Impact of Obesity on miRNA Expression in Human Spermatozoa

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Abstract

Epigenetics is closely linked to the pathogenesis of obesity. Studying the changes in microRNA-mediated processes and its effect on obesity can help us treat and manage obesity more effectively. It was generally perceived that obesity in children was inherited from mothers but it was later discovered that fathers' genetics also played a significant role in their children's obesity. Hence, we hypothesised that obesity does affect micro-RNA (miRNA) expression of spermatozoa. By comparing and processing the miRNA expression values in spermatozoa cells between obese and lean men using R programming, we found that the differentially expressed miRNAs (obese vs lean) with significant changes in fold change value were targeting genes involved in fatty acid (FA) metabolism and actin cytoskeleton pathways that contribute towards a higher risk of developing obesity in the offspring. By identifying the specific miRNA-regulated pathways affected by obesity, potential treatments and preventive measures can be devised.

(149 words)

Introduction

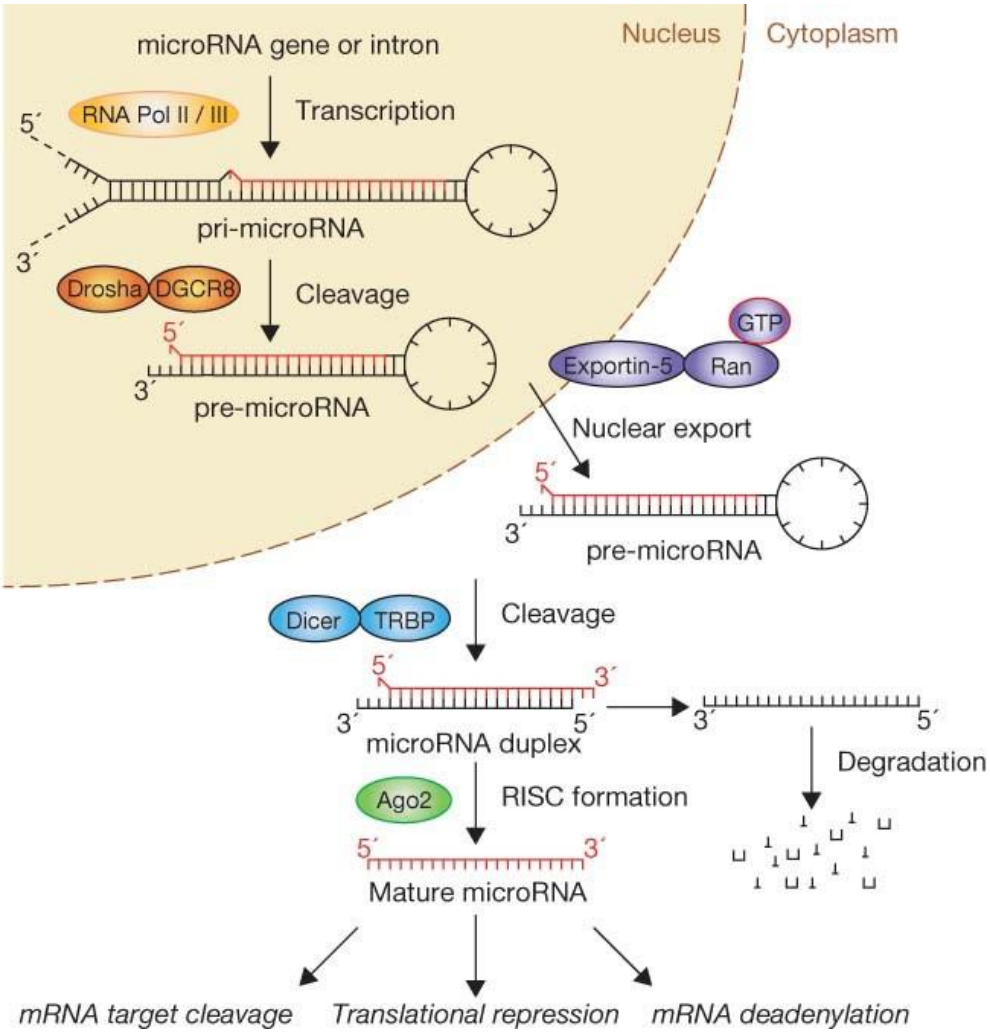
Obesity is the condition of excessive accumulation of adipose tissue in peripheral tissues and is increasingly widespread worldwide, where an estimated 38% of the adult population will be overweight and 20% obese (Hruby and Hu, 2015). Obesity has been closely associated with other metabolic syndromes and chronic health conditions such as diabetes, fatty liver disease and atherosclerosis, highlighting how obesity is a pressing personal and public health burden that demands urgent intervention (Hruby et al, 2015). Understanding the biochemical pathways of obesity would then create opportunities for effective and clearer treatment and preventive measures towards obesity.

Obesity is influenced by genetics and environmental factors, meaning many can inherit the genetic predisposition to develop obesity, but environmental factors such as diet and lifestyle also play an important role (Thaker, 2018). Genome-wide association studies have identified several genetic loci associated with obesity, with an estimated 40-70% of BMI heritability (Herrera et al., 2011), but a large portion of the hereditary still remains unexplained. Hence, attention has turned towards epigenetic change to potentially explain the link between environmental factors and genetic variants to determine the overall risk of obesity (van Dijk et al., 2015).

There has been extensive research on the role of epigenetics in obesity, more specifically, on the roles of DNA methylation, histone modifications, and non-coding RNA processes in regulating energy metabolism (Gao et al., 2021). Small non-coding RNAs (sncRNAs) have been found to be largely involved in regulation of gene expression and play critical roles in several biological processes (Zhang et al., 2019) such as glucose, lipid metabolism, insulin secretion and triglyceride biosynthesis. Specifically, microRNAs (miRNAs) have been found to play an important role in regulating gene expression on the post-transcriptional level. miRNAs help to silence genes by binding to the 3’ untranslated region (3’UTR) of messenger RNAs (mRNAs), thus affecting its stability and degradation (Ying et al., 2008).
During miRNA biogenesis (Figure 1), the complementary RNA sequence is folded into a hairpin-shaped premature miRNA (pre-miRNA) by Drosha enzyme within the nucleus. The pre-miRNA is exported to the cytoplasm before being further processed into mature miRNAs by Dicer protein. Several proteins are then recruited to form RNA induced silencing complex (RISC), which forms complementary bonding with target mRNAs causing translational repression, RNA destabilisation, and mRNA cleavage for post-transcriptional regulation

(Figure 2). The concentration, identity and subcellular distribution of miRNAs can influence adipocyte development, metabolic functions, cell proliferation and differentiation in adipose tissues. Thus, when miRNAs are present in an imbalance they may cause development of obesity and related metabolic complications (Landrier et al., 2019).

A previous paper on Obesity *and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans* with the PubMed ID 26669700 (Appendix A) studied how weight loss between lean and obese men affects the epigentic expression of spermatozoa in human



obesity, concluding that while histone positioning were similar, sncRNA expression and DNA methylation were different. The specific sncRNA expression values differed in

miRNAs, Piwi-interacting RNAs (piRNAs) and transfer RNA Fragments (tRFs), but focus was predominantly placed on the expression of piRNAs and its expression could be linked to obesity (Donkin et al., 2015). Hence, their raw miRNA count data of the study present at GEO under the accession number GSE74426 was used for this project.

Extensive research has already been done on the epigenetic changes from environmental factors in early life, as well as intergenerational relationships. Many studies have established that maternal weight management during pregnancy is crucial in determining the risk of obesity in the child (Herrera et al, 2011; Godfrey et al., 2016), and the correlation between mother and child has been closely followed. However, the relationship between father and child is still poorly understood. There is growing evidence of active transmission of epigenetic states by spermatozoa across generations (Sciamanna et al., 2019), as well as the effect of paternal diet on sncRNA expression (Klastrup et al., 2018). The sncRNAs in spermatozoa can be considered to be the mediators involved in transmission of the acquired metabolic disorder to the offspring. Further research is thus required to investigate the specific mechanism and relationships in the transmission of obesity through sncRNAs. In our project, we intend to study the epigenetic role of spermatozoa miRNAs by investigating the effect of obesity on their expression levels.

Materials & Methods

GEO Raw Data

To investigate the effect of obesity on the miRNA expression, miRNA raw count data was taken from the publicly accessible Gene Expression Omnibus (GEO) database with an accession number of GSE74426 (Appendix A). The data comprises of miRNA expression values of 23 samples, consisting of 10 obese men and 13 lean men.

Data Processing

R programme is a programming language for statistical computing and graphics, and was used to analyse the miRNA expression data and carry out the comparison between 10 obese samples and 13 lean samples.

The data was first filtered to only include those miRNA with an expression value of at least 1 count per million (CPM) across at least 5 different samples. The TMM method, which is the weighted trimmed mean of M-values, from the edgeR package (Robinson and Oshlack, 2010) was then used to normalise the data to remove any bias and technical artefacts, ensuring only biological differences in the data are present for accuracy. The voomwithQualityWeights function (Liu et al, 2015) from the limma package was used to combine voom observational-level weights with sample-specific quality weights before applying linear modelling on the samples using lmFit from the limma package.

miRNA Expression Analysis

Comparisons from obese to lean were made using the makeContrast function and the fold change (FC) was calculated using the eBayes package. Only miRNAs with an absolute $\log_2$FC>log2(1.5) and had an adjusted P value < 0.05 were included in the analysis to ensure the fold change value was significant.

Identification of miRNA-regulated Pathways

To find the miRNA-mediated pathways related to the differentially expressed miRNAs identified, DIANA-mirPath was used (Figure 7). DIANA-mirPath uses a database of experimentally verified miRNA targets (Tarbase v7.0) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) to determine the significant pathways the miRNA gene targets are enriched in.

Results

After filtering (using the cut off of at least 1 CPM in 5 samples), a total of 826 miRNAs were obtained. A total of 31 miRNAs were detected to have a notable change in expression value (obese vs. lean), with 3 miRNAs being downregulated and 27 miRNAs being upregulated respectively and were catergorised under different sections in Table 1. The average logFC of those downregulated is -1.20 and those upregulated had an average of 1.36.

Table 1: Top 10 list of miRNAs identified

miRBase Accession No.	miRBase Name	logFC	Avg Exp	P Val	adj P Val
Downregulated miRNAs					
23712	miR-6087	-0.970362	14.810839	0.000436	0.036396
18926	miR-378d	-1.166752	9.182330	0.001082	0.049211
4917	miR-888-3p	-1.473841	5.394129	0.001204	0.049211
Upregulated miRNAs					
25851	miR-6720-3p	1.499412	2.038735	0.000009	0.003888
27600	miR-6850-5p	1.667793	2.362700	0.000012	0.003888
27484	miR-6792-5p	1.509054	1.969823	0.000013	0.003888
19776	miR-1343-3p	1.692491	2.176558	0.000014	0.003888
4794	miR-551b-5p	1.640324	2.144516	0.000017	0.003888
28231	miR-7160-3p	1.450390	1.969823	0.000027	0.005262
19880	miR-4746-5p	1.545439	2.293789	0.000189	0.030041
19069	miR-4530	1.465209	2.102819	0.000206	0.030041
25486	miR-6515-5p	1.299295	2.087055	0.000253	0.032775
27580	miR-6839-5p	1.584690	2.203772	0.000306	0.035710

Note: Numbers have been rounded to 6dp for readability
Refer to Appendix B for full list of results

These miRNAs were involved in numerous pathways (Table 2) that are involved in the pathogenesis of obesity, suggesting the possible transmission of obesity from men to their offspring through spermatozoa miRNAs. As seen in Table 2, downregulated miRNAs were involved in fatty acid (FA) metabolism, elongation and degradation pathways, as well as the

biosynthesis of unsaturated fatty acids. Upregulated miRNAs are involved in the regulation of actin cytoskeleton and adherens junction.

Table 2: List of Top 15 KEGG pathways identified

KEGG pathway	P Val	No. of genes	No. of miRNAs
Downregulated miRNAs			
Fatty acid metabolism*	4.28E-09	2	2
Fatty acid elongation*	8.18E-09	2	2
MicroRNAs in cancer	4.50E-08	16	2
Biosynthesis of unsaturated fatty acids*	1.34E-06	2	2
Fatty acid degradation*	0.00086701	1	1
Upregulated miRNAs			
Adherens junction*	1.65E-07	16	6
Lysine degradation	0.00051516	9	6
Bacterial invasion of epithelial cells	0.00128639	16	8
Sulfur metabolism	0.00355572	2	1
Hippo signaling pathway*	0.00355572	23	7
Base excision repair	0.00724309	5	4
Axon guidance	0.00724309	21	6
Proteoglycans in cancer	0.00724309	28	6
Glioma	0.00724309	13	7
Regulation of actin cytoskeleton*	0.00724309	31	8

Note: * denotes pathways of interest
Refer to Appendix B for full list of results

Discussion

Fatty Acid Metabolism pathway

The downregulated miRNAs, miR-378d and miR-888-3p, were largely involved in FA metabolism, from FA elongation, FA degradation to FA biosynthesis. Previous studies have already established the close association between the dysregulation of FA metabolism and obesity (Singla et al., 2010), and how it is also closely associated with other obesity-linked health problems like insulin resistance and hypertension (Boden, 2008). The specific genes regulated by miRNA-378d and miR-888-3p are the TECR and the HADHA gene respectively. Particularly, miRNA- 378 has been shown to be involved in the rate of lipid metabolism in the mitochondrion, leading to increased triglyceride levels, and is also involved in a feedback loop for insulin resistance[1] (Xu et al., 2017). The dysregulation of miRNA- 378 is hence largely involved in diet-induced obesity, as well as genetically-induced obesity. Additionally, the HADHA gene has been shown to affect long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) levels[2] (MedlinePlus, 2020), which typically leads to increased risk of obesity and being overweight in children[3] (Haglind et al., 2013), requiring

[1] https://www.ajol.info/index.php/tjpr/article/view/166765/156206
[2] https://medlineplus.gov/genetics/gene/hadha/#conditions
[3] https://pubmed.ncbi.nlm.nih.gov/23430524/

careful monitoring and diet interventions. Additionally, miRNA- 378 has been shown to be involved in the rate of lipid metabolism in the mitochondrion, leading to increased triglyceride levels, and is also involved in a feedback loop for insulin resistance[4] (Xu et al., 2017). The dysregulation of miRNA- 378 is hence largely involved in diet-induced obesity, as well as genetically-induced obesity. This suggests that the decreased expression of the miRNAs found in spermatozoa might be a linking factor in the predisposition and increased risk of obesity in children of obesity patients, hence the hereditary of obesity from father to offspring.

Actin Cytoskeleton pathway

The Actin Cytoskeleton pathway is the key component in the movement of cells, representing the backbone of cells that allow migration and mobility of cells within the body. (Kanaan et al., 2010). Changes in adipocyte size had shown a correlation with a drastic remodeling of the actin cytoskeleton (Hansson et al., 2019). The regulation of actin cytoskeleton by miRNAs (miR-551b, miR-708, miR-505, miR-452, miR-3157, miR-485, miR-4284, miR-658) is linked to other pathways, including adherens junction, and refers to the regulation of the structure and shape of cells. Obesity causes the accumulation of adipose tissues in a process called adipogenesis, where the cytoskeleton is reorganised and there is a change in actin expression. This corresponds with the inhibition of Ras homolog family member A (RhoA) and Rho- associated protein kinases (ROCKs), disrupting the actin cytoskeleton structure and increasing adipogenesis (Roh et al., 2020). With the upregulation of miRNAs (miR-551b and miR-452) inhibiting RhoA and ROCKs in spermatozoa, this may cause the hereditary of increased adipogenesis in the offspring.

Hippo-signalling Pathway

The Hippo-signalling pathway (regulated by miR-708, miR-485, miR-505, miR-452, miR-551b, miR-658, miR-3157) is involved in lipid and glucose metabolism, cell proliferation and apoptosis, which then controls tissue homeostasis (Ardestani et al.,2018). The hippo-signalling pathway has core kinases such as large tumour suppressor 1/2 (LATS1/2), Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), which are controlled by metabolic processes that depend on glucose, amino acids and lipids input (Ibar and Irvine, 2020). YAP proteins can induce cell growth and proliferation by

[4] https://www.ajol.info/index.php/tjpr/article/view/166765/156206

activating transcription of genes such as Myc that is responsible for cell cycle, growth, metabolism and apoptosis (Boopathy and Hong, 2019). These proteins also support maintenance of stem cell or progenitor cells, thus when pathway is dysregulated, YAP proteins which will be present in low concentrations and can result in these cells differentiating into adipocytes (Ibar and Irvine, 2020). Proteins such as MST1 and SAV1 interact to further stabilise and increase the transcriptional activity of adipogenic factor peroxisome proliferator-activated recceptor ɣ (PPARɣ) that results in higher levels of lipid inflammation, differentiation and proliferation (Peters et al., 2012).

Heritability of Obesity

These significant pathways changed in spermatozoa due to obesity, suggesting that these characteristics are likely to pass on to the child through the altered miRNAs, thus creating a predisposition for the child to develop obesity. Studies have already shown that the spermatozoa epigenome is sensitive to environmental changes, and our study helps uncover the effect of obesity on each specific epigenetic expression. Paternal transmission of epigenetic expression to the embryo and subsequently to the offspring's pathogenesis has been shown in previous studies, and it is clear that the paternal miRNA plays an important role in the resultant offspring (Dupont et al., 2019). Hence, the epigenetic change we have identified is likely to be passed down to the child, proving that it is crucial to balance and manage the health of the father as well. Due to the dynamic nature of epigenetics, the effects of obesity on miRNA expressions are likely to be reversible with proper management. This would be significant in helping to devise preventive measures to take care of both the female and male's health to reduce the risk factors of obesity in the offspring, and aid in policies and educational measures formed towards preconception care.

There are also other pathways such as the axon guidance pathway, bacterial invasion of epithelial cells and the sulfur relay systems that may be linked to obesity as well, but through more vague or indirect connections. A dysregulation of the axon guidance pathway could lead to early-onset obesity for the child, with a recent study providing evidence that an imbalance on neural circuit formation could lead to early-onset obesity (Zeltser, 2019), but more evidence is required to clearly establish this relationship. In addition, a decrease in expression of miRNAs in the bacterial invasion of epithelial cells pathway may be a cause of a change in the diversity and concentration of bacteria in the gut epithelial cells, which is linked to increased weight gain independent of energy intake, one's metabolic function and energy homeostasis (Aoun et al., 2020). Thus, this may lead to an increased disposition to

develop obesity in the child, but a more thorough analysis is required to determine if this pathway is related to the gut microbiome.

One limitation of our study includes the low number of 10 samples of obese men and 13 samples of lean men. Additionally, the samples used to obtain the miRNA count files were only from Caucasian men aged 20-40 years old (Donkin et al., 2015), hence the lack of diversity of the samples in terms of ethnicities and age group may affect the results obtained. Including a larger and wide range of samples with diverse backgrounds would have enabled us to identify any outliers and derive more accurate results.

By studying miRNAs, we are able to explore pathways that have already been established with clear links to obesity and with a greater current database of experimentally verified miRNAs. However, future studies can also look further into the effect of obesity on tRFs, which is a more novel sncRNA that has been shown to regulate adipogenesis and fatty acid synthesis. Additionally, whether these epigenetic expressions are influenced by bariatric surgery induced weight loss remains to be clarified, as it has been shown to affect the spermatozoa epigenome by remodelling DNA methylation (Donkin et al, 2015), but its effect on sncRNA expression has yet to be studied.

Conclusion

Understanding the hereditary mechanism of obesity between the father and child would allow us to improve and create more effective measures in order to prevent the high prevalence of obesity in our population today from being passed down to the next generation. By analysing the samples of lean and obese men, certain miRNAs regulating obesity-related pathways were found to have changed significantly. Since these epigenetic changes in spermatozoa due to obesity would likely be passed down to the offspring, this hence creates a future generation at a higher risk of developing obesity. Therefore, it is important for both parents including the father to manage their weight and obesity to ensure the health of their offspring.

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Appendices

Appendix A: Websites used

Figure 1. *Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans* Paper

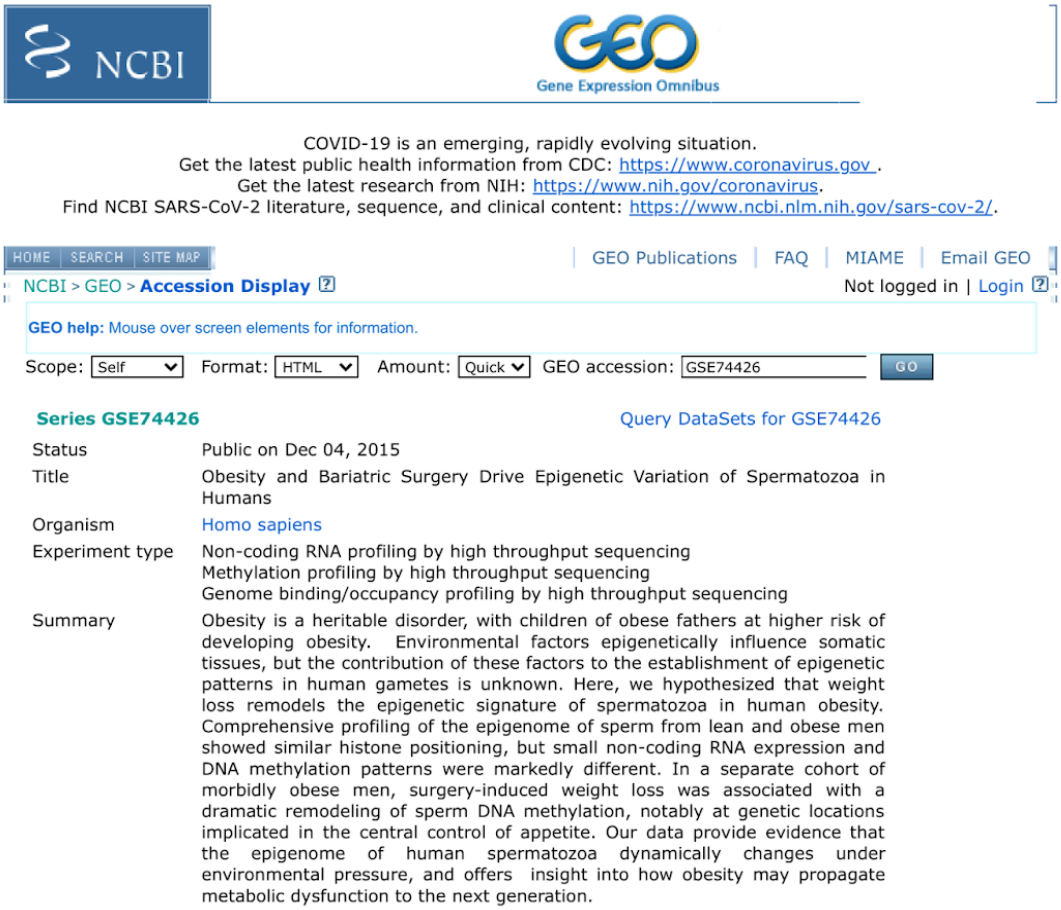
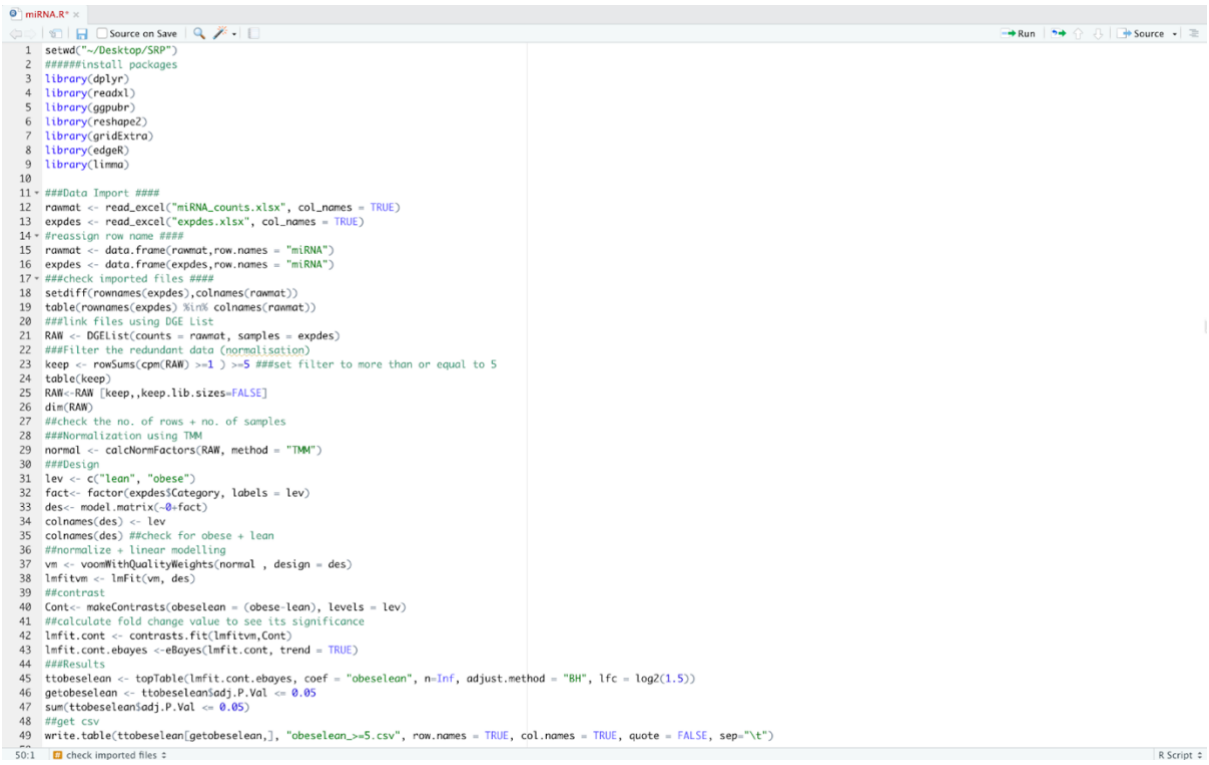


Figure 2. R platform used with relevant codes for the analysis of the miRNA count data



```r
setwd("~/Desktop/SRP")
######install packages
library(dplyr)
library(readxl)
library(ggpubr)
library(reshape2)
library(gridExtra)
library(edgeR)
library(limma)

###Data Import ####
rawmat <- read_excel("miRNA_counts.xlsx", col_names = TRUE)
expdes <- read_excel("expdes.xlsx", col_names = TRUE)
#reassign row name ####
rawmat <- data.frame(rawmat,row.names = "miRNA")
expdes <- data.frame(expdes,row.names = "miRNA")
###check imported files ####
setdiff(rownames(expdes),colnames(rawmat))
table(rownames(expdes) %in% colnames(rawmat))
###link files using DGE List
RAW <- DGEList(counts = rawmat, samples = expdes)
###Filter the redundant data (normalisation)
keep <- rowSums(cpm(RAW) >=1 ) >=5 ###set filter to more than or equal to 5
table(keep)
RAW<-RAW [keep,,keep.lib.sizes=FALSE]
dim(RAW)
##check the no. of rows + no. of samples
###Normalization using TMM
normal <- calcNormFactors(RAW, method = "TMM")
###Design
lev <- c("lean", "obese")
fact<- factor(expdes$Category, labels = lev)
des<- model.matrix(~0+fact)
colnames(des) <- lev
colnames(des) ##check for obese + lean
##normalize + linear modelling
vm <- voomWithQualityWeights(normal , design = des)
lmfitvm <- lmFit(vm, des)
##contrast
Cont<- makeContrasts(obeselean = (obese-lean), levels = lev)
##calculate fold change value to see its significance
lmfit.cont <- contrasts.fit(lmfitvm,Cont)
lmfit.cont.ebayes <-eBayes(lmfit.cont, trend = TRUE)
###Results
ttobeselean <- topTable(lmfit.cont.ebayes, coef = "obeselean", n=Inf, adjust.method = "BH", lfc = log2(1.5))
getobeselean <- ttobeselean$adj.P.Val <= 0.05
sum(ttobeselean$adj.P.Val <= 0.05)
##get csv
write.table(ttobeselean[getobeselean,], "obeselean_>=5.csv", row.names = TRUE, col.names = TRUE, quote = FALSE, sep="\t")
```

Appendix B: Full List of Results

Table 1: List of miRNAs identified

miRBase Accession No.	miRBase Name	logFC	Avg Exp	P Val	adj P Val
Downregulated miRNAs					
23712	miR-6087	-0.970362	14.810839	0.000436	0.036396
18926	miR-378d	-1.166752	9.182330	0.001082	0.049211
4917	miR-888-3p	-1.473841	5.394129	0.001204	0.049211
Upregulated miRNAs					
25851	miR-6720-3p	1.499412	2.038735	0.000009	0.003888
27600	miR-6850-5p	1.667793	2.362700	0.000012	0.003888
27484	miR-6792-5p	1.509054	1.969823	0.000013	0.003888
19776	miR-1343-3p	1.692491	2.176558	0.000014	0.003888
4794	miR-551b-5p	1.640324	2.144516	0.000017	0.003888
28231	miR-7160-3p	1.450390	1.969823	0.000027	0.005262
19880	miR-4746-5p	1.545439	2.293789	0.000189	0.030041
19069	miR-4530	1.465209	2.102819	0.000206	0.030041

25486	miR-6515-5p	1.299295	2.087055	0.000253	0.032775
27580	miR-6839-5p	1.584690	2.203772	0.000306	0.035710
16915	miR-4284	1.577317	3.186461	0.000401	0.036396
9206	miR-2113	1.217955	1.937781	0.000417	0.036396
27601	miR-6850-3p	1.267595	2.139688	0.000434	0.036396
19894	miR-4754	1.359600	2.261747	0.000488	0.037839
19210	miR-3157-3p	1.121745	2.006693	0.000518	0.037839
22942	miR-1229-5p	1.271934	2.208600	0.000555	0.038120
17992	miR-3614-5p	1.180082	1.937781	0.000604	0.039202
23709	miR-6084	1.333014	2.266575	0.000749	0.043728
15022	miR-3149	1.164535	1.969823	0.000748	0.043728
2176	miR-485-3p	1.240502	1.937781	0.000844	0.046928
3336	miR-658	1.335436	2.431612	0.000906	0.047568
4926	miR-708-5p	1.338457	2.663063	0.000937	0.047568
1636	miR-452-3p	1.249353	2.314380	0.001259	0.049211
4776	miR-505-5p	1.398742	2.388633	0.001297	0.049211
5920	miR-1266-5p	1.257341	2.070777	0.001306	0.049211
19361	miR-3976	1.290128	2.468481	0.001348	0.049211
15037	miR-3163	0.998291	1.937781	0.001334	0.049211
27400	miR-6750-5p	1.227186	2.245469	0.001347	0.049211

Note: Numbers have been rounded to 6dp for readability

Table 2: List of Top 10 KEGG pathways identified

KEGG pathway	P Val	No. of genes	No. of miRNAs
Downregulated miRNAs			
Fatty acid metabolism*	4.28E-09	2	2
Fatty acid elongation*	8.18E-09	2	2
MicroRNAs in cancer	4.50E-08	16	2
Biosynthesis of unsaturated fatty acids*	1.34E-06	2	2
Fatty acid degradation*	0.00086701	1	1
Glioma	0.01629867	6	2
Sulfur relay system	0.02714821	2	1
ErbB signaling pathway	0.02714821	5	2
Chronic myeloid leukemia	0.02714821	6	2
Non-small cell lung cancer	0.04249839	5	2
Upregulated miRNAs			
Adherens junction*	1.65E-07	16	6
Lysine degradation	0.00051516	9	6
Bacterial invasion of epithelial cells	0.00128639	16	8
Sulfur metabolism	0.00355572	2	1
Hippo signaling pathway*	0.00355572	23	7
Base excision repair	0.00724309	5	4
Axon guidance	0.00724309	21	6
Proteoglycans in cancer	0.00724309	28	6
Glioma	0.00724309	13	7
Regulation of actin cytoskeleton*	0.00724309	31	8
Salmonella infection	0.00924767	17	8
Dorso-ventral axis formation	0.01082488	9	7
RNA degradation	0.01402541	17	7
Pathogenic Escherichia coli infection	0.02402611	13	8
2-Oxocarboxylic acid metabolism	0.02648436	3	2
Circadian entrainment	0.0285999	10	5
Prostate cancer	0.0285999	17	7
Renin-angiotensin system	0.03048507	4	4
Morphine addiction	0.0334464	10	5
Cell cycle	0.0334464	22	7
Shigellosis	0.0334464	12	7
Bladder cancer	0.0334464	10	7
Chronic myeloid leukemia	0.0334464	14	7

Note: * denotes pathways of interest