).**

The scatter plot and cluster gram data analysis was performed to confirm the miRNA distribution depending on their expression changes in the sperm of HFD and controls (AMC) mice through http://pcrdataanalysis.sabiosciences.com/mirna software (Figure 5.5 and Figure 5.6).

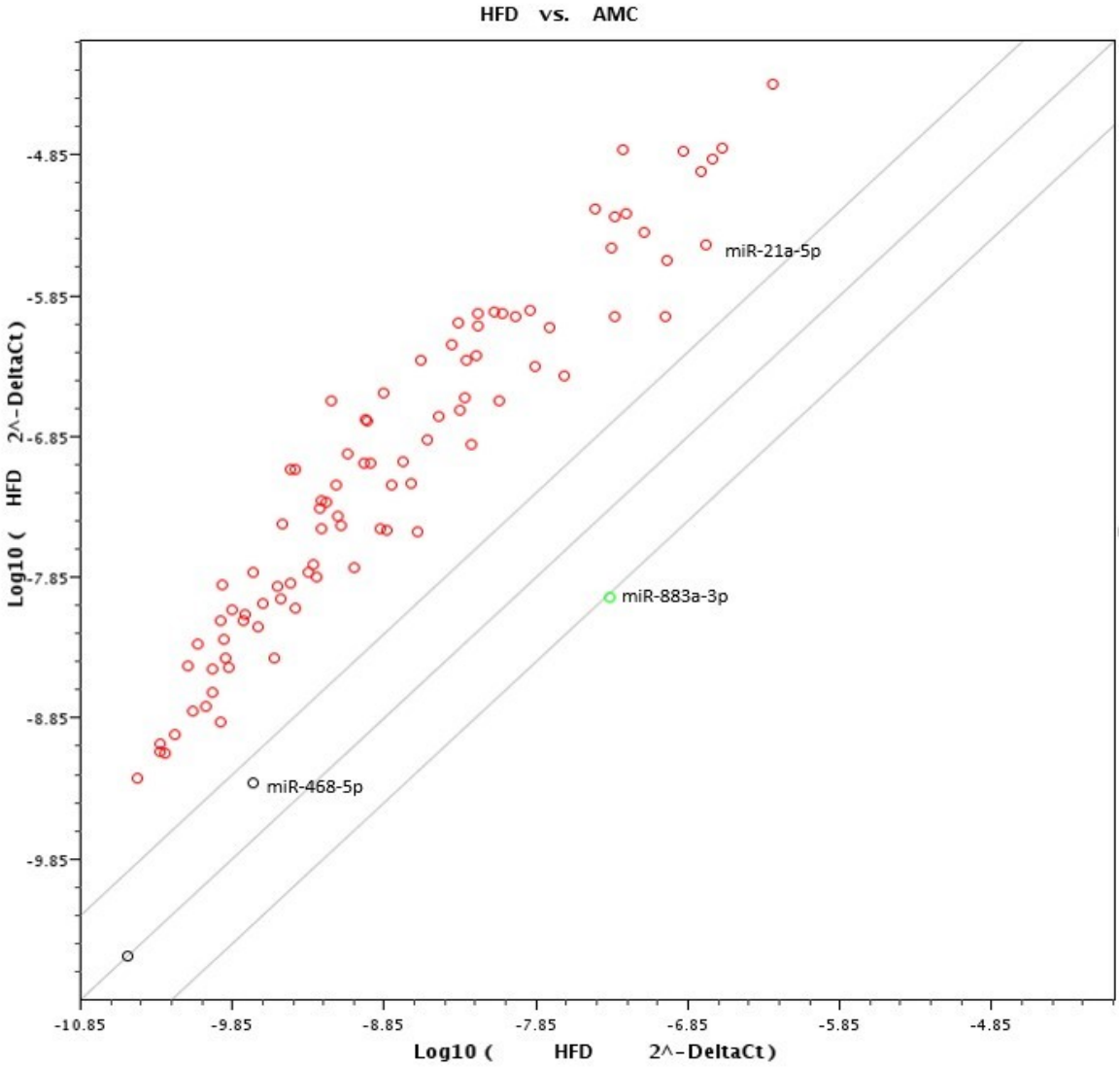

Figure 5.5 Scatter plot of miRNA expression:

log 2 values of relative miRNA expression of mouse sperm versus-Log 10 of the p-value. The red colour is significant and non-significant highly expressed miRNAs, and the black colour is the downregulated miRNAs. The expression profile of 84 different miRNAs is as log10 (2^-Delta Ct) of normalized gene expression level in the sperm of HFD group (X-axis) compared with AMC mice (Y-axis) at P = 0.05. (HDF: High-fat diet mice; AMC: Aged matched control fed mice).

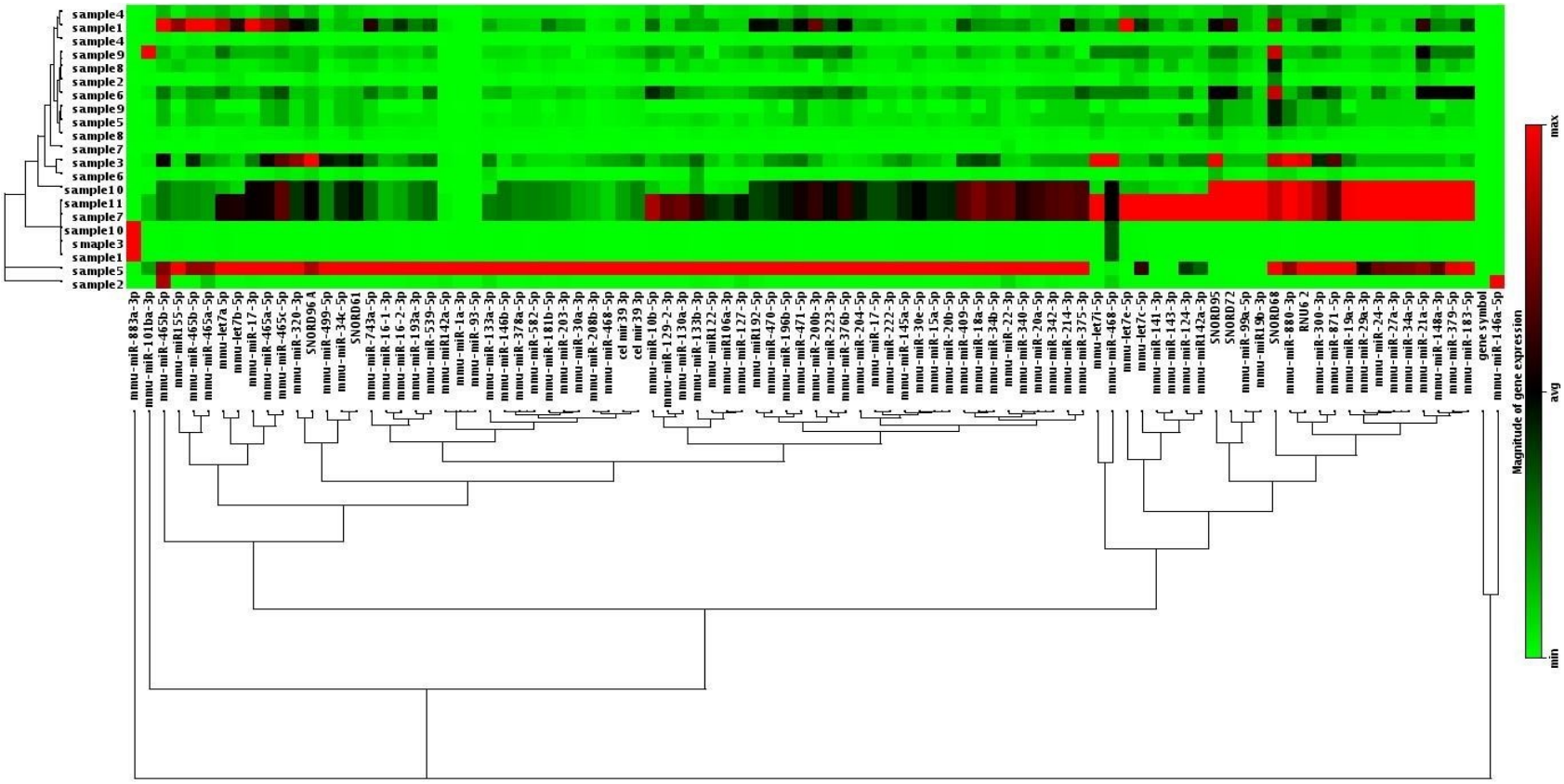

Figure 5.6 **Hierarchical clustering heat map representation of the transcriptome analysis of miRNAs in the spermatozoa of mice.**

Changes in the expression of miRNA from HFD sperm compare to AMC sperm ($p < 0.05$). Each row is displaying the data from different samples. Samples are depicted in columns and miRNAs are clustering in rows. The different colours indicated the miRNAs species upregulation and downregulation of red and green colour indication respectively.

miRNA regulation in the early stages of spermatogenesis has been studied previously (Wang and Xu, 2014) but their role in the later stage of motile sperm remains to be fully appreciated.

After Bonferroni correction, the expression of miR-21a-5p was highly significant (0.000643) in the sperm of HFD mice. The fold regulation is equal to the fold change of 2^ (- Delta Delta Ct) equals the normalized gene expression 2^ (- Delta Ct) in the sperm of HFD mice divided the normalized gene expression 2^ (- Delta Ct) in the sperm of AMC mice. It was decided to explore the epigenetic regulation of this miRNA further.

miR-21a-5p is a ubiquitous oncogene or tumour suppressor; miR-21a (mirBase Accession: MINAT0000530), and is broadly conserved among vertebrates. As this was highly upregulated in HFD mouse sperm, we were interested in investigating the epigenetic regulation of miR-21a by exploring any changes in the methylation of the promoter region responsible for its expression. The Primary transcript (Pri-miR-21a), which lies next to VmP1 (Vacuole Membrane Protein 1) promoter region, also known as Transmembrane Protein 49 (TMEM 49) gene are regulated by binding site “CCTAATAAGG” called CArG box transcription control element within the promoter region (Figure 5.7) according to a previous study(Zhang et al., 2011).

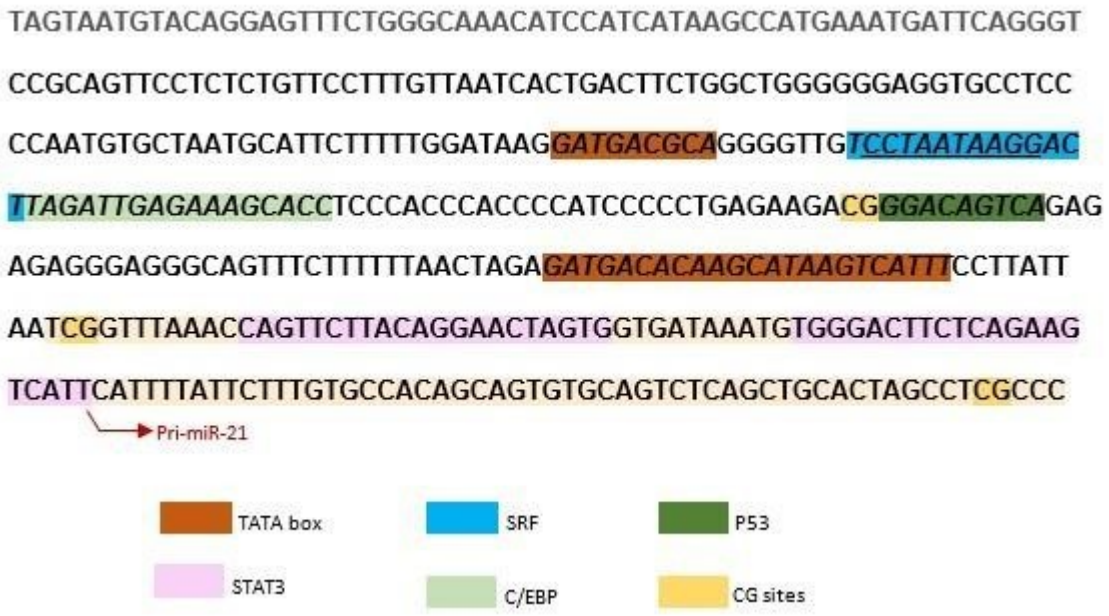

Figure 5.7 Putative region of miRNA-21a structure and function,

The schematic is showing the transcription site of pri-miR-21 with the red arrow and other coloured different transcription sites. Modulated and cited by (Fujita et al., 2008). SRF or CArG is representing the transcription control element.

pri-miR-21 and VmP1 regulate the mature miR-21 independently. Besides, it has been found overlapped with the upstream 3'UTR end of VmP1, of chromosome11qC in mice, in the last protein-coding exon (12 downstream) of the transcript (Figure 5.8).

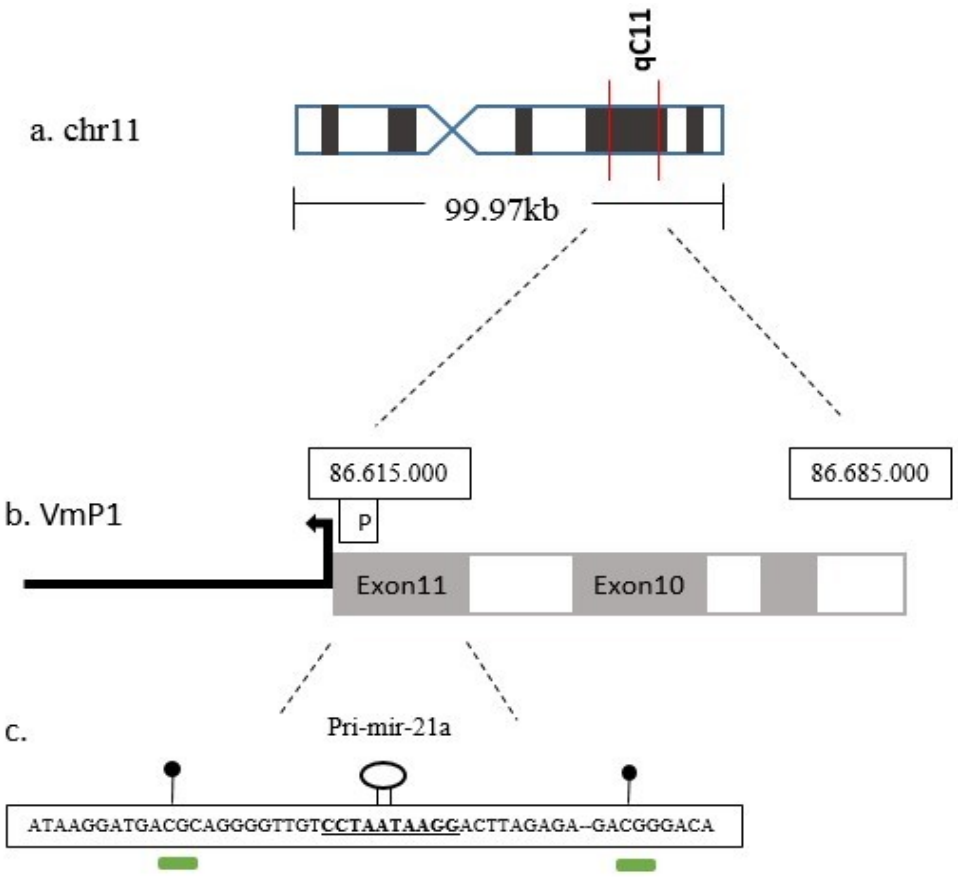

Figure 5.8 Schematic structure of mouse sperm VmP1 transcript and miR-21a.

a. Represents chromosome11 (99.9 /kb size) in mice where VmP1 transcript is located on qc11 between 86.615.0 and 86.685.00 position. b. VmP1 containing 12 highlighted exons, P is the promoter region, the active transcribed site of Pri-miR-21; exon12 adjacent to the promoter region and the site of CpG islands. C. the miRNA and the seed region showed underlined with green marks of the two CpG sites (from UCSC genome browser, genome-euro.ucsc.edu).

5.3.2 DNA methylation analysis

In this study, VmP1 has been found hypermethylated in the sperm of the control mouse sperm when compared to those from HFD mice. miR-21a is one of a small number of miRNAs that are located on exons 12 (Adams et al., 2007), it is a critical regulator of adipogenesis, gluconeogenesis, and glucose uptake (Calo et al., 2016). miR-21a expression level appears to be altered by consumption of obesogenic diet.

5.3.3 VmP1 DNA methylation:

The regulatory region that contains pre-mir-21a has confirmed the presence of two methylated CpG sites in this area (Figure 5.8) at chromosome11qC, a homolog of human chromosome 17q23 (Ribas et al., 2012). Figure 5.9 is exhibiting that site1 of CpG is highly methylated in AMC (89.43%); this site indicates the start of transcription activity of miR-21a, while showed lower methylation level in the HFD sperm (65.73 %). The second site was recorded a relatively low, non-significant methylation value (40-55%) in both groups.

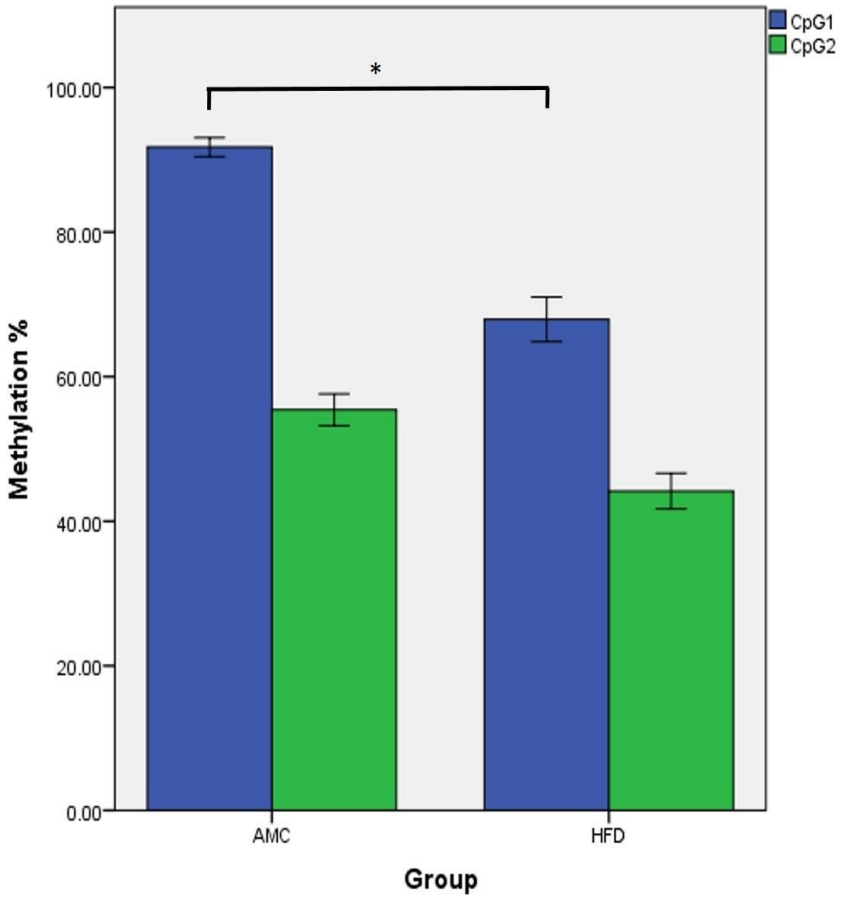

Figure 5.9 CpG site-specific methylation is represented for VmP1 in the sperm of HFD and AMC male mice.

A schematic representation of VmP1 CpG sites from HFD (n=12) and controls (AMC), (n=12). Xaxis is representing the sperm while the Y-axis is the methylation percentage of CpG sites of VmP1. The analysis was performed using one way ANOVA analysis. * P<0.05 compared to HFD from the same site.

5.4 Discussion

The relationship between obesity and male infertility is still ambiguous. Some evidence of semen parameters including sperm density, motility and abnormalities could accompany paternal obesity (Rybar et al., 2011)

The current study shows the miRNA expression differences in C57BL/6 male mice fed on HFD and AMC into gain an overview of how obesity could lead to an improper genes behavior or epigenetic changes on the sperm DNA sequence that may lead to impairing sperm development and function, then cause infertility.

Different studies were carried out to prove the transmission of epigenetic marks from parents to the offspring that make phenotypic changes until the third or fourth generation. For example, let-7 and miR-124 have found to be transferred from the obese father's sperm to the oocyte and reduce the body weight of the newborn (Grandjean et al., 2009; de Castro Barbosa et al., 2016; Fullston et al., 2016; Ornellas et al., 2017). To date, little has been done to understand molecular changes that could take place in the ancestors.

We used a mouse model to study the role of HFD in the male gamete epigenetic reprogramming and the information carried by the sperm that could be induced by dietary changes by interfering with chemical and molecular components of the testes germ cells and mature sperm (Palmer et al., 2012).

We found that the best method of collecting epididymal sperm from mice by the simple swim-up technique that allows the lowest somatic cell contaminations and another artifact that could interfere with the RNA preparation. Good quality RNAs and DNAs from the same samples were isolated to study the miRNA expression and DNA methylation respectively.

PreAmplification for cDNA samples was done in order to improve the miRNA input, which is a low amount in the sperm. After preAMP, we found that we had sufficient miRNAs and also the preAMP did not affect the quality of original RNA samples, which can affect the reverse transcription reaction while the cDNA and the reverse transcription product is already preamplified and not affected.

The expression of 84 mature sperm-specific miRNA can be detected by RTqPCR in all samples of the study and are ready to target pathways vital for sperm maturation, oocyte fertilization, and EED. miRNA were chosen based on previous studies, then some of them were correlated with miRNAs that were expressed and have a vital role in obesity (Appendix1). RT-qPCR confirms that most of sperm-specific miRNA is expressed in the sperm of HFD mice, but only 28 was significant upregulated ($p<0.05$) including let-7 family, miR-10b, miR-34 family and miR-21 family, the only miRNA that records a significant downregulation was miR-883a due to diet changes toward high-fat diet. miR-883a is very important for spermatogenesis by its repression function on the actin reflected protein 5 mRNA (Arpc5) to repress 80S formation in Sertoli cells (Skinner and Griswold 2004; Chang et al., 2012).

The let-7 family has been shown to be targeting genes that have a role in the regulation of lipid, glucose metabolism, and insulin tolerance, miR let-7c is expressed differentially via its targets UcP2 and Ppap2a transcripts in the sperm in response to HFD (de Castro Barbosa et al., 2016). Let-7 family overexpression may relate to excess body fat distribution in the body and develop obesity by PI3K-mTOR signaling and inhibiting translation of multiple gene targets such as, Igf1r, Hmga2, and Igfrbp2, Thus, miR-let-7 are possibly associated with obesity and type2 diabetes (Zhu et al., 2011).

This miRNA expression differences between current and previous studies could be attributed to the concept that sperm miRNA is expressed in a different stage of development, the 29 highly expressed in our mature sperm during its presence in the epididymis and are needed to complete their maturation in the epididymis before being ejaculated. While the other 52 miRNA are also sperm-specific miRNAs but expressed in the different stage during spermatogenesis in the seminiferous tubules or afterward in the oocyte or at fertilization and EED. One example, let-7 was first discovered and needed for the early stage of development by targeting lin-14 mRNA (Bartel and Chen, 2004). Thus, each miRNA is important for a specific time of life and exert their functions by switching genes on or off.

miRNAs have different expression pattern depends on the type of cells they are expressing in and its condition. Some miRNAs were downregulated during adipogenesis and others upregulated depending on the genes they are targeting. miR-27 significantly upregulated ($p>0.02$) in the current study, it is known to be downregulated with adipogenesis and it is increased in fat cells of HFD mice by targeting PPAR family. miR-126-3p has not expressed significantly in our study, however, it recorded a significant increase in the sperm of HFD mice by targeting DNMT1 and cause its hypomethylation in a previous study (Fullston et al., 2016).

Another known mechanism that leads to P53 dysregulation in adiposity is when Six1 targets miR-27a and PRL26 that lead to cell cycle arrest and apoptosis (Towers et al., 2015).

HFD activates the P53 pathway with target genes to cause DNA damage, P53 is known to regulate adipogenesis and enhance the expression and maturation of miR-145a and miR-143. miR-145 expression, mostly occurs in reproductive

organs like ovary and prostate, lead to mTOR signalling pathway inhibition which is for regulation of insulin, ATP, and amino acid secretion as well as for embryogenesis, this will cause a PI3K, AKt3 pathway important for most sperm activity and inhibition of RPS6 then apoptosis (Zhou et al., 2017; Twenter 2016).

miR-34c is upregulated in our data, this miRNA has been proposed to be very critical for male fertility by impairing spermatogenesis to cause oligospermia (Wu et al., 2014) and required in mouse embryo for the first cleavage division (Liu et al., 2012) by targeting Bcl2 mRNA to start embryo development (Chen et al., 2017).

Our study has confirmed some of the miRNAs that are expressed a non-significant differences in the sperm of HFD mice like miR-133b-3p, miR -196a-5p and miR -340-5p. This is in-line with a previous study by Fullston 2016 using Taqman assay and microarray analysis (Fullston et al., 2016) while we used the SyberGreen RT-PCR with PreAmp of acceptable Ct value less than 30 for miRNA detection. The miR-133 family is highly expressed in muscles and called myomiRNAs by targeting SFR, HDAC4, and cyclin D2 mRNA (Yu et al., 2014). miR-130 is upregulated in HFD mice by targeting the same mRNA PPARγ2 (A McGregor and S Choi, 2011; Peng et al., 2014) while it is not expressed in the sperm of our study.

Also, we found the upregulation of miR-20, miR-21 and miR-106 which been approved that they target STAT3 and Ccnd1 to stimulate spermatogonial stem cells renewal in mice(He et al., 2013). Another overexpression of miR-34c, miR-204, and miR-465b which found enriched in testicular cells (Niu et al., 2011). miR-17/92 family (miR-17/18a,18b, 20b, 93, 106a and 106b) upregulation been found related to spermatogenesis and targeting bcl2 and its inhibition leads to male

infertility (Tong et al., 2012) also they have a role in adipogenesis (Wang et al., 2008; An et al., 2016; Deiuliis 2016). The significant upregulation of miR-222 and miR-223 ($P<0.04$ and 0.005 respectively) in our data and both are located on chromosome X where they related to metabolic disorders were confirmed previously, miR-222 is regulating GLUT4, ERα and miR-223 is targeting Pknox1 in the fat of mice epididymis (Deiuliis, 2016). miR-200 also significantly ($P<0.003$) overexpressed in our study have previously approved its regulation in obesity (Benoit et al., 2013). miR-29a ($P<0.04$) from mir-29 family (a, b-1, b-2, and c) has been reported to target genes like FOXA2 in the insulin signaling pathway of fat cells (He et al., 2007). miR-375 ($P< 0.01$) has had a role as well in obesity via the ERK1/2 pathway (Ling et al., 2011). The highly significant ($p< 0.045197$) downregulated in the sperm of HFD mice was miR-883a, which was reported that it is vital in the gene expression of testicular germ cells (Song et al., 2009).

miR-21a was highly upregulated ($p>0.0006$) in the sperm of HFD, this may be due to the hypomethylation of miRNA transcripts and its promoter, the transcription process is increasing as well. It has already been approved that miR21a is upregulated in different tissues when associated with HFD, our study revealed for the first time its upregulation in sperm (Zhang et al., 2014).

To understand the miR-21a regulation and function we search the transcript regulators and its promoters to study the methylation pattern in the sperm of HFD and AMC mice. miR-21a in mice was found in the opposite direction on chromosome 11 on the antisense, not like the human which usually located on the sense DNA. Chromosome 11 has found that it contains genes that control high-fat diet-induced metabolic syndrome like STAT3/5 (Ermakova et al., 2011).

miR-21 abnormal expression may cause abnormal cell proliferation, invasion or growth and metastasis depending on the function of miR-21 in specific cells. It could be up or down-regulated by targeting several mRNAs, including MMP9, PTEN, PDCD4, and TGF-B suggesting that balancing miR-21 has importance in cell function, cell growth and proliferation (Krichevsky and Gabriely, 2009).

miR-21 has been found to be the best hit in the detection of miRNA regulation in cancer and cardiac diseases (Zhang et al., 2011). Also has found it is a key regulator of PPARγ mRNA that direct metabolic diseases (Rodrigues et al., 2017).

miR-21a is targeting TGBR2 pathway which is the principal pathway for adipocyte differentiation and development (Kim et al., 2009) at the same time targeting SMAD3 to inhibit TGF-β pathway. On the other side, P53 pathway plays a role by signalling miR-27, miR-145a (Cui et al., 2014) and miR143 (Feng et al., 2011) to participate in adipocyte and sperm function through multiple targets and can cause apoptosis and affect early embryonic development (Napoletano et al., 2017) (Figure 5.10).

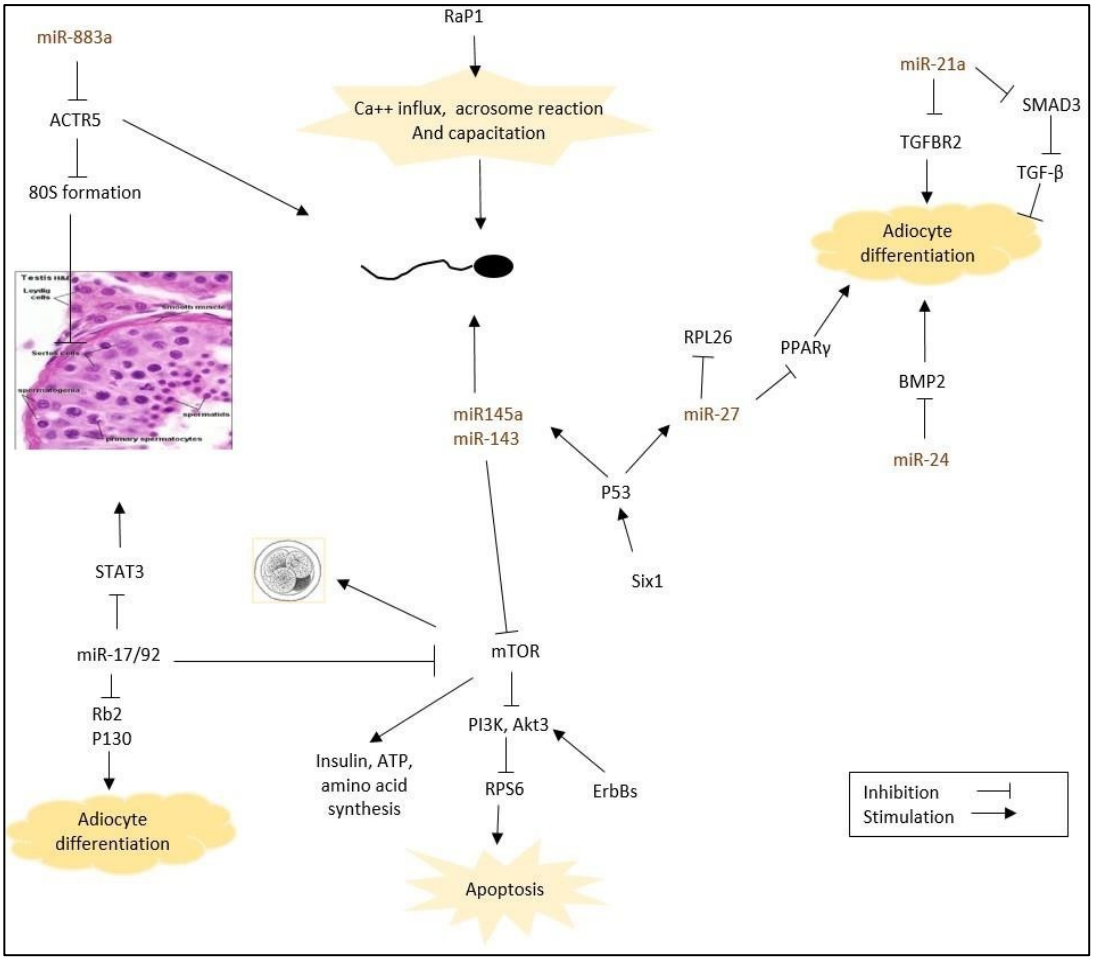

Figure 5.10 miRNA pathways that affect spermatogenesis and sperm activity and related to hypertrophy of adipocytes

DNA methylation has been evaluated using bisulfite conversion method to treat the DNA of interest and perform PCR analysis.

The pattern of reprogramming in germ cells where demethylation followed by *de novo* methylation is fundamental for embryogenesis as well as plays a vital role in initiation and development of diseases and cancer (Piperi et al., 2008). The sperm DNA could be methylated or non-methylated depending on specific loci on the sperm that affected by environmental stimuli (Bohacek and Mansuy, 2015).

The sperm genes are mostly methylated and the transcription sites are acting similarly to pluripotent cells genes like Nanog and Smarcd1 suggesting that the

sperm cells can highly reprogram and could change after fertilization (Farthing et al., 2008). The VmP1 is the promoter region of pri-miRNA-21a and found more methylated in AMC mice than obese male mice, which are accurate that most sperm genes are transcriptionally silent and increase methylation leads to gene transcription inhibition while miR-21a found highly expressed that also concludes that it is by targeting the VmP1 leads to translation inhibition.

Some promoters are highly methylated in sperm like Nanog, Oct4, and Sox2 key regulators transcripts, while in other tissues were hypomethylated or not methylated. Changes in methylation level may refer to a reprogramming process at spermatogenesis and gene transcription sites, are protected from *de novo* methylation (Farthing et al., 2008; Jones, 2012). Also, the variation of sites of CpG that could be some of them methylated while the others are not may referred to a dynamic state of that sites or not maintained in EED or due to inheriting abnormalities (Jones, 2012).

Here we provide clear evidence that obesity has an impact on the sperm epigenome in specific miRNAs expression and DNA methylation. Further investigation may need to confirm firstly, the relationship between these changes that we found with the pregnancy rate success and ART outcomes. Secondly, to investigate in depth miRNA targets in sperm and their role in sperm development and function with diseases and reproduction.

5.5 Conclusion and future directions

In conclusion, miRNA expression is altered in an obese mouse model, which indicate that obesity may regulate miRNA expression. Additionally, changes in the methylation status at CpG islands within the promoter region of the gene coding miR-21 demonstrates that obesity can alter the epigenetic control of both DNA methylation and miRNA expression.

Although this study revealed some interesting data concerning the relative expression of miRNA – only a pre-selected set of miRNAs were investigated. Further work would involve expanding miRNAs of interest and more shotgun sequencing to discover novel miRNAs. Additionally, more focus on the transcript and protein targets of these miRNAs would be explored.

Strenghts and limintaitons of mouse models in reproductive biology

The house mice (*Mus musculus)* is a commonly used animal model to study reproductive biology because of their phylogenetic kinship and physiological similarity to humans. Moreover, in light of the moral, ethical, and legal issues involved in working with human gametes, as well as their limited availability the mouse model has proven to be an appropriate replacement. The mouse has many favourable traits making it a suitable model – including the ease of maintaining and breeding them in the laboratory, and the availability of many inbred strains. Mice have long served as models of human biology and disease including fertility studies.

The mouse has been a useful model to study reproductive biology – in particular sperm-egg interactions and fertilisaiton studies. Mouse studies on sperm-egg binding (Clarke and Dell, 2006), egg activation (Saunders et al, 2002) and

embryogenesis, and *in vitro* culture (Ménézo and Hérubel, 2002) have been pivitol in our understanding of fundamental reproductive physiology and biology – many findings such as PLC zeta (Amdani, Jones Coward, 2013) and sperm binding protiens (Izumo / juno) (Young, Aitken and Baker, 2015) have been subsequently found active in human – with clinical relavence. Furthermore, the early work on mouse embryo culture by McLaren and Biggers (1958) paved the pathway for Assisted Reproductive Technology and IVF.

The process of spermatogenesis in rodents and humans have features are conserved between the species. For example, spermatogenesis emerges from isolated spermatogonia that give rise to clones of interconnected of cells that become more differentiated with each successive division, to the final mature spermatozoon and as mentioned above many similar cellular precess of fertilisation are shared.

However, differences exist between the two species that are limit the use o of the mouse system a poor model, for instance, methods of centrosome inheritance. During human fertilization the spermatozoon restores the zygotic centrosome whereas the mouse follows a maternal method of centrosome inheritance. Mouse sperm biology are very different to humans. Mouse sperm are more morphologically homogenous than human sperm (where the lower limits for normal forms are considered to be 4%: WHO, 2010).

Furthermore, a potential bias when using mice is the use of epididymal spermatozoa in the mouse system and the use of ejaculated spermatozoa in the human system. Although the use of mouse models is ideal for *in vivo* physiological studies, caution with interpretation and extrapolation to human

reproductive biology is warrented. Having said that – the mouse has been an invaluable model for the study of reproction.

Chapter 6: General discussion

Summary of findings

The first findings of this thesis describe that sperm contains a myriad of a significant amount of RNA. mRNA expression in motile and immotile sperm of normal cohorts, in order to study the RNA contents that may be altered due to different sperm activity. mRNA pathways and different software were applied to study the expression differences and functions, as well as find the miRNAs involved in such pathways. Ribosomal RNAs were the most significant mRNA that expressed differentially between motile and immotile sperm. This confirms the importance of these transcripts in spermatogenesis and fertilization despite the notion that sperm is void of ribosomes during spermatogenesis (Johnson et al., 2011). However here we can confirm that sperm still contains some active transcripts that have an impact on its activity.

Next, to study the miRNA that targeted the mRNA in the same cohorts, miRNA were studied and found that miRNAs were expressed differentially between different motility sperm groups. This could be used as a non-invasive method to diagnose male infertility.

In chapter 5, we utilised mouse model of obesity to explored miRNA expression (Takao and Tsuyoshi, 2015; Perlman, 2016). This study would highlight potential modulations in miRNA expression in sperm from obese and control mice. In addition, miRNA are highly conserved between mice and human in most conditions (Chen et al., 2019). Here we find that miR-21 was the most abundant miRNA in the sperm of HFD mice, and it is located on the promoter region of

VmP1 gene. VmP1 was less methylated in the same group of study (sperm of HFD mice). Normally, miRNA targets mRNAs transcripts and cause degradation of that gene, but in abnormal conditions, they can be expressed differently causing genes switched on/ off depending on expression level. DNA methylation can interfere with transcription site of miRNA leading to repressing genes at that site (Li et al., 2019). These findings could explain how changes in diet toward fat content can modulate the genetic content in the sperm and lead to infertility.

This is important research that correlates the sperm function, miRNA content and its targets, and the mRNA, and the region where they originate from that called promoter region where DNA methylation can augment the previous findings.

Strenghts and Limitaitons

The apllicaiton of cutting edge technology such as Next Generation sequencing (NGS) and the Illulumina platforms to explore the transcriptome and miRNA content of sperm has become more available and afforadable in recent years. These methods offers a rich resource for investigations in to the molecular andrology of fertility and reproductive biology. The present study applied this approach to investigate the relative expression of mRNA in human sperm. The use of NGS does however come with challenges, a significant one being to complete transcript analysis of low quantity and/or degraded samples is the amplification of minimal input RNA to enable sequencing library construction. The content of total RNA in sperm is small and the % of quality mRNA derived from sperm nuclear acid extracts limited. High quality sperm RNA extraction and the method of amplification strategy was an important limiting factor in this approach.

However, through testing a number of RNA isolation kits, suffiucient RNA was obtained to run a successful sequence analysis.

In the attempt to isolate and amplify sperm miRNA form human sperm – the repeated technical challenges was the gel purification for library preparation, where sufficient and quality cDNA could not be obtained. Due to time contratints and cost, this approach had to be abandoned. With more time and resources, this approach could be improved through exploring the use of different RNA preparation protocols to improve miRNA yield and alternative cDNA isolation techniques.

Sample size was another limitation of this study. Unlike clinical research programmes, where samples surplus to clinical use can be consented for research - alturistic donation of samples for reaearch provide resources, however the number of volunteers were limited.

The use of animal models to study reproductive biology is a standard approach –due to the ease of controlled experimental conditions and tissue extraction. However, caution is warrented with extrapolations to human studies. The mouse model of offered a controlled approach to explore the epigenetic regulation of miRNA expression. Additionally, as the tissue dertrived from these mice were surplus to use, this approach was fitting with the 3Rs remit of reducing the use of animals in research.

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Appendices

Appendix1:

Table 1 Previous studies of miRNA differential expressed in motile and immotile human sperm

Mature miRNA	Group D		Group A		References	comments
	Fold change	P value	Fold change	P value		
hsa-let-7a-5p	0.217 ↓	0.0005**	0.437↓	0.685	(Liu, 2012) (Zhu, 2011) (Tong, Mitchell et al., 2011)	Involved in the regulation of insulin resistance genes, obesity, diabetes and aging. Highly expressed in sperm and important in early development stages.
hsa-let-7b-5p	0.68↓	0.759	0.94↑	0.435		
hsa-let-7c-5p	undetermined		5.143↑	0.322		
hsa-let-7d-5p	undetermined		4.50↑	0.361		
hsa-let-7e-5p	0.533 ↓	0.0127*	1.48↑	0.431		
hsa-miR-100-5p	0.589 ↓	0.418	1.22↑	0.494	(Liu, 2012) (Abu Halima, 2013)	Related to fertile and infertile cohorts
hsa-miR-125b-5p	0.616 ↓	0.55	1.931↑	0.362	Not expressed in sperm in previous studies	
hsa-miR-132-3p	undetermined		2.89↑	0.082		
hsa-miR-148a-3p	undetermined		3.130↑	0.336		
hsa-miR-15b-5p	0.389 ↓	0.082	0.66↓			Highly expressed in astheno and oligospermic patients

hsa-miR-16-5p	0.152 ↓	0.001**	0.286↓	0.045*	(Abu-Halima, Hammadeh et al., 2013)	Highly expressed in asrheno and oligospermic patients

hsa-miR-191-5p	undetermined		3.759↑	0.020*	Not expressed in sperm in previous studies	
hsa-miR-195-5p	0.103 ↓	0.0006***	0.218↓	0.0001***		
hsa-miR-19a-3p	0.084 ↓	0.006**	0.309↓	0.0039***	(Abu-Halima et al., 2013)	Highly expressed in astheno and oligospermic patients
hsa-miR-19b-3p	0.152 ↓	0.030*	0.342↓	0.0009***	(Liu, 2012)	Related to fertile and infertile cohorts
hsa-miR-21-5p	0.834 ↓	0.528	1.848↑	0.391	(Zhang et al., 2011)	
hsa-miR-23a-3p	0.665 ↓	0.739	1.606↑	0.426	(Liu, 2012) (Krwatz et al., 2011)	Related with fertile and infertile cohorts
hsa-miR-23b-3p	0.490 ↓	0.079	3.13↑	0.623	(Abhari, Zarghami et al., 2014)	Expression of mir21 and mir 22 is much higher in oligospermic patients AKT1 gene been targeted by miRNAs and is important for survival and metabolism in cells.
hsa-miR-24-3p	0.473 ↓	0.389	undetermined		(Marcon et al., 2008	/
hsa-miR-25-3p	0.362 ↓	0.107	undetermined		(Abu-Halima et al., 2013)	Highly expressed in astheno and oligospermicpatients
hsa-miR-26a-5p	0.509 ↓	0.0009***	1.361↑	0.459↑	(Liu, 2012)	Related to fertile and infertile cohorts

hsa-miR-26b-5p	0.328 ↓	0.00003***	0.811↑	0.656↑	Not expressed in sperm in previous studies	
hsa-miR-142-5p	7.890 ↑	0.241	undetermined		(Abu Halima, 2013)	Highly expressed in astheno and oligospermic patients
hsa-miR-223-3p	5.655 ↑	0.149	undetermined		Not expressed in sperm in previous studies	
hsa-miR-29c-3p	undetermined		1.506↑	0.438	(Yan et al., 2012) (Yan, Shah et al., 2012)	/
hsa-miR-30a-5p	0.766 ↓	0.803	1.511↑	0.438	(Liu, 2012)	Related to fertile and infertile cohorts
hsa-miR-30c-5p	0.511 ↓	0.142	0.905↓	0.761	(Marczylo, Amoako et al., 2012)	Expressed differentially in sperm of smokers compared with nonsmokers
hsa-miR-30d-5p	undetermined		5.356↑	0.271	(Abu Halima et al., 2013)	/
hsa-miR-30e-5p	0.781 ↓	0.865	1.889↑	0.296	(Yan et al, 2012)	/
hsa-miR-342-3p	undetermined		4.365	0.341	Not expressed in sperm in previous studies	
hsa-miR-92a-3p	undetermined		2.717	0.340		
hsa-miR-99a-5p	undetermined		1.99↑	0.333	Krwatz et al., 2011	Enriched in spermatogonial cell population

Table 2 Some of applicable web- based tools analysis for miRNA prediction sites

Tool of analysis	Brief description	Web server
miROrtho	Computational catalogue analysis of miRNA sequence for multiple species (Gerlach, Kriventseva et al.,,, 2008)	(http://cegg.unige.ch/mirort ho)
TargetScan		http://www.targetscan.org/
miRBAse	A database search of published miRNA sequences.	ww.mirbase.org
miRTar	An integrated web server for detecting miRNA-target interactions in human	http://mirtar.mbc.nctu.edu.tw/human/
miRscan	A web server to allocate scores for sequence of hairpains based on their parallels to 50 pairs of experimentally confirmed *C.elegans / C.briggsae* miRNA hairpains	http://genes.mit.edu/mirsca n/
DIANA tool	To provide software of databases and algorithms to analyse the deep sequencing data expression and find targets involved in diverse diseases and pathways.	http://diana.imis.athena- n innovation.gr/DianaTools/index.php
miRanda	To recognise the miRNA targets in numerous mammalian cells	http://www.microrna.org/microrna/getGeneForm.do
PicTar	An algorithm tool to detect miRNA targets	http://pictar.mdc-berlin.de/

Table.3 The expressed genes from both mRNA and miRNA in the current study via Target scan tool analysis

No.	Gene symbol	miRNA targets
1	NKX3-1	miR-142-5p, miR-26-5p, miR-129-5p, miR-302-3p/ 372-3p/ 373-3p/ 520-3p, miR-155-5p
2	RPS4X	miR-125a/b-5p, miR-23a/b/c-5p, miR-130a-5p
3	RPLP0	Poorly conserved
4	EEF1A1	miR-33-5pa/b, miR-143a-5p, miR-133a/b-3p
5	RPLP26	miR-216a/b-5p
6	RPL27	miR-143-3p, miR-141-3p, miR200a-3p, miR365a/b-3p
7	RPLP1	
8	TPT1	miR-520c/b/d-3p, miR-302a/b/d, miR-372-3p, miR-150-5p
9	KLK2	miR-33a/b, miR-26a/b, miR-143, miR-9, miR-135a/b, miR-216b, miR-15a/b, miR-16, miR-21-5p, miR-590, miR-212,miR-132
10	FTH1	miR-142-3p, miR-365a/b-3p, miR-590-5p, miR-21-5p
11	HSP90	miR-23a (Wenhao, De-Feng et al.,,, 2015)

Appendix 2

Faculty Ethics number: SE111229A03

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

MEMORANDUM

TO Dr Michael Carroll

FROM Elanor Henry

DATE 10th April 2014

SUBJECT Application for Ethical Approval (amendment) **(SE111229A01)**

On the 10th April 2014 the Head of Ethics for Science & Engineering considered the amendment made to your application for Ethical Approval (SE111229A01)) entitled "Investigating the lifestyle and environment on human sperm". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Prof Bill Gilmore) and the Administrator (Elanor Henry). Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence (currently Prof Bill Gilmore).

If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the attached form and submit it to the Administrator.

Regards

Elanor Henry
Assistant Research Administrator
All Saints North

Page **1** of **1**

6.1.1.1 Participant information Sheet

Title of Study: *Investigating the effects of lifestyle and environment on human sperm*

Study Background:

Male factor infertility can account for 40% of infertility experienced by couples trying to conceive. As sperm is produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage. This damage can occur in the sperm cell membrane and the DNA and RNA. In addition to damage to sperm nucleic acids, alterations in epigenetic status can occur, which include aberrant DNA methylation and microRNA expression.
We will investigate how environmental compounds, various drugs and lifestyle exposures can cause this damage. The information will offer potential therapeutic options that may improve infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.
The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. The samples will be stored at -80°C for further analysis.

Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes. You will also be asked to provide a blood sample and saliva (optional). The blood will be taken by an experienced phlebotomist and is a quick and painless procedure. For the saliva sample, you will just spit in a sterile container. Both blood and saliva will be prepared and stored at -80°C until required. These samples will be used for biochemical and molecular analysis.

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

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

2.

Participant informed consent. ID code .....

Name: ..... Sex: Male / Female

Date of Birth: ..... Age: .....

Project title:

Investigating the effect of lifestyle and environment on human sperm.

Principal Investigator:
Dr Michael Carroll

Investigator/Collaborators:
Dr Nesser Ahmed
Dr Christopher Murgatroyd
Dr Oliver Sutcliffe
Prof. Daniel Brison (external collaborator)

Ethics approval number:

I have read and understood the information sheet and all verbal explanation outlining the purpose of the study and the assessments involved.
Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without having to give a reason. I understand that my sperm will not be used, at any time, for any form of assisted reproductive technique, such as fertilising human eggs or the creation of human embryos. I understand that my semen may be also used for teaching purposes. My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of the School of Healthcare Sciences, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

Signed ..... Date .....

I give my consent for semen collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis.

Date

Signed ....

Semen Procurement Form.

Introduction

Please follow the instructions concerning the production of a semen sample.

As a fresh sample is essential for this test, it must be brought to the laboratory within one hour of being passed at home.

NOTE: USING A PUBLIC TOILET TO PASS YOUR SAMPLE IS AGAINST THE LAW.

Instructions for collecting the semen sample

You should abstain from intercourse or masturbation for three to four days prior to providing the specimen.

The sample must be obtained by masturbation (manual stimulation) and should be collected directly into the specimen container provided. A condom or artificial lubrication must not be used for semen collection, as it will kill the sperm.

The complete specimen is needed for this study, so if any is spilt you must tell us, as a repeat specimen may be required.

Label the specimen container with your full name, date of birth and the date and time the specimen was passed.

Delivery of your sample

Deliver the sample to the School of Healthcare Science within **one hour** of passing the sample.

Keep the sample at body temperature while being transported to the laboratory, for example by carrying it in an inside pocket. Excessive cold or heat can damage the sperm.

For further information contact:
Dr M. Carroll (michael.carroll@mmu.ac.uk)
Phone: 0161 247 1231

Specimen details

Please complete this form and bring it with you to the laboratory along with your sample and the request form.

Name:_____

Date of Birth: _____

Date of specimen Time passed:_____

Time specimen passed: _____

Abstinence (days): _____

I confirm that this semen specimen is mine and I consent to its use for the purpose of research and education.

Signed

_____

Appendix 3: High-Fat mouse Diet formula

Open formula purified diets for lab animals

OpenSource DIETS®

Report ▸ Repeat ▸ Revise

Product Data - D12492

Description
Rodent Diet with 60% kcal% fat.

Used in Research
Fatty Liver
Inflammation
Obesity
Diabetes

Packaging
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

Lead Time
IN-STOCK.
Ready for next day shipment.

Gamma-Irradiation
Yes. Add 10 days to delivery time.

Form
Pellet, Powder, Liquid

Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.
Because of the high fat content is best if kept frozen.

Control Diets
D12450B, D12450J, D12450K

Formula

Product #D12492		gm%	kcal%
Protein		26.2	20
Carbohydrate		26.3	20
Fat		34.9	60
	Total		100
	kcal/gm	5.24	

Ingredient	gm	kcal
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	245	2205
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye #1	0.05	0
Total	**773.85**	**4057**

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 216.4
Cholesterol (mg)/kg = 279.6

RESEARCH DIETS INC.
www.ResearchDiets.com
Where NutriPhenomics Begins

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
Tel: 732.247.2390
Fax: 732.247.2340
info@researchdiets.com

References - D12492

Open formula purified diets for lab animals

D12450B, D12451, D12492 The "Original High Fat Diets"

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Appendix 4: Age-matched control mouse diet formula

Open formula purified diets for lab animals

OpenSource DIETS®

Report ▸ Repeat ▸ Revise

Product Data - D12450J

Description
Rodent Diet with 10% kcal% fat.

Used in Research
Obesity
Diabetes

Packaging
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

Lead Time
5-7 business days.

Gamma-Irradiation
Yes. Add 10 days to delivery time.

Form
Pellet, Powder, Liquid

Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.

Control Diets
Used as a control diet for D12492

Sucrose Content
D12492 Match
7% Sucrose

Formula

Product #D12450J		gm%	kcal%
Protein		19.2	20
Carbohydrate		67.3	70
Fat		4.3	10
	Total		100
	kcal/gm	3.85	

Ingredient	gm	kcal
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	506.2	2024.8
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	20	180
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Yellow Dye #5	0.04	0
FD&C Blue Dye #1	0.01	0
Total	1055.05	4057

*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 54.4
Cholesterol (mg)/kg = 51.6

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20 Jules Lane
New Brunswick, NJ 08901 USA
Tel: 732.247.2390
Fax: 732.247.2340
info@researchdiets.com

Appendix 5 : Collaboration letter of HFD mouse study

MANCHESTER 1824

The University of Manchester

Adam Greenstein MBChB PhD MRCP

Senior Lecturer in Cardiovascular Medicine
BHF Intermediate Clinical Research Fellow
Institute of Cardiovascular Sciences
Room 3.22,
Core Technology Facility
46 Grafton Street,
University of Manchester
Manchester M13 9NT
United Kingdom

Honorary Consultant Physician
Division of Acute and Rehabilitation Medicine
Manchester Royal Infirmary
Central Manchester Foundation Hospitals Trust

Direct Line: 0161 275 1202
Fax: 0161 275 2703
Email adam.greenstein@manchester.ac.uk

30th November 2017

Dear Michael,

Many thanks for giving our group the opportunity to collaborate with you. I am very happy to provide the surplus tissue from our high fat fed mice and their normal chow counterparts for your studies.

Good luck with your experiments!

Yours sincerely

Adam Greenstein

Dr. Adam Greenstein
University of Manchester