INVESTIGATING MOLECULAR ADAPTATIONS IN ADIPOSE TISSUE AND SKELETAL MUSCLE IN RESPONSE TO INTERMITTENT FASTING AND EXERCISE TRAINING

by

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ABSTRACT

The rapid rise in obesity prevalence appears to be a reflection of the changes in dietary and behavioural patterns, with eating habits shifting to higher consumption of energy-dense foods which are rich in fats and sugars, while at the same time, levels of physical activity are decreasing. These differences in energy intake and expenditure, often referred to as energy balance, have direct implications for weight regulation, with even small deviations in daily energy balance resulting in large body weight changes over the long term. Diet and exercise interventions aiming to shift the energy balance towards negative by either decreasing caloric intake and/or increasing physical activity have shown to be effective for weight loss. Many iterations of such dietary and physical activity interventions have been proposed, but intermittent fasting (IF) and high intensity interval training (HIIT) have recently been purported as effective strategies. Despite their effectiveness, the molecular mechanisms by which these lifestyle interventions induce their effects are unclear. Therefore, the purpose of this thesis was to examine the effects of IF and HIIT, alone and in combination, on anthropometric and metabolic health parameters in a model of diet-induced obese mice. To elucidate possible mechanisms of action, we investigated the impact of both lifestyle interventions on mRNA-miRNA regulatory networks, but more importantly, how such changes may translate into exercise-induced and diet-induced improvements in body composition and metabolic health. The findings from the thesis demonstrate that intermittent fasting with or without high intensity interval training resulted in significantly less weight gain in male mice despite concurrently consuming a high fat and sugar diet. The reduced weight gain was predominantly in the form of lower fat mass accumulation, with no significant loss in lean mass. These observations were supported by enhanced expression of adipose tissue genes

relating to fragmentation of unilocular lipid droplets, lipolysis, fatty acid oxidation and efflux. Moreover, lower expression levels of leptin, pro-inflammatory markers and markers of hypoxia were also observed. These changes were also reflected by changes in miRNA-24, -222, -145 and -143. Within the skeletal muscle, the combination of diet and exercise demonstrated minimal impact on mRNA and miRNA expression markers relating to energy metabolism, however IF alone displayed significantly lower expression of all markers compared to control mice or other intervention groups. Interestingly, the changes in body composition, glycaemic control, lipid panels and expression of mRNA and miRNA seem to be gender specific with different responses, independent of intervention, demonstrated in male mice compared to the female mice. In conclusion, the novel results from this thesis have demonstrated superior effects on body composition and lipid profiles following combined IF and HIIT compared to either diet or exercise intervention alone while concurrently consuming a high fat and sugar diet. These observations are likely due to the physiological and biochemical changes that occur within the adipose and skeletal muscle tissue when creating a negative energy balance shift. The gender specific responses to the same diet and/or exercise intervention could indicate potential hormonal differences influencing metabolic control/adaptation in mice. Identification of important regulatory miRNAs through this thesis could provide potential therapeutic targets for obesity treatment and management.

DECLARATION

"I, Robin Alvin Wilson, declare that the PhD thesis entitled "The effect of intermittent fasting and exercise training on body composition and molecular markers of adipose and skeletal muscle tissue physiology in diet-induced obese mice" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

28-06-2018

Signature Date

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1	Effect of intermittent fasting and high intensity interval training on body composition and metabolic health in diet-induced obese mice.	**Robin A. Wilson,** William Deasy, Christos Stathis, Alan Hayes and Matthew Cooke.	Victoria University/ University of Texas El Paso Health Research Symposium, 9th February 2017, Melbourne, Victoria, Australia. (*Oral presentation*)
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3	Effect of intermittent fasting and high intensity interval training on body weight and metabolic health markers in diet induced obese mice.	**Robin A. Wilson**, William Deasy, Ben Harvey, Christos Stathis, Alan Hayes and Matthew Cooke.	Victoria University College of health and biomedicine postgraduate research student conference, 1st December 2016, Victoria, Australia. (*Oral presentation*)

TABLE OF CONTENTS

ABSTRACT ii
DECLARATION iv
ACKNOWLEDGEMENTS v
COMMUNICATION vi
CONFERENCE PRESENTATIONS vii
TABLE OF CONTENTS viii
LIST OF FIGURES xiv
LIST OF TABLES xxiii
LIST OF ABBREVIATIONS xxv
CHAPTER 1: INTRODUCTION 1
1.1 Aims and hypothesis 3
1.2 Thesis Outline 4
CHAPTER 2 LITERATURE REVIEW 5
2.1 Causes of obesity 5
2.1.1 Environmental 6
2.1.2 Genetic and epigenetic 7
2.2 Effect of obesity on Adipose tissue 7
2.2.1 Formation of unilocular lipid droplet and adipose tissue expansion 8
2.2.2 Hypoxia 11
2.2.3 Extracellular matrix and cytoskeletal remodelling 12
2.2.4 Metabolic dysregulation (reduced oxidative capacity and insulin sensitivity) 13
2.2.4.1 Lipogenesis 13
2.2.4.2 Lipolysis 15
2.2.4.3 Lipid handling 16
2.2.5 Inflammation 17
2.2.6 Endocrine changes 18
2.3 Effect of obesity on Muscle tissue 21
2.3.1 Fibre type switching 22
2.3.2 Incomplete Fat oxidation and insulin resistance 24
2.3.3 Insulin signalling 25
2.3.4 Master regulators-PGC-1α 26
2.3.5 Metabolic sensors- AMPK and SIRT1 28
2.3.6 Inflammation 31
2.4 MicroRNA 34
2.4.1 Biogenesis of miRNA 35
2.4.2 Regulation of gene expression by miRNA 37
2.4.2.1 mRNA Degradation 37
2.4.2.2 Blocking translation initiation 37
2.4.2.3 Translocation to processing bodies 38
2.4.3 MiRNA, energy homeostasis and obesity 38
2.4.4 miRNAs: the effect of diet and exercise 40
2.5 Lifestyle interventions 43

2.5.1 Intermittent Fasting 43
2.5.1.1 Effect of IF on Body composition 45
2.5.1.2 Effect of IF on glucose homeostasis 46
2.5.1.3 Effect of IF on lipid profiles 48
2.5.1.4 Molecular changes induced by IF 49
2.5.2 High Intensity Interval Training 50
2.5.2.1 Effect of HIIT on Body composition 51
2.5.2.2 Effect of HIIT on glucose homeostasis 53
2.5.2.3 Effect of HIIT on lipid profiles 54
2.5.2.4 Molecular changes induced by HIIT 54
CHAPTER 3 MATERIAL AND METHODS 57
3.1 Overview 57
3.2 Animals 57
3.2.1 Procurements and Housing: 57
3.2.2 Diet 59
3.2.3 Assessment of Caloric Intake 60
3.3 Interventions 60
3.3.1 Intermittent Fasting Protocol 60
3.3.2 High Intensity Interval Training Protocol 61
3.3.2.1 Acclimatization to treadmill exercise 61
3.3.2.2 Top Speed Test 62
3.3.2.3 HIIT Training 62
3.4 Body Weight and Composition Assessment 63
3.5 Culling and tissue collection 63
3.6 Isolation of RNA 64
3.6.1 Assessment of RNA quality 65
3.7 mRNA Reverse transcription and assessment of gene expression 66
3.7.1 Synthesis of cDNA-reverse transcription 66
3.7.2 Gene expression 66
3.8 MiRNA Reverse transcription and assessment of miRNA expression 67
3.8.1 Synthesis of cDNA-reverse transcription 67
3.8.2 MiRNA expression 67
3.9 Statistical Analysis 68
CHAPTER 4- INTERMITTENT FASTING WITH OR WITHOUT EXERCISE PREVENTS WEIGHT GAIN AND IMPROVES LIPIDS IN DIET-INDUCED OBESE MICE 69
4.1 Introduction 69
4.2 Materials and methods 72
4.2.1 Animals 72
4.2.2 Assessment of Caloric Intake 72
4.2.3 Interventions 72
4.2.4 Body Weight and Composition Assessment 72
4.2.5 Assessment of Glucose Tolerance (AUC) 73
4.2.6 Blood and organ collection 73
4.2.7 Assessment of Plasma Insulin, TAG, LDL and HDL Levels 74
4.2.7.1 Plasma insulin 74

4.2.7.2 Triglycerides 74
4.2.7.3 Low density lipoproteins 75
4.2.7.4 High density lipoproteins 75
4.2.8 Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) 75
4.2.9 Assessment of Muscle Strength 76
4.2.10 Statistical analysis 77
4.3 Results 78
4.3.1 Effects of 12-weeks of the HF/S diet on body weight of all animals 78
4.3.2 Effects of IF and/or HIIT on body weight and composition 79
4.3.2.1 Body weight and fat mass 81
4.3.2.2 Lean mass 81
4.3.3 Effects of IF on caloric intake 82
4.3.4 Effect of IF and/or HIIT on blood glucose, plasma insulin, HOMA-IR and AUC 83
4.3.4.1 Fasting blood glucose 83
4.3.4.2 Plasma Insulin 83
4.3.5 Insulin Resistance (HOMA-IR) and Glucose Tolerance (AUC) 85
4.3.6 Effect of IF and/or HIIT on lipid profiles 88
4.3.7 Effect of IF and/or HIIT on muscle strength 89
4.3.8 Effect of IF and/or HIIT on organ weight 90
4.4 Discussion 91
4.4.1 Effect of IF and/or HIIT on body composition 92
4.4.2 Effect of IF and/or HIIT on glucose homeostasis 96
4.4.3 Effect of IF and/or HIIT on plasma lipids 97
4.4.4 Effect of IF and/or HIIT on muscle strength 98
4.4.5 Effect of IF and/or HIIT on organ weight 98
4.4.6 Sexually dimorphic response to IF and/or HIIT 99
4.5 Conclusion 100
CHAPTER 5- MOLECULAR BASIS OF REDUCED FAT MASS AND ASSOCIATED BENEFITS IN ADIPOSE TISSUE INDUCED BY INTERMITTENT FASTING WITH OR WITHOUT HIGH INTENSITY INTERVAL TRAINING IN DIET-INDUCED OBESE MICE 102
5.1 Introduction 102
5.2 Materials and methods 105
5.2.1 Animals 105
5.2.2 Culling and tissue collection 105
5.2.3 Adipose tissue gene and miRNA expression 105
5.2.4 Statistical analysis 107
5.3 Results 108
5.3.1 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to structure and function 108
5.3.1.1 CIDEC 108
5.3.1.2 Leptin 109

5.3.1.3 PPARγ 110
5.3.1.4 FOXO1 111
5.3.2 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to Fatty acid oxidation and trafficking 112
5.3.2.1 HADH 112
5.3.2.2 FABP4 113
5.3.2.3 Hormone sensitive lipase 114
5.3.3 The effect of 12 weeks of IF and/or HIIT on WAT gene expression of SIRT1 115
5.3.4 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to inflammation 116
5.3.4.1 TRPV4 116
5.3.5 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to hypoxia 117
5.3.5.1 HIF1α 117
5.3.6 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to white adipose tissue beiging 118
5.3.6.1 UCP1 118
5.3.7 The effect of 12 weeks of IF and/or HIIT on miRNA expression relating to adipogenesis and glucose handling in adipose tissue 119
5.3.7.1 miR-27a 119
5.3.7.2 miR-24 120
5.3.7.3 miR-222 121
5.3.7.4 miR-143 122
5.3.7.5 miR-145 123
5.3.8 Correlation analysis between mRNA and miRNA expression 124
5.4 Discussion 126
5.4.1 Adipose tissue structure and function 126
5.4.2 Fatty acid oxidation and trafficking 128
5.4.3 Adipose tissue SIRT1 expression and systemic insulin sensitivity 130
5.4.4 Inflammation 131
5.4.5 Hypoxia 132
5.4.6 Adipose tissue browning 133
5.4.7 Expression of miRNAs 133
5.5 Conclusion 135
CHAPTER 6- INTERMITTENT FASTING AND HIGH INTENSITY INTERVAL TRAINING DISPLAY OPPOSING EFFECTS ON MARKERS OF SKELETAL MUSCLE OXIDATIVE METABOLISM, INSULIN SIGNALLING, INFLAMMATION, AND ATROPHY IN DIET-INDUCED OBESE MICE 136
6.1 Introduction 136
6.2 Materials and methods 138
6.2.1 Animals 138
6.2.2 Culling and tissue collection 139

6.2.3 Myosin ATPase Fibre typing 139
6.2.4 Skeletal muscle gene and miRNA expression 140
6.2.5 Statistical analysis 141
6.3 Results 143
6.3.1 The effect of IF and/or HIIT on skeletal muscle mass and morphology 143
6.3.1.1 Muscle weight 143
6.3.1.2 Muscle fibre type 144
6.3.2 The effect of IF and/or HIIT on gene expression of AMPK-SIRT1-PGC1α axis 146
6.3.2.1 AMPK 146
6.3.2.2 SIRT1 147
6.3.2.3 PGC1α 148
6.3.3 The effect of IF and/or HIIT on gene expression related to oxidative metabolism 149
6.3.3.1 CPT1 149
6.3.3.2 Citrate Synthase 150
6.3.3.3 COX-IV 151
6.3.3.4 UCP3 152
6.3.4 The Effect of IF and/or HIIT on gene expression related to Inflammation 153
6.3.4.1 TNF 153
6.3.5 The effect of IF and/or HIIT on gene expression related to Insulin signalling 154
6.3.5.1 AS160 154
6.3.6 The effect of IF and/or HIIT on gene expression related to muscle atrophy 155
6.3.6.1 MAFbx 155
6.3.6.2 MURF1 156
6.3.7 The effect of IF and/or HIIT on expression of myomiRs 157
6.3.7.1 miR-696 157
6.3.7.2 miR-133a 158
6.3.7.3 miR-133b 159
6.3.8 Correlation analysis between mRNA and miRNA expression 160
6.4 Discussion 161
6.4.1 AMPK-SIRT1-PGC1α signalling axis 161
6.4.2 Oxidative metabolism 164
6.4.3 Insulin signalling 167
6.4.4 Inflammation 167
6.4.5 Muscle atrophy 168
6.4.6 Muscle mass and fibre type 169
6.4.7 MyomiR expression 170
6.5 Conclusion 172
CHAPTER 7- CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS 173
7.1 Key findings 173

7.1.1 Combination of IF and HIIT prevents weight gain by less accumulation of fat mass and maintenance of lean mass 173
7.1.2 Combination of IF with HIIT leads to less fat accumulation due enhanced fatty acid oxidation and efflux. 174
7.1.3 Intermittent fasting and high intensity exercise had opposite effect on skeletal muscle molecular markers 175
7.1.4 Intermittent fasting perturbed the systemic glucose homeostasis 176
7.1.5 Sexually dimorphic response to IF and/or HIIT 176
7.2 Limitations 176
7.3 Future directions 177
REFERENCES 179

LIST OF FIGURES

No	Figure title	Page no
Figure 2.1.	Obesity by age, United States, 1971-1974 through 2005-2006 (CDC, 2008).	6
Figure 2.2.	Mouse epididymal white adipose tissue (10x -H&E stained).	8
Figure 2.3.	Transverse section of soleus muscle showing type 1 (dark) and type 2a (light) fibres.	22
Figure 2.4.	Insulin-signalling pathway for insulin-stimulated GLUT4 translocation and glucose transport in skeletal muscle (Cartee, 2015).	25
Figure 2.5.	Proposed mechanism of PGC1α activation by cooperation between AMPK and SIRT (Ruderman et al., 2010).	31
Figure 2.6.	MiRNA biogenesis pathway. MiRNAs are transcribed by RNA polymerase II resulting in pri-miRNA transcripts. Pri-miRNAs are processed by Drosha leaving Pre-miRNAs which are exported from the nucleus via Exportin5. Pre-miRNAs are processed by Dicer, the mature miRNA is then permanently incorporated into the RNA-induced silencing complex and will bind to the 3′-UTR of a target mRNA (Peng et al., 2014).	36
Figure 2.7.	An example of a HIIT protocol (Cassidy et al., 2017).	50
Figure 3.1.	General outlay of the study.	58
Figure 3.2.	Animal diets-(A) chow diet and (B) High fat diet.	59
Figure 3.3.	Mice treadmill (A), Treadmill control (B), mouse on treadmill (C).	61
Figure 3.4.	EchoMRI machine (A) and mouse tunnel (B).	63
Figure 3.5.	Representative image of agarose gel showing two rRNA bands.	65
Figure 4.1.	Grip strength apparatus (A) mouse performing grip test (B).	76

Figure 4.2.	Weight gain (g) over the 12-weeks high fat/sugar diet feeding period in (A) male and (B) females. Data is presented as mean of each group and error bars represent standard deviation.	77
Figure 4.3.	Change in body weight over the 10-weeks intervention period in (A) male and (B) females. Data is presented as mean of each group and error bars represent standard deviation.	80
Figure 4.4.	Change in body composition (g) in males (A), Females (B), after 10-weeks of intervention. Bars indicate means and error bars represent standard deviation. * significant change over time, € significantly different from CON, # significantly different from HIIT, § significantly different from IF, ‡ significantly different from IF+HIIT.	81
Figure 4.5.	Caloric intake over the 12-weeks intervention period in (A) males and (B) females. Data is presented as mean of each group and error bars represent standard deviation.	82
Figure 4.6.	Change in (A) fasting glucose and (B) insulin over the 10 or 12-weeks period in males and females. Bars represent means, error bars represent standard deviation and * indicates significant change over time.	84
Figure 4.7.	HOMA-IR changes from 'obese' baseline in males and females after 12-weeks of diet and/or exercise intervention. Bars represent means, error bars represent standard deviation and * indicates significant change over time.	85
Figure 4.8.	Glucose tolerance curves (A,C,E,G) and glucose are under the curve (B,D,F,H) of different intervention groups in males before and after 10-weeks of intervention. Bars represent means, error bars represent standard deviation and * indicates significant change over time.	86
Figure 4.9.	Glucose tolerance curves (A,C,E,G) and glucose are under the curve (B,D,F,H) of different intervention groups in females before and after 10-weeks of intervention. Bars represent	87

means, error bars represent standard deviation and * indicates significant change over time.

Figure 4.10. Change in glucose area under the curve (AUC) in males and females after 10-weeks of diet and/or exercise intervention. Bars represent means, error bars represent standard deviation and * indicates significant change over time. 88

Figure 5.1. Effect of IF, HIIT and IF+HIIT on *CIDEC* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively. 108

Figure 5.2. Effect of IF, HIIT and IF+HIIT on *Lep* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively. 109

Figure 5.3. Effect of IF, HIIT and IF+HIIT on *PPARγ* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between groups at $p<0.05$ level of significance. 110

Figure 5.4. Effect of IF, HIIT and IF+HIIT on *FOXO1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance. 111

Figure 5.5. Effect of IF, HIIT and IF+HIIT on *HADH* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively. 112

Figure 5.6. Effect of IF, HIIT and IF+HIIT on *FABP4* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance. 113

Figure 5.7. Effect of IF, HIIT and IF+HIIT on *HSL* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. 114

Figure 5.8. Effect of IF, HIIT and IF+HIIT on *SIRT1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance. 115

Figure 5.9. Effect of IF, HIIT and IF+HIIT on *TRPV4* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance. 116

Figure 5.10. Effect of IF, HIIT and IF+HIIT on *HIF1α* gene expression (mRNA fold change) relative to CON group in (A) males and 117

(B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between groups at p<0.01 of significance.

Figure 5.11. Effect of IF, HIIT and IF+HIIT on *UCP1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. 118

Figure 5.12. Effect of IF, HIIT and IF+HIIT on miR-27a expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. 119

Figure 5.13. Effect of IF, HIIT and IF+HIIT on miR-24 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at p<0.01 level of significance. 120

Figure 5.14. Effect of IF, HIIT and IF+HIIT on miR-222 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between groups at p<0.01 level of significance. 121

Figure 5.15. Effect of IF, HIIT and IF+HIIT on miR-143 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at p<0.01 level of significance. 122

Figure 5.16. Effect of IF, HIIT and IF+HIIT on miR-145 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively. 123

Figure 5.17. Correlation between (A) HIF1α and miR-222a in HIIT group ($R=-0.68$, $p<0.05$) and (B) FOXO1 and miR-145a in males from IF+HIIT group ($R=-0.81$, $p<0.016$) in males. 125

Figure 6.1. Effect of IF, HIIT and IF+HIIT on weight (mg) of soleus muscle in (A) males and (B) females and EDL muscle in (C) males and (D) females. Bars represent means, error bars represent standard deviation. 143

Figure 6.2. Effect of IF, HIIT and IF+HIIT on soleus muscle type I fibre in (A) males and (B) females and type IIa fibre in (C) males and (D) females. Bars represent means, error bars represent standard deviation. 144

Figure 6.3. Representative images of (A) soleus and (B) EDL muscle showing fibre type distribution. Effect of IF, HIIT and IF+HIIT on ED muscle type IIa fibre in (C) males and (D) females and type IIb fibre in (E) males and (F) females. Bars represent means, error bars represent standard deviation. 145

Figure 6.4. Effect of IF, HIIT and IF+HIIT on *AMPK* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance. 146

Figure 6.5. Effect of IF, HIIT and IF+HIIT on *SIRT1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* 147

gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance.

Figure 6.6. Effect of IF, HIIT and IF+HIIT on *PGC1α* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at p<0.05 level of significance. 148

Figure 6.7. Effect of IF, HIIT and IF+HIIT on *CPT1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance. 149

Figure 6.8. Effect of IF, HIIT and IF+HIIT on citrate synthase *(CS)* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at p<0.05 level of significance. 150

Figure 6.9. Effect of IF, HIIT and IF+HIIT on *COX-IV* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at p<0.05 level of significance. 151

Figure 6.10. Effect of IF, HIIT and IF+HIIT on *UCP3* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent 152

standard deviation. Asterisk (*) and (**) bracket represent significant difference between group at p<0.05 and p<0.01 level of significance respectively.

Figure 6.11. Effect of IF, HIIT and IF+HIIT on *TNFα* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance. 153

Figure 6.12. Effect of IF, HIIT and IF+HIIT on *AS160* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance. 154

Figure 6.13. Effect of IF, HIIT and IF+HIIT on *MAFbx* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance. 155

Figure 6.14. Effect of IF, HIIT and IF+HIIT on *MURF1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) bracket represent significant difference between group at p<0.05 and p<0.01 level of significance respectively. 156

Figure 6.15. Effect of IF, HIIT and IF+HIIT on miR-696 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. 157

Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

Figure 6.16. Effect of IF, HIIT and IF+HIIT on miR-133a expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance. 158

Figure 6.17. Effect of IF, HIIT and IF+HIIT on miR-133b expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance. 159

LIST OF TABLES

Table No	Table title	Page No.
Table 2.1.	Factors secreted by adipose tissue into the bloodstream and respective function/effect in their targets	20
Table 3.1.	Calculated nutritional parameters of diets	59
Table 4.1.	The effect of 12-weeks of intermittent fasting and high intensity exercise combined or individual on plasma TAG, HDL and LDL in male and female mice	89
Table 4.2.	The effect of 10-weeks of intermittent fasting and high intensity exercise combined or individual on absolute and relative muscle strength in both male and female mice	90
Table 4.3.	The effect of 12-weeks of intermittent fasting and high intensity exercise combined or individual on liver and pancreas weight in both male and female mice.	91
Table 5.1.	List of targeted genes in adipose tissue with full official names, NCBI reference sequence numbers and Qiagen catalogue number	106
Table 5.2.	List of miRNAs analysed in adipose tissue, miRbase accession numbers and Qiagen miScript Primer Assay Catalog Numbers	106
Table 5.3.	Correlation between mRNA and miRNA expression in skeletal muscle in males.	124
Table 5.4.	Correlation between mRNA and miRNA expression in skeletal muscle in females.	124
Table 6.1.	List of targeted genes in skeletal muscle with full official names, NCBI reference sequence numbers and Qiagen catalogue number	141
Table 6.2.	List of miRNAs analysed in skeletal muscle, miRbase accession numbers and Qiagen miScript Primer Assay Catalog Numbers	141

Table 6.3. Correlation between mRNA and miRNA expression in skeletal muscle in males. 160

Table 6.4. Correlation between mRNA and miRNA expression in skeletal muscle in females. 160

LIST OF ABBREVIATIONS

ADF	Alternate day fasting
AMP	Adenosine monophosphate
AMPK	Protein kinase AMP-activated catalytic subunit alpha 2
ANOVA	Analysis of variance
AS160	Akt substrate of 160 kDa
ATGL	Adipose tissue triglyceride lipase
ATP	Adenosine triphosphate
AUC	Area under the curve
B2M	Beta-2 microglobulin
BMI	Body mass index
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CIDE	Cell Death Inducing DFFA like effector
CIDEC	Cell death-inducing DFFA-like effector c
$CoCl_2$	Cobalt chloride
CON	Control group
COX-IV	Cytochrome c oxidase subunit 4 isoform 2
CPT1	Carnitine palmitoyltransferase Ib
CR	Calorie restriction
CS	Citrate synthase
C_T	Cycle threshold values
DNA	Deoxyribonucleic acid
DPX	Dibutylphthalate Polystyrene Xylene
ECM	Extracellular matrix
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FABP4	Fatty acid binding protein 4, adipocyte
FABPs	Fatty acid binding proteins
FAT/CD36	Fatty acid translocase

FATPs	Fatty acid transport proteins
FFA	Free fatty acid
FOXO1	Forkhead box O1
FSP27	Fat specific protein 27
GLUT4	Glucose transporter type 4
GWAS	Genome wide association studies
HADH	Hydroxyacyl-Coenzyme A dehydrogenase
HDL	High density lipoproteins
HF/S	High fat-high sugar
HFD	High fat diet
HIE	High intensity exercise
HIF1α	Hypoxia inducible factor 1, alpha subunit
HIIT	High intensity interval training
HOMA-IR	Homeostatic model assessment
HSL	Hormone sensitive lipase
HSP70	Heat shock protein 70
IF	Intermittent fasting
IF+HIIT	Intermittent fasting with High intensity interval training
IGF1	Insulin like growth factor-1
IGTT	Intraperitoneal glucose tolerance test
IL-6	Interleukin-6
IMAT	Inter-myocellular or intermuscular adipose tissue
IRS	insulin receptor substrate
JNK	c-JUN N-terminal kinase
JNK	c-Jun N-terminal kinase
LDL	Low density lipoproteins
LEP	Leptin
LIPE	Lipase, hormone sensitive
MAFbx	F-box protein 32
MCP1	Macrophage chemo attractant protein-1
miR	microRNA
miRNA	MicroRNA

mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING finger 1
MyHC	Myosin heavy chain
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-kappa B
OBC	Obese baseline control
OCT	Optimal cutting temperature compound
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PKA	Protein kinase A
PMAT	Perimuscular adipose tissue
PPARγ	Peroxisome proliferator-activated receptor-γ
PPARγ	Peroxisome proliferator activated receptor gamma
Ppia	Peptidylprolyl isomerase A
Pri miRNA	Primary miRNA
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT- PCR	Real time polymerase chain reaction
SD	Standard deviation
Sec	Second
SIRT1	Sirtuin 1
SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1
SOD	Superoxide dismutase
SREBP1	Sterol regulatory element-binding protein 1
TAE	Tris-acetate-EDTA buffer
TAG	Triacylglyceride
TNFα	Tumour necrosis factor-α
TNFα	Tumor necrosis factor-alpha
TRIS-HCl	Trisaminomethane hydroxychloride
TRPV4	Transient receptor potential cation channel, subfamily V, member 4
UCP1	Uncoupling protein 1 (mitochondrial, proton carrier)
UCP3	Uncoupling protein 3
VEGF	Vascular endothelial growth factor

VLDL	Very low density lipoproteins
WAT	White adipose tissue

CHAPTER 1

INTRODUCTION

Obesity is a growing health issue around the globe and has recently been classified as a disease by the American Medical Association (Kushner, 2014). Since 1975, global obesity has approximately tripled, with more than 1.9 billion adults (18 years and older) currently classified as overweight and obese. Moreover, more than 41 million children below the age of 5 years and 340 million children and adolescents aged 5-19, are classified as overweight or obese in 2016. Overall, 13% of the world's adult population is obese (WHO 2017). In Australia, 63% (11.2 million) of population aged 18 and above was overweight or obese in 2014-15. Approximately, 36% (1 in 3 adults) and ~28% (1 in 4 adults) of population is overweight and obese respectively (Australian Institute of Health and Welfare 2017). Such figures impose a substantial economic burden on the Australian health care system. Obesity is associated with various comorbidities such as type 2 diabetes, hypertension, dyslipidemia, and certain types of cancers (Kim et al., 2014a) and the incidence of such diseases have also risen worldwide.

Although genetics, physiological and environmental factors contribute to weight gain and obesity (Wright and Aronne, 2012), the combination of high calorie intake and low physical activity, is a major contributing factor (Meldrum et al., 2017, Moore et al., 2012). Minor deviations in energy balance as a result of high energy intake and low energy expenditure can manifests itself into large increases in body weight over time (Romieu et al., 2017). Increased weight is also accompanied by various other changes at systemic level and cellular level in most of the metabolically important tissues like adipose, skeletal muscle, liver, brain etc. (Kim, 2016). Obesity alters various regulatory

molecular signals at transcriptional and translational levels which contributes to the changes at systemic level. Recent addition to these molecular signalling molecules is microRNAs which regulate various cellular functions at transcriptional or translational levels (Cui et al., 2017).

Lifestyle interventions aimed at shifting the energy balance towards negative side are the first line of defence for prevention and cure of obesity (Wilson et al., 2018). Intermittent fasting (IF) has recently gained attention, supported by several human and animal studies (Patterson and Sears, 2017). Intermittent fasting refers to fasting (100-75% reduced caloric intake) for 2-3 alternate days in a week with *ad libitum* food intake on non-fasting days (Mattson et al., 2018). Intermittent fasting has been suggested to be effective not only for improving body composition but also improves glucose homeostasis, plasma lipid profiles (Patterson and Sears, 2017) and cognitive function (Shojaie et al., 2017). High intensity interval training (HIIT) has gained popularity as an alternative to moderate intensity exercise, which is currently recommended by American college of sports medicine (ACSM) for weight loss (Wewege et al., 2017). High intensity interval training involves, small bouts of high intensity exercise interspersed by active rest and is considered to improve body composition, cardiopulmonary fitness (Türk et al., 2017), reduces plasma lipids (Ouerghi et al., 2017) and improves glucose homeostasis (Batacan et al., 2017). This mode of exercise is much less time intensive and is therefore more preferred by clinical population (Ribeiro et al., 2017).

Combination of dietary and exercise interventions has been reported to be more effective than either of them alone (Foster-Schubert et al., 2012). Few studies have reported the effects of IF or HIIT individually on obese human population or animal models. However, combined effect of these two less intensive but efficient interventions

has not been explored and warrants investigation. In addition, very few studies have reported the effects of IF or HIIT in both males and female cohorts and it is unknown if there is any sexual dimorphic response to these lifestyle interventions. Moreover, given changes in body composition reported by previous studies are mainly due to changes in adipose tissue mass, very few studies, both on IF and HIIT, have investigated the wide range of molecular changes at mRNA and especially at miRNA level in adipose tissue that underpin the systemic changes induced by these interventions. Since obesity has various detrimental effects on skeletal muscle function, it is important to know if IF and/or HIIT can reverse those damages. Finally, diet restriction has been reported to cause loss in lean mass (Heymsfield et al., 2014). It is unknown how IF affects molecular markers of muscle atrophy or growth and such knowledge will provide a foundation for its application in future research and clinical population.

1.1 AIMS AND HYPOTHESIS

The aims and hypothesis of the thesis are:

1. To investigate the effect of combining IF and HIIT on changes in body composition, systemic glucose homeostasis and plasma lipids in diet induced obese mice. It is hypothesised in this study that combination of IF with HIIT will have synergistic effects on these anthropometric or physiological parameters.
2. To explore molecular changes at mRNA and miRNA expression level induced by IF and/or HIIT on structural and functional aspects of adipose tissue like unilocular lipid droplet formation, fatty acid oxidation and trafficking, inflammation, hypoxia and beiging. It has been hypothesised that IF with HIIT will synergistically reduce fat accumulation by increasing fatty acid oxidation and efflux, prevent/reduce obesity induced inflammation and hypoxia.

3. To examine changes in fibre type distribution and molecular changes at mRNA expression levels induced by IF and/or HIIT related to skeletal muscle oxidative metabolism, insulin signalling, energy sensing molecules and muscle atrophy as well as alterations in muscle specific miRNAs. It is hypothesised that IF and/or HIIT will enhance oxidative metabolism, improve insulin signalling, enhance the expression of energy sensing molecules and reduce inflammation and muscle atrophy.

1.2 THESIS OUTLINE

Chapter 2 provides the review of current scientific knowledge on the causes of obesity, its manifestations on adipose tissue and muscle as both of them are considered important for maintenance of systemic energy homeostasis. Also discussed, how lifestyle intervention, IF and HIIT, impact systemic and cellular homeostasis in obesity. A part of the review talks about miRNAs, from basic synthesis to their role in regulating diet and exercise induced changes at cellular level. Chapter 3 details the general materials and methods used in this thesis. Chapter 4 reports the effects of IF and/or HIIT on body composition, systemic glucose homeostasis and plasma lipids in diet induced obese mice. Chapter 5 presents the underpinning molecular adaptation that derived the reduction in adipose tissue mass induced by IF and/or HIIT including alterations in miRNAs. Chapter 6 describes the molecular changes induced by IF and/or HIIT on muscle energy sensors, oxidative metabolism, inflammation, insulin signalling, and muscle atrophy, including changes in muscle specific miRNAs. Chapter 7 presents the study conclusion, limitations and future directions.

CHAPTER 2

LITERATURE REVIEW

Obesity is a condition in which excess body fat has accumulated to an extent that may adversely affect the health of an individual. According to World Health Organization, individuals with a body mass index (BMI) greater than 25 and 30 are considered overweight and obese, respectively. There are multiple causes of obesity, for example genetic, physiological, alterations in neurotransmitter functions, changes in epigenome, dysbiosis of gut microbiota, effect of certain nutrients on mitochondrial function that promote fat accumulation, environmental, socio-economic, and psychological which either alone or in combination with another leads to obesity. While many factors may influence an individual's weight, overweight and obesity are due mainly to an imbalance of energy intake from the diet and energy expenditure (through physical activities and bodily functions). During onset and development of obesity, various changes occur at morphological, physiological, cellular and molecular levels both systemically and in metabolically active tissues like adipose tissue, muscle, liver, brain and pancreas etc. (Wilson et al., 2017, Rogge and Gautam, 2017).

2.1. CAUSES OF OBESITY

The etiology of obesity is quite complex and involves many factors for example, genetic, physiological, alterations in neurotransmitter functions, changes in epigenome, dysbiosis of gut microbiota, effect of certain nutrients on mitochondrial function that promote fat accumulation, environmental, socio-economic, and psychological which either alone or in combination with another leads to obesity (Wright and Aronne, 2012). Although multifactorial in origin, it is evident from the literature that increased

availability of food supplies (increased energy intake) and a reduction in physical activity (decreased energy expenditure) have been the major contributor to the increased prevalence of obesity (Olivo-Marston et al., 2014).

2.1.1 Environmental

As per United States centre for disease control, incidence of obesity in USA increased after 1980's (Figure 2.1). This increase occurred within a decade of huge changes in lifestyle (Swinburn, 2008). Within the last few decades' advancement in technology has reduced the need for physical activity such as elevators, escalators and other labour saving devices, in addition to passive entertainment such as video games, television, online socialization and entertainment. It has been noticed that average daily physical activity has gone down in the last few decades around the globe (Moore et al., 2012). In conjunction to this, availability of inexpensive highly processed and sugar laden food has increased which is considered as an important factor responsible for soaring increase in obesity (Meldrum et al., 2017). The average consumption of sugar in 1800s was 4-6 pounds per year which has gone up to 150-170 pounds per year today. Sugar has been reported to be highly addictive with both short term and long term adverse health effects (Ifland et al., 2009, Davy and Jahren, 2016). Environmental and behavioural factors alone and in combination create what is known as "obesogenic environment" of modern times (Meldrum et al., 2017).

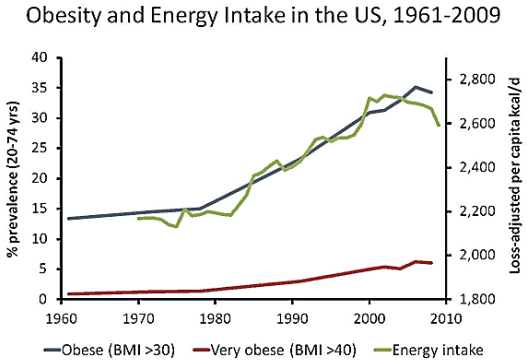

Figure 2.1. Obesity by age, United States, 1971-1974 through 2005-2006 (CDC, 2008).

2.1.2 Genetic and epigenetic

Despite the huge impact of environment on the recent surge in obesity, huge variation has been found among individual's susceptibility to obesity in the population. Heritability plays an important role in determining the susceptibility to obesity and accounts for approximately 40-70% to variation in susceptibility (Locke et al., 2015). Genome wide association studies (GWAS) have identified a number of genetic loci associated with obesity, however these genetic loci can explain only 20% of variation in susceptibility and large portion of it still remains unexplained (van Dijk et al., 2015, Locke et al., 2015).

Notwithstanding, genetic and environmental factors, emerging research suggests epigenetics may play an important role in development of overweight or obese phenotype (Milagro et al., 2013). Epigenetics is the heritable change in gene expression without changes in the DNA sequence. Epigenetic modifications are affected by various external and internal environmental factors and are now considered as important players in the etiology of many diseases like obesity, diabetes and cancers (Choi and Friso, 2010, Ehlert et al., 2013). Epigenetic changes include DNA methylation, histone modification, chromatin remodelling and most recently known microRNA expression (Choi and Friso, 2010).

2.2. EFFECT OF OBESITY ON ADIPOSE TISSUE

Adipose tissue is found under the skin (subcutaneous), around internal organs (visceral) (figure 2.2), in muscle fibres (intramuscular) and in the breast tissue. Adipose tissue consists of adipocytes and non-adipocytes also called vascular-stromal fraction, which is made up of macrophages, fibroblasts, endothelial cells and pre-adipocytes (Hajer

et al., 2008). Classically, adipose tissue is considered important for providing insulation and cushion to the body and as a reservoir for storage of excessive energy after food intake and its release during fasting state (Hajer et al., 2008). However, over the last few decades, experimental evidences have shown that adipose tissue is an important endocrine organ, which secretes various bioactive compounds to maintain systemic energy homeostasis (Coelho et al., 2013, Choe et al., 2016). Alterations in energy status induce dynamic changes in adipose tissue like numerical and functional changes in adipocyte and extracellular matrix, collectively called adipose tissue remodelling (Choe et al., 2016), manifested as adipose tissue hypoxia, decline in insulin sensitivity, enhanced autophagy, apoptosis and tissue inflammation. Consequently, adipose tissue releases certain signals in the form of adipokines, cells and metabolites which induce pro-inflammatory, diabetogenic and atherogenic serum profile. These signals in turn further promote adipose tissue inflammation and damage other organs like skeletal muscle, liver, brain, endothelium, vasculature and endocrine organs (Klöting and Blüher, 2014). Some important events that leads to adipose tissue dysfunction have been discussed underneath.

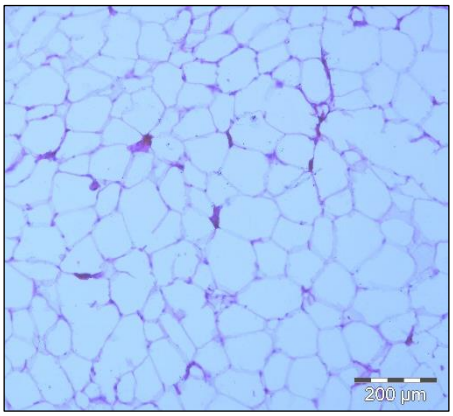

Figure 2.2. Mouse epididymal white adipose tissue (10x -H&E stained).

2.2.1 Formation of unilocular lipid droplet and adipose tissue expansion

The excess lipids like free fatty acids and sterols are stored after esterification in lipid droplets as neutral lipids (Thiam et al., 2013). Although adipocytes in adipose tissue are the primary storage organs, lipid droplets are also found in other cells like hepatocytes, enterocytes, macrophages and adrenocortical cells (Krahmer et al., 2013). Structurally,

lipid droplet is composed of a non-polar core which is mainly triglycerides or sterol esters surrounded by a polar phospholipid monolayer (Bartz et al., 2007). Phospholipid monolayer is mainly composed of phosphatidylcholine, phosphatidylinositol, lyso-phosphatidylcholine and lyso-phosphatidylethanolamine (Krahmer et al., 2011, Guo et al., 2008, Fei et al., 2011, Szymanski et al., 2007). In addition to phospholipids, various proteins are also attached to the lipid droplet surface which helps regulating the lipid droplet size, number and lipid metabolism. These include PAT proteins (Perilipin 1, Perilipin2/adipophilin (ADRP), Perilipin3/TIP47, Perilipin4/S3-12, Perilipin 5 (OXPAT)), Cell Death Inducing DFFA like effector (CIDE) proteins, including CIDEA, CIDEB, CIDEC/FSP27) and several lipases. However, the lipid droplet proteome is large with around 100 proteins identified so far (Thiam et al., 2013, Krahmer et al., 2013). Adipose tissue has one large unilocular lipid droplet with a diameter of about 100μm, which is in contrast to other cell types where numerous small lipid droplets are found with diameter ranging from 100nm to 5μm. CIDE proteins promote the formation of unilocular lipid droplets (Krahmer et al., 2013). CIDEC also FSP27/regulate the triglyceride storage in lipid droplet and influence the development of metabolic syndrome (Krahmer et al., 2013). Perilipin1 also regulate triglyceride storage and is an important regulator of lipolysis. It protects the lipid droplets from basal lipolysis but regulates access of lipases to lipid droplets when the lipolysis is stimulated (Sztalryd et al., 2003, Zhai et al., 2010). In state of excess energy intake, owing to the massive accumulation of lipid droplets, the expression of lipid droplet proteins changes which in turn influence the pathological state. For instance, in human adipose tissue expression, of *FSP27*/CIDEC inversely correlate with insulin resistance (Puri et al., 2008). Higher expression of perilipin1 and CIDEC have been found in adipose tissue of insulin sensitive subjects in comparison to insulin

resistant subject of same weight indicating that elevated expression of these proteins enhance triglyceride storage in adipose tissue and protect from lipotoxicity. Polymorphism in FSP27/CIDEC has also been reported to influence metabolic risk (Zhang et al., 2008, Dahlman et al., 2005). However, these human findings are in contrast to the findings in mice/animals. Mice lacking FSP27 have higher energy expenditure and exhibit protection from diet induced obesity and insulin resistance (Nishino et al., 2008). Likewise, perilipin1 knockout mice also remained lean and protected from high fat diet induced obesity (Tansey et al., 2001, Martinez-Botas et al., 2000). These finding indicate the difficulty in translating the animal findings into human pathologies.

The expansion of adipocytes due to enlargement of lipid droplets results in adipose tissue expansion. Initially the adipose tissue expands by increasing the size of existing adipocytes to accommodate incoming triglycerides, a phenomenon called hypertrophy. This is followed by increase in the number of adipocytes, a phenomenon called hyperplasia (Sun and Scherer, 2010). Although previously hypertrophy was considered as the sole mechanism but later it was established that hyperplasia also contributes to the adipose tissue expansion (de Ferranti and Mozaffarian, 2008). During hyperplasia, initially number of pre-adipocytes increases which is followed by differentiation of pre-adipocytes into mature adipocytes. Through some animal studies, it has been revealed that hypertrophy is followed by hyperplasia and the latter is associated with increased severity and reduced irreversibility of metabolic consequences (Hirsch et al., 1989, Bjorntorp et al., 1982), however this has yet to be demonstrated in humans. Although the factors regulating adipose tissue expansion (hypertrophy and hyperplasia) have still remained elusive but it has been seen that insulin and glucocorticoid stimulate differentiation of pre- adipocytes (Avram et al., 2007). Hypertrophied adipocytes have

also been reported to secrete tumour necrosis factor-α (TNFα) and insulin like growth factor-1 (IGF1), both of which stimulate hyperplasia (Avram et al., 2007). In addition to this, Peroxisome proliferator-activated receptor-γ (PPARγ), an important transcription factor has also been reported to stimulate hyperplasia (Tontonoz et al., 1994).

2.2.2 Hypoxia

Adipocytes can expand beyond 100μm in size whereas, the diffusion capacity of oxygen is only 100μm (Trayhurn, 2013). Lack of oxygen supply causes hypoxia in adipocytes at cellular level which leads to induction of Hypoxia-inducible factor 1α (HIF1α) (Ban Jae-Jun et al., 2014). HIFα is a transcription factor induced by hypoxia and is ones of the adipose tissue hypoxia marker genes (He et al., 2011). HIF1α is constantly expressed in the cell and increase in mRNA level is not necessary for elevation of HIF1α protein level (He et al., 2011). HIF1α rapidly degrades under normoxic conditions (Rutkowski et al., 2015) however under hypoxic conditions degradation is inhibited (He et al., 2011) leading to elevated level of HIF1α protein. In adipocytes, elevated HIF1α fails to induce pro-angiogenic response, albeit the underpinning reason is not completely understood. Several studies have demonstrated that even though the HIF1α level is effectively stabilized under hypoxia, obese adipose tissue lack other co-factors like vascular endothelial growth factor (VEGF) necessary for vascularization (Sun and Scherer, 2010). Selective inhibition of HIF1α improves adipose tissue metabolism indicating that HIF1α responsive genes are more important for adipose tissue dysfunction than vascularization (Sun et al., 2013a). In addition to this, deletion of HIF1α in adipocytes protect against inflammation, insulin resistance (Lee et al., 2014). These effects have been related to the protective signalling of estrogen receptor α which limits hypoxia and fibrosis by HIF1α ubiquitination and deactivation (Kim et al., 2014b). It has

been suggested that inhibition of HIF1α has a therapeutic potential to prevent the detrimental effect of obesity (Ban Jae-Jun et al., 2014). Overexpression of HIF1α in adipocytes did not exhibit pro-angiogenic response per se rather it induced another transcriptional panoply involved in remodelling of extracellular matrix (Halberg et al., 2009).

2.2.3 Extracellular matrix and cytoskeletal remodelling

A unique extracellular matrix (ECM) surrounds adipocytes which provides mechanical support to the cells and also participates in variety of signalling events (Khan et al., 2009). ECM surrounding adipose tissue is an isotropic matrix of collagen and elastic fibres with extracellular fibronectin and laminin forming networks with collagen fibres (Pope et al., 2016). The components of ECM are regulated by matrix metalloproteinases, a family of neutral endopeptidase that cleaves ECM components and facilitates remodelling of ECM (Sun et al., 2011). The health of adipocyte is regulated by interaction with ECM and matrix composition per se (Rutkowski et al., 2015). Flexibility of ECM is important for healthy expansion of adipocytes. However, during the course of development of obesity, ECM loses its flexibility and plasticity due to enhanced interstitial fibrosis in white adipose tissue, resulting in adipose tissue dysfunction. Excessive synthesis of fibrillar components of ECM like collagen I, III and VI and reduced breakdown of these components leads to their accumulation and adipose tissue fibrosis (Sun et al., 2013b). Adipocytes in fibrotic ECM lose proper function and exhibit increased rate of fibrosis. Once the necrotic cell dies, the large lipid droplets remain in the tissue. The surrounding dysfunctional live adipocytes induce infiltration of macrophages, neutrophils, lymphocytes, and mast cells, initiating a local pro-inflammatory environment (Sun et al., 2013b). Destabilised ECM due to lack of an

important component, Collagen VI, does not affect the phenotype of mice in comparison to wild type. On challenging these mice with high fat diet or with *ob/ob* mutation, despite increase in adipocyte size, the tissue metabolic health and systemic metabolic phenotype of mice radically improved. Which indicates that, expansion of adipocytes itself does not leads to adipose tissue dysfunction and ECM plays an important role in adipose tissue dysfunction rather than merely an epiphenomenon associated with obesity (Khan et al., 2009, Sun and Scherer, 2010). HIF1α has been found to play a critical role in inducing the fibrotic response. Enhanced expression of HIF1α under expanding hypoxic adipocytes induce increased transcription of ECM components leading to development of fibrosis (Sun et al., 2013b). HIF1α also alters cellular redox state which affects enzymes associated with collagen crosslinking and stabilization like lysyl oxidase and prolyl-4-hydroxylase (Mariman and Wang, 2010). PPARγ has also been found to exhibit antifibrotic activity by supressing the transcription of collagen (Khan et al., 2009).

The cytoskeleton of adipocytes is also remodelled during adipogenesis which alters the mechanical properties of the tissue. During the expansion of lipid droplet, the vimentin (type III intermediate filament protein) expression increases to increase the size of vimentin cage surrounding each lipid droplet, whereas the expression of actin and tubulin decreases (Pope et al., 2016).

2.2.4 Metabolic dysregulation (reduced oxidative capacity and insulin sensitivity)

2.2.4.1. Lipogenesis

The extra energy stored in adipose tissue as triglycerides are synthesized from fatty acids either by *de novo* lipogenesis from non-lipid precursors or taken up from circulating triglycerides in chylomicron and very low density lipoproteins (VLDL) (Lafontan, 2008, Luo and Liu, 2016). For *de no* lipogenesis, glucose provides acetyl-

coenzyme A (acetyl CoA), induces the rate limiting enzyme acetyl-CoA carboxylase and also stimulates the release of insulin from pancreatic β cells which boosts lipogenesis (Luo and Liu, 2016). Insulin stimulated uptake of glucose by adipocyte, activate glycolytic and lipogenic enzymes and induce the expression of transcription factor sterol regulatory element-binding protein 1 (SREBP1) which regulates the expression of fatty acids, triglycerides, phospholipids and cholesterol synthesis genes (Assimacopoulos-Jeannet et al., 1995, Ferre and Foufelle, 2007). In addition to SREBP1, carbohydrate response element-binding protein (ChREBP) also stimulates *de novo* lipogenesis and regulates glucose and lipid metabolism in adipocytes and systemic insulin sensitivity (Eissing et al., 2013, Herman et al., 2012). In adipose tissue, the *de no* lipogenesis is low in comparison to liver under normal conditions in rodents and is even lower in humans (Letexier et al., 2003, Swierczynski et al., 2000). However this does not rule out the possibility of enhanced *de novo* lipogenesis under high carbohydrate intake state (Lafontan, 2008).

The majority of fatty acids incorporated into triglyceride stored in adipose tissue are supplied by circulating lipids (Luo and Liu, 2016). Lipoprotein lipase (LPL), secreted by adipocytes, translocated to the lumen of adipose tissue capillaries and catalyses hydrolysis of one fatty acid from circulating triglycerides (Frayn, 2002, Kersten, 2014). LPL activity is regulated by insulin and Angiopoietin-like 4 (Angptl4) (Luo and Liu, 2016). During adipocyte hypertrophy the LPL activation is impaired (Lafontan, 2008), which can lead to increased availability of fatty acids in non-adipose tissues and ectopic lipid accumulation (Mittendorfer, 2011).

2.2.4.2. Lipolysis

During the period of energy demand triglycerides in adipose tissue are hydrolysed by lipolysis, which supplied free fatty acids as fluids for other organs and glycerol for hepatic gluconeogenesis (Kuriyama et al., 2002). Adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) are the major lipases besides 20 other lipases (Saponaro et al., 2015). ATGL catalyses the first step of conversion of triglycerides to diglycerides and a free fatty acid. ATGL belongs to patatin like phospholipase family, also called PNPLA2. Besides adipose tissue, ATGL is also present in other tissue forms like liver, muscle and heart, however its function is slightly different types of tissue. ATGL is activated by fasting, glucocorticoids, PPAR agonists and act in the presence of coactivator protein named comparative gene identification-58 (CGI-58) (Haemmerle et al., 2006, Shaw et al., 2013, Smirnova et al., 2006). HSL although can act on triglycerides, diglycerides, monoglycerides and cholesteryl esters but is mainly involved in hydrolysis of diglycerides to monoglycerides and monoglycerides to free fatty acid and glycerol (Kraemer and Shen, 2002). During fasting, elevated glucagon level activates cAMP dependent protein kinase A (PKA) which phosphorylates perilipin which in turn translocates HSL to the lipid droplet for lipolysis (Marcinkiewicz et al., 2006, Lafontan and Langin, 2009, Brasaemle, 2007). Insulin promotes lipogenesis and inhibit lipolysis (Gastaldelli, 2011). Insulin resistant individuals exhibit higher level of plasma free fatty acids in comparison to insulin sensitive subjects at the same plasma insulin level. Elevated plasma free fatty acids inhibit insulin signalling, reducing basal and plasma stimulated glucose uptake (Boden et al., 2001). Free fatty acids decline muscle ATP synthesis (Brehm et al., 2006), and nitric oxide production (Wang et al., 2006), and impair insulin-stimulated activation of phosphoinositol-3 kinase (PI3K), pyruvate dehydrogenase

kinase-isozyme1(PDK1), RAC-alpha serine/threonine-protein kinase (also known as proto-oncogene c-AKT), and endothelial nitric oxide synthase (eNOS) (Wang et al., 2006). Also, elevated level of plasma free fatty acids is related to higher level of cellular diglycerides, the first step of triglyceride synthesis. In addition to this, excess free fatty acids also increases insulin secretion and impedes β- cell function (Gastaldelli, 2011, Kashyap et al., 2003).

2.2.4.3. Lipid handling

The increased plasma free fatty acid concentration under obese or insulin resistant state could be due to increased adipose tissue lipolysis and free fatty acid secretion, increased hepatic VLDL triglyceride synthesis and secretion, and reduced peripheral free fatty acid uptake (Gertow et al., 2004). The cellular uptake of free fatty acids is facilitated by membrane associated fatty acid transport proteins (FATPs) and fatty acid translocase (FAT/CD36) (Rutkowski et al., 2015, Gertow et al., 2004). Intracellularly, the free fatty acids transport is facilitated by a family of lipid chaperone protein called fatty acid binding proteins (FABPs) (Furuhashi et al., 2014). FABPs have been suggested to play an important role in lipid trafficking with in the cell facilitating fatty acid transport to mitochondria and peroxisomes for oxidation, to nucleus for fatty acid mediated transcriptional regulation, to endoplasmic reticulum for signalling and membrane synthesis, and to lipid droplets for storage (Zimmer et al., 2004, Spiegelman et al., 1983). The level of FABPs in the cell reflects the rate of fatty acid handling and metabolism (Veerkamp and van Moerkerk, 1993, Furuhashi et al., 2014). FABPs have several isoforms namely FABP1, FABP2, FABP3, FABP4, FABP5, FABP6, FABP7, FABP8 and FABP9. FABP4 also known as aP2, is abundantly expressed in adipose tissue and constitutes 1% of all soluble proteins in adipocytes (Baxa et al., 1989, Spiegelman et al.,

1983). FABP4 is transcriptionally regulated by PPARγ, insulin, fatty acids and dexamethasone (Furuhashi et al., 2014). FABP4 deficient mice exhibit enhanced body weight and reduced insulin resistance in both high-fat diet fed and genetically obese mice but its effect on insulin sensitivity was not observed in lean mice (Hotamisligil et al., 1996, Uysal et al., 2000). FABP4 has been reported to physically interact with HSL, stimulates its activity and regulates lipolysis (Hertzel et al., 2002). FABP4 deficient mice exhibit impaired lipolysis in vitro and vivo (Scheja et al., 1999). FABP4 deficient adipocytes (*in vitro*) exhibit reduced lipolysis, indicating its role in FA efflux from adipocytes (Hertzel et al., 2002, Furuhashi et al., 2014). Loss of FABP4 is compensated by FABP5 which is mainly expressed in epidermal cells but also expressed in other tissues including adipose. Although, the amount of FABP5 is 100 times lower than the FABP4, both of them contribute to the development of insulin resistance and atherosclerosis (Furuhashi et al., 2014).

2.2.5 Inflammation

Adipose tissue remodelling under positive energy balance is accompanied by infiltration of immune cells into adipose tissue resulting in chronic low grade adipose tissue inflammation (Wang and Ye, 2015). Various immune cells like macrophages, granulocytes, and lymphocytes gets accumulated in the stromal vascular fraction of adipose tissue (Esser et al., 2014). Although enlarged adipocytes secrete pro-inflammatory cytokines and chemokines, macrophages have been reported to make significant contribution to the adipose tissue derived pro-inflammatory cytokines (Skurk et al., 2007, Esser et al., 2014). Macrophages in adipose tissue are of two types namely M1 and M2 macrophages. M1 macrophages secrete TNF-α, interleukin-6 (IL-6) and thus enhance inflammation whereas M2 macrophages secrete IL-10 which is an anti-

inflammatory cytokine (Chawla et al., 2011). Macrophage infiltration correlates directly with adiposity, systemic inflammation, insulin resistance (Weisberg et al., 2003) and metabolic syndrome (Esser et al., 2013). Large adipocytes release saturated free fatty acids which binds to toll like receptors 4 (TLR4) on macrophages, activating nuclear factor-kappa B (NF-κB) and enhancing production of TNF-α. TNF-α in turn activate human adipocytes, inducing lipolysis and enhancing expression of genes like IL-6, intracellular adhesion molecule-1 (ICAM1) and macrophage chemo attractant protein-1 (MCP1). ICAM1 and MCP1 facilitates diapedesis of monocytes from blood to adipose tissue and their differentiation into macrophages (Hajer et al., 2008).

These immune cells release pro-inflammatory cytokines including TNF-α, IL-1, IL-6 (Wang and Ye, 2015), which act in autocrine or paracrine manner, activating c-JUN N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) pathways in peripheral tissues and promote insulin resistance by interfering in insulin signalling (Shoelson et al., 2006)

2.2.6 Endocrine changes

Apart from storing energy, adipose tissue releases various molecules, called adipokines (Table.1), which acts as endocrine, paracrine and autocrine signals, controlling various cellular functions in several tissues like hypothalamus, pancreas, liver, skeletal muscle, kidneys, endothelium, and the immune system (Coelho et al., 2013, Booth et al., 2016, Leal and Mafra, 2013). It has been suggested that most adipokines secreted by adipose tissue are produced by cells other than adipocytes. Leptin, an adipokine released by white adipose tissue is the first one discovered in 1994 (Zhang et al., 1994). Leptin controls adipose tissue growth through its effect on central nervous system. Leptin targets γ-amino butyric acid (GABA)-nergic neurons to reduce appetite

and enhance energy expenditure via its receptors (Vong et al., 2011, Leal and Mafra, 2013). Leptin receptors are located in various hypothalamic sites (Berglund et al., 2012), liver, kidney, lung, pancreas and adipose tissue (Hoggard et al., 1997, Lollmann et al., 1997, Leal and Mafra, 2013) . Under obese conditions, hyperphagia and increased adipose tissue mass with hyperleptinemia indicates leptin resistance (Vazquez-Vela et al., 2008, Coppari and Bjorbaek, 2012). Leptin mRNA expression and plasma concentration has been found to be directly proportional to the severity of obesity (Considine et al., 1996, Vidal et al., 1996). Hyperleptinemia is associated with insulin resistance (Howard and Flier, 2006), inflammation (Loffreda et al., 1998), activation of macrophages, TNFα, reactive oxygen species production, MCP-1 expression and endothelial cell proliferation and migration (Loffreda et al., 1998, Konstantinides et al., 2001, Cooke and Oka, 2002).

Adiponectin, another adipokine secreted by adipose tissue is considered as "good adipokine" as it improves insulin sensitivity through activation of AMPK in liver and skeletal muscle (Yamauchi et al., 2002). Adiponectin lowers gluconeogenesis enzyme expression (Kadowaki and Yamauchi, 2005), reduce inflammation by inhibition of TNFα induced activation of Nuclear factor-κB (Ouchi et al., 2000). Adiponectin concentration decreases with increase in adiposity (Yatagai et al., 2003, Turer et al., 2011) and level comes to normal with weight loss.

As mentioned in the previous section, obesity is associated with low grade inflammation along with activation of macrophages and enhanced production of proinflammatory cytokines like TNFα and IL-6 (Lumeng et al., 2007). TNFα is considered as an important player in activation and recruitment of inflammatory cells (Clark, 2007). It has also been associated with insulin resistance in skeletal muscle and adipose tissue by abnormal phosphorylation of IRS1 and declining glucose transporter

type 4 (GLUT4) signal transduction and translocation (Lorenzo et al., 2008). TNFα also activates Nuclear factor-κB (NF-κB) (Collins et al., 1995), which in turn is vital transcriptional factor of an array of inflammatory genes (Antunes and Han, 2009). IL-6 is another proinflammatory factor produced by monocytes, fibroblasts and the stromal vascular fraction of visceral white adipose tissue (Fain et al., 2004). Plasma levels of IL-6 increases with adiposity and is stimulated by TNFα and IL-1. Increased circulatory levels are associated with increase of coronary artery disease, atherosclerosis and instable angina (Coelho et al., 2013). Il-6 inhibits insulin signalling by activating Suppressor of cytokine signalling 3 (SOC3) expression, which in turn hampers insulin induced insulin receptor and insulin receptor substrate-1 phosphorylation in adipocytes and hepatocytes (Coelho et al., 2013). Both TNFα and IL-6 are involved in regulation of other adipokines and both reduce the expression of adiponectin (Bruun et al., 2003). However, TNFα stimulates the production of leptin and chemerin (Wang and Trayhurn, 2006, Sell et al., 2009).

Table 2.1. Factors secreted by adipose tissue into the bloodstream and respective function/effect in their targets (Coelho et al., 2013).

Molecule	Function/effect
Leptin	Signals to the brain about body fat stores. Regulation of appetite and energy expenditure. Wide variety of physiological functions
Adiponectin	Plays a protective role in the pathogenesis of type 2 diabetes and cardiovascular disease
Resistin	Hypothetical role in insulin resistance
TNF-α	Affects insulin receptor signalling, possible cause of the development of insulin resistance in obesity
IL-6	Pro-inflammatory, lipid and glucose metabolism, regulation of body weight
PAI-1	Inhibitor of the fibrinolytic system by inhibition of activation of plasminogen
Angiotensinogen	Precursor of angiotensin II; regulator of blood pressure and electrolyte homeostasis
FFA	Oxidized in tissues to produce local energy. Serve as a substrate for triglyceride and structural molecular synthesis. Involved in the development of insulin resistance
ASP	Influences the rate of triacylglycerol synthesis in adipose tissue
VEGF	Stimulation of angiogenesis
Adipsin	Potential relation between the complement pathway and adipose tissue metabolism
Glycerol	Structural component of the major classes of biological lipids and gluconeogenic precursor
IGF-1	Stimulates proliferation of a wide variety of cells and mediates many cells and many of the effects of growth hormone

2.3. EFFECT OF OBESITY ON MUSCLE TISSUE

Skeletal Muscle constitutes approximately 40% of body weight of a lean non-obese individual (male or female) (Pedersen and Febbraio, 2012). It is an important metabolic organ, plays an important role in energy expenditure, physical strength, locomotion (Pattanakuhar et al., 2016) and is a highly plastic organ (Loenneke and Loprinzi, 2016). Skeletal muscle is composed of different types of muscle fibres and ratio between these different types of fibres is determined by various genetic and environmental factors (Simoneau and Bouchard, 1995). In resting conditions, lipids contribute 90% of energy requirement of skeletal muscle, indicating that skeletal muscle determines the fate of circulating lipids (Bonen et al., 2006). Under positive energy balance, level of circulatory lipids increases, resulting in accumulation if lipids in adipose tissue, liver and skeletal muscle (Stern et al., 2016). Accumulation of lipids in skeletal muscle is associated with lower lipid oxidation as it was found in obese individuals before and after weight loss (Kelley et al., 1999, Kim et al., 2000). Lower lipid oxidation is attributed to lower activities of enzymes involve in fatty acid oxidation (Kim et al., 2000). Accumulation of lipids and their metabolites perturbs the metabolic functions of muscle which may leads to muscle insulin resistance in obesity and development of metabolic syndrome (Wells et al., 2014). Increasing evidence have indicated that skeletal muscle is an important organ regulating the systemic glucose homeostasis (Baskin et al., 2015). Disturbance in muscle metabolic function due to lipid accumulation in muscle is a mediator or marker of insulin resistance remains questionable (Goodpaster and Kelley, 2002). Nevertheless, it is clear that positive energy balance leads to structural and functional changes in skeletal muscle. Some aspects of obesity induced changes in skeletal muscle relevant to this study are discussed in this section.

2.3.1 Fibre type switching

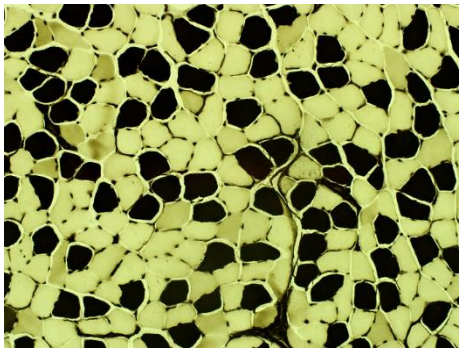

Figure 2.3. Transverse section of soleus muscle showing type 1 (dark) and type 2a (light) fibres.

Skeletal muscle is composed of different types of muscle fibres, broadly classified as slow twitch or type I fibre and fast twitch or type II fibre. Based on the expression of myosin heavy chain (MyHC), muscle fibres are further classified as type I, IIa, IIx and IIb (Figure 2.2) expressing MyHC7, MyHC2, MyHC1 and MyHC4 respectively. Type IIb fibres are only found in rodents and not in humans as humans do not express MyHC4 (Talbot and Maves, 2016). Slow twitch fibres exhibit low fatigability, high oxidative capacity and preference for fatty acids as substrate for ATP production. On the other hand, fast twitch fibres exhibit moderate (IIa) to high (IIb) fatigability, high or medium (IIa) to low (IIb) oxidative capacity and preference for glucose as substrate for ATP production (Bassel-Duby and Olson, 2006, Baskin et al., 2015). Both genetics and environmental factors are responsible for the ratio of oxidative to glycolytic muscle fibre in the body (Simoneau and Bouchard, 1995). In the state of positive energy balance, characteristic of obesity and diabetes, muscle fibres switch from slow twitch to fast twitch which lowers the oxidative capacity of muscle (Mootha et al., 2003).

Two different mechanisms have been hypothesized for obesity induced alterations in muscle fibre type (Pattanakuhar et al., 2016). One of the mechanism, also called metabolic hypothesis proposes that, the obesity induced changes in muscle fibre type are induced by metabolic changes in muscle fibres due to oxidative stress and inflammation. Muscle fibre type phenotype is controlled by several proteins and transcriptional factors

involved in various signalling pathways like p38 mitogen-activated protein kinase (MAPK) (Murgia et al., 2000), calcium/calmodulin-dependent protein kinase IV (CaMKIV) (Wu et al., 2002), calcineurin (Naya et al., 2000) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)-peroxisome proliferator-activated receptor delta (PPARδ) pathway (Wang et al., 2004, Schuler et al., 2006b, Rockl et al., 2007), however, the PGC-1α-PPARδ pathway is the considerably supported by several experimental evidences (Stuart et al., 2013, Rockl et al., 2007, Kaneko et al., 2011, Couturier et al., 2013, Ringseis et al., 2013, Shortreed et al., 2009). PGC-1α is a coactivator interacting with all forms of PPAR isoforms and more details of its role in metabolic regulation will be discussed in the later section 2.2.3. As for muscle phenotype, genetic modification studies in mice have indicated that PGC-1α is a key regulator of muscle fibre type alterations (Wang et al., 2004, Schuler et al., 2006a). Myocyte enhancing factor 2 (MEF2) is the main downstream transcription factor of PGC-1α responsible for oxidative type muscle fibre transformation (Rockl et al., 2008). A systemic oxidative stress and inflammation reduces PGC-1α expression in skeletal muscle, which results in relatively reduced oxidative muscle fibres (Liang and Ward, 2006).

Second mechanism, also called mechanical hypothesis, relates muscle fibre type to workload. Weight gain during obesity increases the muscle workload particularly in weight bearing muscles (Kaneko et al., 2011). Increased muscle work load promotes glycolytic muscle fibre phenotype (Scott et al., 2001). Oxidative muscle fibres are associated with walking ability (Zierath and Hawley, 2004) which is reduced in obese individuals owing to reduced oxidative fibres and more glycolytic fibres induced by increased muscle work load (Healy et al., 2011). This hypothesis suggests that fibre type

switching is an indirect repercussion of obesity. However long term clinical studies are required to prove this hypothesis.

2.3.2 Incomplete Fat oxidation and insulin resistance

Obesity is associated with development of muscle insulin resistance. Although the exact mechanism remains elusive but according to the model hypothesised by Muoio et al. (2008) fatty acid influx and β-oxidation increases in muscle mitochondria owing to both increase in transcriptional regulation and fuel oversupply under obese conditions. However, in state of positive energy (over nutrition and low physical activity), there is no co-ordinated increase in downstream TCA cycle and electron transport chain to fully oxidize acetyl-CoA. Downstream cycles are not only inactivated at transcriptional level but are also hampered due to high energy redox state (increasing NADH/NAD and acetyl-CoA/free CoA ratios). As a result, the by-products of incomplete fatty acid oxidation (acylcarnitines, ketones and reactive oxygen species) accumulate in muscle, which leads to accumulation of long chain-CoA species and subsequent production of other lipid derivatives like diacylglyceroles, ketones, ceramides, intramuscular triacylglycerols and yet some unidentified metabolites. Accumulation of these lipid species affects insulin signal transduction and sensitivity, impeding glucose uptake and metabolism (Muoio et al., 2008).

Besides accumulated lipid derivatives affecting insulin signalling, growing number of evidence suggest that inflammation of skeletal muscle in obesity adversely affect muscle metabolism and contributes to insulin sensitivity (Wu and Ballantyne, 2017). This aspect will be discussed later in inflammation section.

2.3.3 Insulin signalling

After a meal insulin is released into the blood stream. Insulin binds to insulin receptor, a dimeric protein also called insulin receptor kinase. This bonding leads to closing of the protein dimer and cross-phosphorylation of tyrosine residues of insulin receptors changing its conformation and activates insulin receptor kinase (Di Meo et al., 2017). One of the phosphorylated tyrosine residue attract a protein called insulin receptor substrate (IRS), which has two isoforms (IRS1 and IRS2). Upon binding to insulin receptor, IRS also gets phosphorylated at tyrosine residues. IRS acts as an attachment point for PI3K (Cheatham et al., 1994), which phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that travels along membrane to activate phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 is not membrane bound and travels around the cell and stimulate a cascade of signalling events that results in the movement of glucose transporter 4 to cell membrane to facilitate glucose transport (White, 2002) (Figure 2.4).

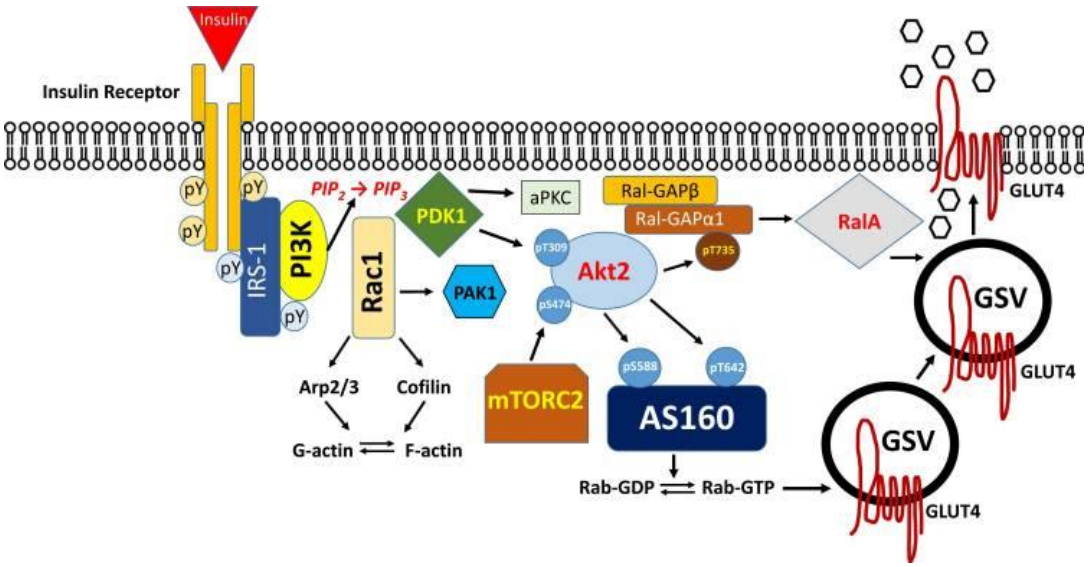

Figure 2.4. Insulin-signalling pathway for insulin-stimulated GLUT4 translocation and glucose transport in skeletal muscle (Cartee, 2015).

In obese and type 2 diabetes subjects, IRS1 tyrosine phosphorylation and PI3K activity has been found to be reduced (Goodyear et al., 1995, Björnholm et al., 1997). Reduced IRS1 tyrosine phosphorylation has been related to enhanced serine threonine phosphorylation(Paz et al., 1997) by serine/threonine kinases including IκB kinase (IKK), c-JUN N-terminal kinase (JNK) and mammalian target of rapamycin (mTOR) (Gao et al., 2002, Aguirre et al., 2002, Li et al., 1999). Consequently, level of PI3K decreases, affecting the downstream targets leading to lower activity of Protein kinase B (AKT) (Kim et al., 1999), atypical Protein kinase C (PKC) (Kim et al., 2003) and lower glucose uptake probably due to reduced GLUT activity/translocation (Shulman, 2000). It is important to mention that a number of studies have provided evidence to support dysfunctionality of IRS in insulin resistance, however there have been some inconsistencies in the model and it has also been observed that insulin resistance can occur independent of IRS (Hoehn et al., 2008).

2.3.4 Master regulators-PGC-1α

PGC-1α has been extensively described as a master regulator of metabolic programming in skeletal muscle, both in health and disease (Chan and Arany, 2014). In addition to fibre type switching mentioned above, PGC-1α regulates the expression of various genes related to lipid oxidation and mitochondrial metabolism (Canto and Auwerx, 2009). Besides muscle, PGC-1α is highly expressed in tissues with high oxidative capacity lie heart, liver, brown adipose tissue and brain (Puigserver et al., 1998, Mootha et al., 2004). PGC-1α increases energy expenditure by co-ordinately enhancing mitochondrial biogenesis, respiration rates and substrate uptake and utilization for energy production (Chan and Arany, 2014, Wu et al., 1999, St-Pierre et al., 2003,

Lehman et al., 2000). These wide range of functions of PGC-1α are exerted by directly co-activating multiple transcription factors such as PPARs (Vega et al., 2000, Wang et al., 2003), myocyte enhancer factor 2 (MEF2) and the family of fork head o-box (FOXO) and nuclear receptors such as thyroid hormone receptor (Puigserver et al., 1998), glucocorticoid receptors (Vega et al., 2000), estrogen receptor (Vega et al., 2000, Puigserver et al., 1998) and estrogen related receptor (Schreiber et al., 2003, Huss et al., 2002). Several studies have demonstrated using genetically modified organisms to demonstrate the role of PGC-1α at systemic and skeletal muscle level. Muscle specific PGC-1α knock out mice displayed abnormal glucose homeostasis owing to reduced oxidative muscle fibre type I, decreased endurance capacity and mitochondrial gene expression (Handschin et al., 2007). Contrary to this, mice with muscle specific overexpression of PGC-1α exhibit increased insulin resistance in response to high fat diet. However, same PGC-1α overexpressing mice exhibit improvement in insulin sensitivity in response to exercise in comparison to exercising wild type mice, indicating the insulin sensitizing effect of PGC-1α, though the mechanism is still unclear (Finley et al., 2012). PGC-1α expression was found to be lower in humans with metabolic syndromes in conjunction with lower oxidative capacities and reduced expression of metabolic genes (Richardson et al., 2005, Mootha et al., 2003, Tanner et al., 2002). However, PGC-1α expression is restored after weight loss or interventions to maintain glucose homeostasis [36]. Expression of PGC-1α is not only controlled at transcriptional level but its activity is affected by various post-translational modifications like phosphorylation, acetylation and methylation etc. (Canto and Auwerx, 2009, Mensink et al., 2007). However how these various modifications regulate PGC-1α activity at any time point is still unclear.

Owing to the role of PGC-1α as master regulator of metabolism, its activity is regulated by cellular mechanisms sensing metabolic perturbations and substrate availability. In response to these metabolic changes, these sensors determine which enzymatic and transcriptional pathways needs to be tweaked to adapt to the changing environment. Two such metabolic sensors, AMP-activated protein kinase (AMPK) and Sirtuin 1, also known as NAD-dependent deacetylase sirtuin-1 (SIRT1), play a major role in metabolic regulation and have also been reported to regulate the activity of PGC-1α (Canto and Auwerx, 2009).

2.3.5 Metabolic sensors- AMPK and SIRT1

AMPK and SIRT1 are energy-sensing molecules present in all eukaryotes and probably coexisted thorough out evolution. It has recently become apparent that AMPK and SIRT1 have common targets like PGC-1α, regulate cellular metabolism in similar way and surprisingly regulate each other (Ruderman et al., 2010). The expression and activity of both AMPK and SIRT1 is affected under metabolic syndromes (Pfluger et al., 2008, O'Neill et al., 2013).

AMPK is a serine/threonine kinase that plays a key role in maintaining skeletal muscle and systemic energy homeostasis (Ferraro et al., 2014). AMPK is a heterotrimer, consisting of catalytic α subunit and regulatory β and γ subunits. The γ subunit has several cystathione β-synthase domains that under normal conditions primarily bind ATP. However, glucose deprivation, fasting, exercise, cellular and oxidative stress depletes energy stores and increases AMP/ATP and creatine/phosphocreatine (Cr/PCr) ratios and AMP is replaced by ATP on two of these domains (Kahn et al., 2005, Hardie, 2011, Winder and Hardie, 1996, Wojtaszewski et al., 2000). It leads to a conformational change in AMPK, which increase AMPK activity by two to ten folds and phosphorylation of α

subunit at Threonine^{172}, which increases the activity by several folds. In muscle, an upstream kinase, serine threonine liver kinase B1 (LKB1) is required for phosphorylation of AMPK (Carling et al., 2008). However, LKB1 has been reported to be constitutively expressed in skeletal muscle and AMPK activation under energy deficit conditions is proposed to be a result of conformational changes that makes it resistant to protein phosphatases (Sanders et al., 2007, Ruderman et al., 2010). Nevertheless, to restore energy stores, activated AMPK induce catabolic pathways by upregulating the transcription of enzymes involved in glucose, lipid and mitochondrial oxidative metabolism and mitochondrial biogenesis (Jager et al., 2007, Bergeron et al., 2001, Long et al., 2005, Winder and Hardie, 1996). In addition to this, AMPK inhibits anabolic processes like glycogen synthesis and protein synthesis to spare ATP (Carling and Hardie, 1989).

SIRT1 is an enzyme that catalyses deacetylation of wide range of substrate (Lin et al., 2004) and its activity is tightly regulated by NAD^+. NAD^+ is an essential coenzyme in all cellular organisms. It participates in wide array of redox reactions which involve electron transfer and quickly change from electron accepting form (oxidising) NAD^+ to electron donating form (reducing) NADH and is therefore an important indicator of cellular energy status (Houtkooper et al., 2010, Bordone and Guarente, 2005). Several studies have indicated that like AMPK, SIRT1 senses changes in NAD^+ availability which changes with nutrient availability (Bordone and Guarente, 2005, Cohen et al., 2004, Nemoto et al., 2004) and energy expenditure (Canto et al., 2009, Suwa et al., 2008). SIRT1 activity increases under low energy conditions that increases cellular NAD^+ level like fasting and calorie restriction and exercise (Rodgers et al., 2005, Chen et al., 2008b, Hayashida et al., 2010, Chabi et al., 2009, Canto et al., 2009, Canto et al., 2010). However,

activity decreases with high fat diet feeding and acute inflammation due to decrease in cellular NAD^+ level (Yoshino et al., 2011, Kim et al., 2011, Kendrick et al., 2011, Tao et al., 2011). Besides NAD^+ level, SIRT activity is also regulated by other factors that affect cellular NAD^+ level, for example, concentration of nicotinamide (NAM) (Ruderman et al., 2010) and activity of NAM phosphoribosyltransferase (Nampt), the rate-limiting enzyme in the salvage pathway of NAD^+ biosynthesis (Li, 2013).

AMPK and SIRT1 regulate each other and are proposed to be the components of a regulatory cycle (Ruderman et al., 2010). It has been suggested that activated AMPK phosphorylates PGC-1α and activates SIRT1, later in turn deacetylate PGC-1α, an important step for its activation (Figure 2.5). Although several in vitro and in vivo studies have provided the evidence for the link between the AMPK and SIRT 1, existence of a regulatory cycle has yet to be proven and many gaps needs to be filled. One such gap is link between the action of AMPK and SIRT one as AMPK activation is an early event in contracting muscle and SIRT1 activation is a let event. Nevertheless, it has been

hypothesised that in muscle, late activation of SIRT1 helps to maintain the activation of AMPK (Ruderman et al., 2010).

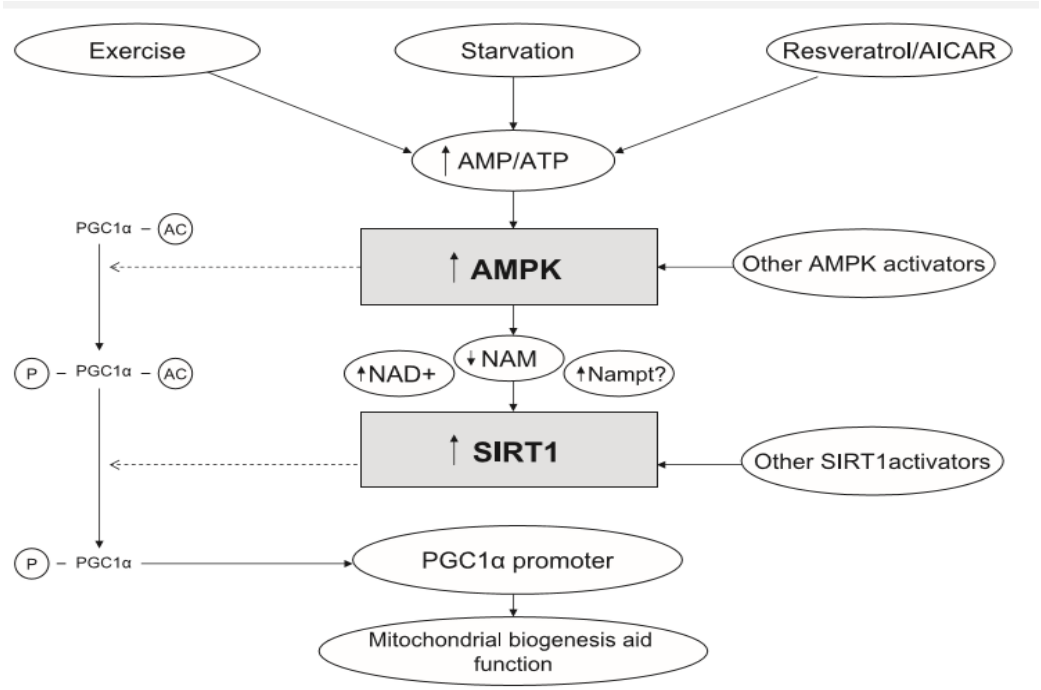

Figure 2.5. Proposed mechanism of PGC1α activation by cooperation between AMPK and SIRT (Ruderman et al., 2010).

Several human and animal studies have reported that AMPK and SIRT 1 activity decreases in obese state and this dysregulation is related to pathological changes like altered lipid metabolism, hyperinsulinemia, insulin resistance, ectopic lipid accumulation, inflammation, oxidative stress and mitochondrial dysfunction. However, dysregulation of AMPK and SIRT1 accompany or precedes these pathological changes remains elusive (Ruderman et al., 2010).

2.3.6 Inflammation

Obesity induced inflammation is well documented in adipose tissue, however number of studies have suggested that inflammation also occurs in skeletal muscle (Warfel et al., 2016, Hommelberg et al., 2010, Kewalramani et al., 2010). Increasing

evidence suggest that obesity is associated with immune cell infiltration into skeletal muscle that contribute to the predominant inflammatory cells in muscle (Khan et al., 2015, Lackey and Olefsky, 2016, Olefsky and Glass, 2010). Density of macrophages and T cells have been reported to increase after high fat diet feeding in both humans (Boon et al., 2015, Tam et al., 2014) and mice (Khan et al., 2015, Patsouris et al., 2014, Olefsky and Glass, 2010). Some immune cells like mast cells and eosinophils displayed no change in muscle with obesity (Fink et al., 2014, Altintas et al., 2012), however others like neutrophils, B cells, NK cells and invariant NKT cells have not been reported in SM in obesity (Wu and Ballantyne, 2017).

In muscle, adipose tissue is found between the myocytes called inter-myocellular or intermuscular adipose tissue (IMAT) and surrounding muscle called perimuscular adipose tissue (PMAT) (Wu and Ballantyne, 2017). Both IMAT and PMAT are different from subcutaneous adipose tissue and their amount in muscle increases with obesity and decreases with weight loss (Santanasto et al., 2015). Additionally, they are highly correlated to insulin resistance and inflammation (Kelley and Goodpaster, 2001, Albu et al., 2005, Goodpaster et al., 2000, Haam et al., 2016, Scott et al., 2015). Histologically, macrophages and T cells reside both in muscle adipose tissue (IMAT/PMAT) and between myofibres, however, their density is much higher in muscle adipose tissue (Togashi et al., 2000, de Alvaro et al., 2004, Fink et al., 2014) than between myofibres (Fink et al., 2014, Khan et al., 2015, Patsouris et al., 2014). Obesity linked changes in immune cells in skeletal muscle is much higher in adipose tissue depots than in muscle, which helps to explain no alteration in immune cell number in muscle after weight loss in some studies (Tam et al., 2012, Bruun et al., 2006).

Although the exact trigger for skeletal muscle inflammation remains elusive, fatty acids, particularly dietary saturated fatty acids have been proposed to induce expression of inflammatory molecules like chemokines in both skeletal muscle and adipose tissue (Ciaraldi et al., 2016, Togashi et al., 2000). Myocytes secrete number of cytokines like IL-6, IL-8, IL-15 and other molecules like FGF21, irisin, myonectin, myostatin, also known as myokines (Pedersen and Febbraio, 2012, Eckardt et al., 2014). In comparison to adipokines which are considered as proinfammatory and are involved in development of metabolic syndromes, myokines have beneficial effects on glucose and lipid metabolism and counteract the detrimental effects of adipokines (Lumeng and Saltiel, 2011, Osborn and Olefsky, 2012, Olefsky and Glass, 2010, Eckardt et al., 2014). Myokines can act in autocrine and paracrine manner affecting myocytes and immune cells and can also act in endocrine manner affecting other cells like adipocytes and hepatocytes (Wu and Ballantyne, 2017). IL-6 is most extensively studied myokine. Its secretion and plasma concentration increases with exercise and muscle contraction (Pedersen and Febbraio, 2012, Eckardt et al., 2014, Fischer, 2006). Although considered important but its function is highly arguable in muscle inflammation owing to the inconsistency in results reported by different studies. After acute exercise or acute treatment of myocytes or acute infusion of IL-6 in healthy humans enhance basal and insulin stimulated glucose uptake and systemic insulin sensitivity (Carey et al., 2006, Pedersen and Febbraio, 2012). Positive effect of IL-6 is attributed to phosphorylation of AS160 through AMPK (Kewalramani et al., 2010). IL-6 also enhances lipolysis, fatty acid oxidation, induce expression of anti-inflammatory cytokines such as IL-10 and inhibit proinflammatory cytokines TNF1α (19, 26). However under chronic conditions like obesity, it acts as proinflammatory and promotes insulin resistance (Wu and Ballantyne, 2017). Negative

effects of IL-6 are attributed to activation of JNK1/2, suppression of cytokine signalling 3 expression and activation of IRS1 tyrosine phosphatase and protein tyrosine phosphatase-1B (Kewalramani et al., 2010). Inconsistencies does not only lie among the acute and chronic studies, even among chronic studies contradictory results have been reported. For instance, IL-6 expression decreased in skeletal muscle of obese rats (Shin et al., 2015), however, its expression level increased in cultured skeletal muscle myocytes of obese type 2 diabetic humans in comparison to healthy cohort (Green et al., 2011). Also, increase in IL-6 expression have been reported in obese type 2 diabetes subjects in comparison to healthy cohort (Reyna et al., 2008, Corpeleijn et al., 2005, Ciaraldi et al., 2016). Taken together, the exact role of IL-6 in muscle insulin sensitivity needs further investigation.

TNFα is a proinflammatory cytokine, its expression increases in obesity with insulin resistance or with type 2 diabetes, inhibits insulin sensitivity and insulin stimulated glucose uptake and impairs mitochondrial structure and function (Wu and Ballantyne, 2017). TNFα manifests these effects through number of mechanisms including activation of extracellular signal regulated kinase 1/2 (ERK1/2), JNK or the NFκβ pathway (Kewalramani et al., 2010). TNFα also activates sphingomyelinase which produces ceramides (Haus et al., 2009) and reduces expression of sortilin (Kaddai et al., 2009) that apparently controls GLUT4 sorting (Shi and Kandror, 2005).

2.4. MICRORNA

MicroRNAs (miRNA) are noncoding, endogenous, highly conserved (in most instances), 18-25 nucleotide RNAs (Hilton et al., 2013, Flowers et al., 2013, Palmer et al., 2014). MiRNA binds with specific mRNA guided by Watson-Crick complementary base pairing rules and regulate gene expression at post transcriptional level either by

cleavage of mRNA or blocking the translation process. MiRNAs control various cellular processes like proliferation, differentiation and apoptosis (McGregor et al., 2014). In humans, more than 1000 miRNAs have been identified which regulate 60% of human genome (Pasiakos and McClung, 2013). Expression of miRNA is developmental stage, cell and tissue specific (Sood et al., 2006).

2.4.1 Biogenesis of miRNA

The miRNAs are transcribed from miRNA genes, introns of protein coding genes or from polycistronic transcripts that encode multiple, closely related miRNAs (Peng et al., 2014). Biogenesis begins with synthesis of long RNA transcript which is generally several thousand nucleotide long stem loop and is called primary miRNA transcript (pri miRNA) (Figure 2.6) (Hilton et al., 2013). Primary miRNA is cleaved near the base of pri miRNA stem loop into 60-70 nucleotide intermediates known as miRNA precursor or pre miRNA, with the help of enzyme Drosha and RNase III endonuclease. Cleavage of primary miRNA by Drosha is staggered, typical of RNase III endonuclease, leaving a 5′ end and approximately 2 nucleotide overhanging at the 3′ end, defining one end of the miRNA (Basyuk et al., 2003, Lee et al., 2003). These pre miRNAs are actively transported by RAN-GTP and export receptor, Exportin-5 from nucleus to cytoplasm (Bartel, 2004).

In the cytoplasm, pre miRNA is cleaved, two helical turns away from the loop by Dicer (an RNase III endonuclease) with a staggered cut, leaving a 5′ end and approximately 2 nucleotide overhanging at the 3′ end, defining other end of the miRNA (Bartel, 2004). This results in a double stranded RNA, with about 2 nucleotide overhang at each 3′ end. One of the strand from the duplex is called “guide strand (miRNA)” will

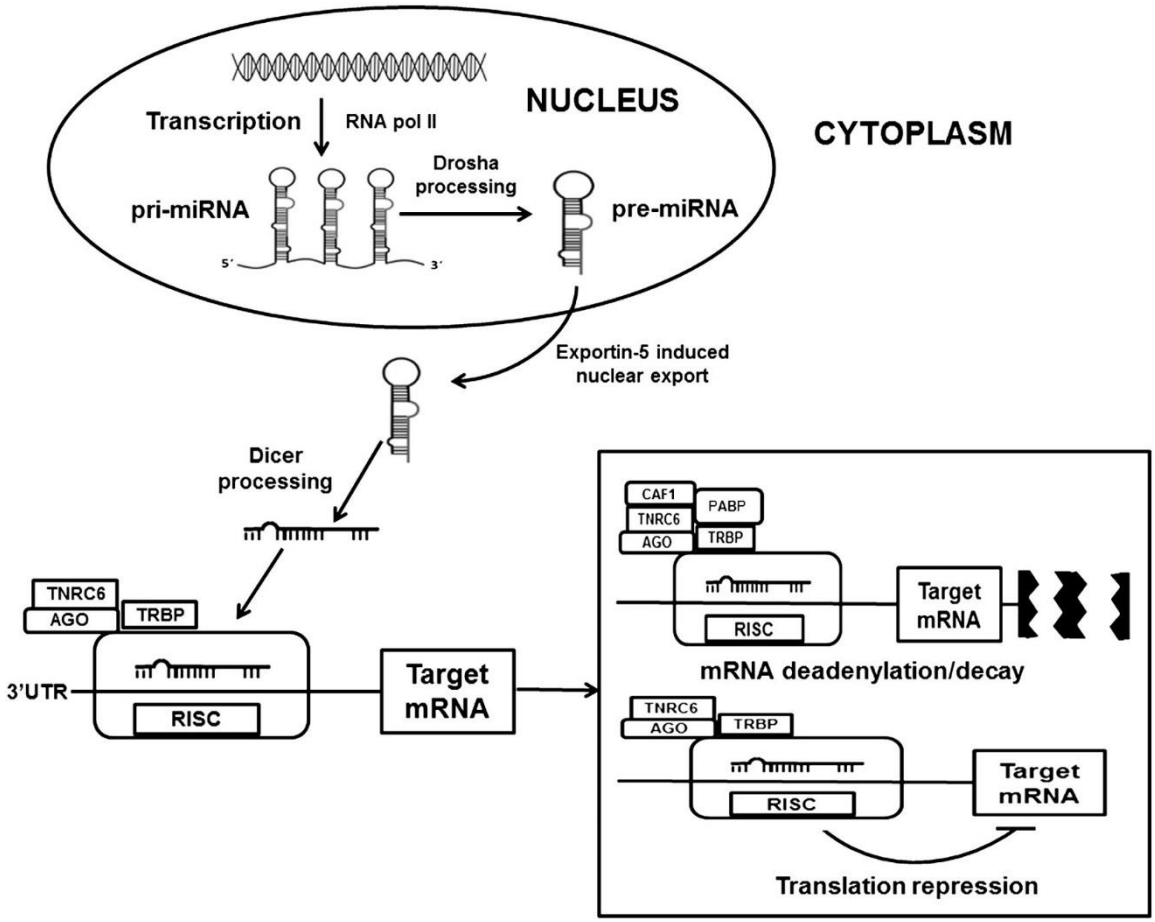

Figure 2.6. MiRNA biogenesis pathway. MiRNAs are transcribed by RNA polymerase II resulting in pri-miRNA transcripts. Pri-miRNAs are processed by Drosha leaving Pre-miRNAs which are exported from the nucleus via Exportin5. Pre-miRNAs are processed by Dicer, the mature miRNA is then permanently incorporated into the RNA-induced silencing complex and will bind to the 3′-UTR of a target mRNA (Peng et al., 2014).

act as a mature miRNA and the complementary strand is called "passenger strand (miRNA*)". Out of the two complementary strands of miRNA, the strand which acts as guide strand is the one whose 5′ end is less tightly paired (Khvorova et al., 2003, Schwarz et al., 2003). Helicase like enzyme (yet to be identified) separates the guide strand from the passenger strand and the latter is degraded by DiGeorge syndrome critical region gene 8 (DGCR8) protein, commonly called Pasha (Yeom et al., 2006). Now the single stranded mature miRNA is incorporated into ribonucleoprotein complex known as ribonucleoprotein miRNA induced silencing complex (RISC).

RISC is a complex ribonucleoprotein consisting of miRNA, Argonaute protein, DICER and human immune deficiency virus-1 transactivating response element RNA binding protein (McDaneld, 2009). The Argonaute protein plays a major function in RISC by acting as catalytic component of endonucleolytic cleavage (Liu et al., 2004).

2.4.2 Regulation of gene expression by miRNA

There are three mechanisms by which the gene expression is regulated by miRNA namely mRNA degradation, blocking translation initiation and translocation to processing bodies.

2.4.2.1. mRNA Degradation

RISC is directed by miRNA to target mRNA by base pairing interactions. The miRNA has 7-8 nucleotides sequence called seed sequence, at the 5′ end which binds with mRNA (Heneghan et al., 2010, McDaneld, 2009). The binding of miRNA to mRNA, induce endonucleolytic cleavage between the nucleotide pairing to residue 10 and 11 of miRNA (Elbashir et al., 2001). The cleaved mRNA is then recognized and degraded by general cellular degradation machinery (Valencia-Sanchez et al., 2006). Additionally, miRNA deadenylates poly A tail followed by decapping of mRNA, resulting in unstable mRNA sequence which is susceptible to degradation by cellular mRNA degradation machinery (Giraldez et al., 2006, Wu et al., 2006).

2.4.2.2. Blocking translation initiation

Gene regulation by miRNA does not always results in decrease in the level of mRNA indicating that besides degradation/cleavage or deadenylation, miRNAs regulate gene expression by other alternative mechanisms. It has been reported that in certain cases, interaction of miRNA with target mRNA blocks translation initiation by binding to the 5′ cap of mRNA instead of inducing cleavage (Chendrimada et al., 2007, Kiriakidou

et al., 2007). Some Argonaute proteins in RISC have similar binding domains as translation initiation factors, competing with the latter for binding with 5′ cap of mRNA, blocking translation initiation (McDaneld, 2009). Thus translation initiation is halted, lowering the level of protein without altering the level of mRNA.

2.4.2.3. Translocation to processing bodies

Processing bodies (P bodies) are aggregation of translationally repressed mRNA and mRNA decay machinery in cytoplasmic foci (Parker and Sheth, 2007). It has been hypothesized that miRNA-mRNA complex is translocated to P bodies. There are some evidences that support this hypothesis like the presence of enzyme and factors for translational repression. However, certain evidences also suggest that p bodies may only serve as temporary storage units for mRNA because disrupting P bodies does not disrupt the miRNA pathway or degree of translational repression (McDaneld, 2009).

2.4.3 MiRNA, energy homeostasis and obesity

MiRNA play important role in energy homeostasis by regulating the expression of various key players of regulatory cascade involved in glucose, lipid and protein metabolism. They are expressed in various tissues involved in energy homeostasis for example adipose tissue, liver, muscle and pancreas and have been implicated to regulate pancreatic islet development, β-cell differentiation, insulin secretion, insulin signalling, insulin sensitivity in muscle, adipose tissue and liver (Dumortier et al., 2013).

Several miRNAs have been reported to play regulatory role in lipid metabolism like miR-14, miR-33, miR-122, miR-370, miR-378/378*, miR-302a, miR-613, miR-168a, miR-758, miR-10b, miR-224 (Peng et al., 2014). MiR-122 is besides being liver specific, is the most abundant miRNA in liver and is the first miRNA identified, which directly regulates lipid metabolism (Rottiers and Näär, 2012, Esau et al., 2006).

Downregulation of miR-122 leads to decreased plasma cholesterol and triglyceride levels associated with altered cholesterol biosynthesis and increased fatty acid oxidation (Dumortier et al., 2013). Although, several genes have been reported to be regulated by miR-122, target validation needs further investigation. Number of studies have shown that bifunctional SREBP2-miR-33a locus regulates lipid metabolism (Rottiers and Näär, 2012). The *Srebf2* gene encodes both SREBP2 which regulated cholesterogenic gene expression and miR-33a, located in the intronic region of the gene. Likewise, *Srebf1* express SREBP1a/c and miR-33b, found in the intronic region of the gene. SPREBP1a/c regulates lipogenic gene expression. Interestingly, Mir-33a and b regulate cholesterol efflux and fatty acid degradation. Taken together, SREBP proteins and miR-33 together regulate the cholesterol and lipid homeostasis (Dumortier et al., 2013).

Glucose homeostasis is maintained by the action of two antagonistic hormones, glucagon and insulin, secreted by α and β cells of pancreas respectively. Although there haven't been much reported about the miRNA regulation of glucagon secretion, number of miRNAs have been implicated in insulin secretion (Özcan, 2014). For instance, miR-375 have been implicated in regulating α and β cell growth and proliferation and also influences the physiology of β cells (Poy et al., 2009). In addition to pancreas, miRNAs regulate glucose homeostasis at systemic level. The Lin28/let-7 axis regulates glucose metabolism by targeting various components of insulin signalling pathway (Zhu et al., 2011). Let-7 over expression induce insulin resistance and impaired glucose tolerance (Frost and Olson, 2011). Similarly, downregulation of miR-103/107, improves insulin action and glucose homeostasis, however, upregulation of miR-103/107 impairs insulin action and glucose homeostasis (Trajkovski et al., 2011). Improvement in glucose homeostasis by these miRNAs has been attributed to the inhibition of caveolin-1 by these

miRNAs. Likewise, miR-143/145 cluster has been implicated to regulate glucose homeostasis through activation of insulin stimulated Akt (Jordan et al., 2011). Recently, miR-802 has been found to decrease insulin action and hampers glucose tolerance by inhibiting Hnf1b in liver (Kornfeld et al., 2013).

MiRNAs also regulate amino acid metabolism (Dumortier et al., 2013). MiR-29b control amino acid catabolism by targeting mRNA of dihydrolipoamide branched chain acyltransferase component of branched-chain keto acid dehydrogenase, which catalyses first step in branched-chain amino acid breakdown. Branched-chain amino acid homeostasis is important since they contribute 20% of the amino acid found in proteins, regulate hormone secretion (e.g. insulin), protein synthesis, and autophagy, through modulation of the mTOR pathway (Mersey et al., 2005).

2.4.4 miRNAs: the effect of diet and exercise

Recent studies have suggested that dietary intake can alter the expression of miRNAs (McGregor et al., 2014). Our recent review has reported that diet rich in fats alters the expression of miRNAs in various tissues like adipose, liver, skeletal muscle, intestine, nervous tissue, heart and corporal tissue (Wilson et al., 2017). For instance, expression of miR-143 has been reported to be elevated in adipose tissue of mice fed high fat diet (45% of total calories from fat) in comparison to the group on standard chow for 8 weeks (Takanabe et al., 2008). The expression of miR-143 was found to be positively correlated with body weight, mesenteric fat weight, plasma leptin concentration levels, peroxisome proliferator-activated receptor-γ (PPAR-γ) expression and adipocyte fatty acid-binding protein (aP2). PPAR-γ is a nuclear receptor which controls adipocyte differentiation and hypertrophy, while aP2 regulates systemic glucose and lipid metabolism and is highly expressed in obesity (Maeda et al., 2005). High dietary fat intake

also upregulates, miR-221 and 222 in adipose tissue (Chartoumpekis et al., 2012). miR-222 targets hypoxia-inducible factor 1-alpha (Hif-1α) and tumour necrosis factor alpha (TNF-α), which have been reported to regulate cell differentiation and may also be linked to inflammatory processes (Ortega et al., 2010, Xie et al., 2009b, Xu et al., 1999, Yun et al., 2002) and also associated with arterial smooth muscle angiogenesis (Felli et al., 2005). In skeletal muscle, high fat diet feeding down-regulated expression of miR-1, miR-133a, miR-133b and miR-206, known as the myomiRs and upregulated expression of whereas miR-144 and miR-106b (Chen et al., 2012). Subsequent signalling mapping revealed 19 significant signalling pathways related to insulin resistance. These included signal transduction pathways related to cell proliferation [(Mitogen-activated protein kinases (MAPK), Wnt, neurotrophic, hedgehog, vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGF-β)], metabolism (lysine degradation, heparin sulphate biosynthesis and glycan degradation), protein degradation (ubiquitin mediated proteolysis) and oncogenic (colorectal) pathways. Few of these pathways, for instance MAPK have already been implicated in skeletal muscle insulin resistance (Gehart et al., 2010) and key proteins within the MAPK pathway such as p38 and pJUK have been found to be up-regulated in high fat diet-induced diabetic mice (Chen et al., 2012). Down-regulation of miR-1, miR-206, and miR-133 levels has also been reported in adipose tissue of high fat diet-induced obese model (Chartoumpekis et al., 2012) and in the vastus lateralis muscle of type 2 diabetic patients (Gallagher et al., 2010). Also, expression of miR-1 was negatively correlated with glycemia in high fat diet-induced diabetic mice (Frias Fde et al., 2016) and thus could be a prospective target for management of skeletal muscle insulin resistance.

In terms of exercise, several studies have reported alteration in miRNA expression in response to resistance versus cardiorespiratory training, acute versus prolonged exercise and acute changes versus chronic changes in miRNA expression in response to long term training in both animal and human studies (Flowers et al., 2015). Resistance training has been reported to result in differential expression of miR-378, miR-299, miR-269 and miR-451 in skeletal muscles of low and high responding human males. Downregulation of these miRNAs in low responders correlated with failure to activate muscle growth and remodelling genes (Davidsen et al., 2011). Acute resistance exercise has also been reported to alter muscle specific primary miRNA (miR-1, miR-133a and miR-206), implicated to regulate muscle cell differentiation and development (Koutsoulidou et al., 2011)), pre-miRNA expression and miRNA biogenesis enzyme, mRNA expression in skeletal muscles of young and old adults (Drummond et al., 2008). Same study also reported that miR-1 expression reduced in young adults but remained unaltered in older adults. Similar increase in expression of miR-1, miR-133a, miR-133b and miR- 181a following acute endurance exercise bout have been reported in human skeletal muscle. Increased expression of miR-1 and miR-29b following short term endurance training has also been reported by the same study (Russell et al., 2013). Likewise, another study examining the effect of endurance exercise on miRNA profile of human skeletal muscles revealed alterations in profile of miR-1 and miR-133 with endurance exercise and its reversal to the basal level 14 days after training period (Nielsen et al., 2010). However, no negative correlation between miRNAs and protein involved in endurance adaptation (Transforming growth factor beta (TGFβ) and mitogen activated protein kinase (MAPK)) was observed in this study. In C57BL/6J mice, endurance exercise increased the expression miR-1, miR107and mi-181 and decreased expression

of miR-23. Decreased expression of miR-23 has been concluded to negatively regulate PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) (Safdar et al., 2009) which is a transcriptional activator of energy metabolism genes. Similar study on C57BL/6 mice demonstrated that expression of miR-696 is dependent on physical activity and is involved in regulation of PGC-1 alpha and skeletal muscle metabolism (Aoi et al., 2010). In addition to skeletal muscle, plasma miR-486 associated with energy metabolism have been reported to decline by both acute and chronic exercise in young human males (Aoi et al., 2013). In a human study, different circulatory/plasma miRNA were reported to alter their expression profiles in response to different exercise regimes. Acute exhaustive exercise before and after sustained aerobic training upregulate miR-146a and miR-222 associated with anti-inflammation and anti-angiogenesis respectively. The expression of miR-21 (associated with anti-apoptosis, anti-proliferation and anti-inflammation) and miR221 (associated with anti-angiogenesis) was increased by acute exhaustive exercise before but not after sustained aerobic training. Plasma level of miR-20a (associated with anti-angiogenesis) was not affected by Acute exhaustive exercise before but increased after sustained aerobic training only (Baggish et al., 2011). Besides skeletal muscle and plasma, impact of exercise on miRNA expression in nervous and cardiac tissue have also been addressed in few animal studies as these kind of experiments are highly invasive and therefore only animal models have been used (Denham et al., 2014).

2.2 LIFESTYLE INTERVENTIONS

2.4.5 Intermittent Fasting

The concept of intermittent fasting (IF) or more commonly known as 5:2 diet was popularized by two British authors, Michael Mosley and Mimi Spencer. Broadly, IF

refers to calorie restriction by 75%-100% on 1-3 consecutive or alternate days, called fast days, per week and ad libitum food consumption on other days (Patterson et al., 2015, Tinsley and La Bounty, 2015). More precisely IF can be categorized into 3 groups: alternate day fasting, whole day fasting and time restricted eating. Alternate day fasting (ADF) refers to alternating fasting days with only 25% calorie intake and ad libitum feeding days. Whole day fasting refers to 1-2 complete fasting days and ad libitum feeding on other days of the week. However, some protocols allow 25% calorie intake and are therefore referred to as modified fasting (Brown et al., 2013). Time restricted eating included a daily routine with a restricted period called feeding window of 4-8 hours devoted to eating with remaining hours of no calorie intake called fasting window (Rothschild et al., 2014). Although time restricted feeding comes under the umbrella of IF, this section of the review mainly focuses on whole day modified fasting and alternate day fasting, as this study is based on whole day fasting.

Although only few scientific (preclinical and clinical) studies have been reported on IF, still it has gained a lot of attention with in the last decade owing to its health benefits like improvement in body composition, glucose homeostasis and cardiovascular health and delayed aging. Besides that, IF has also been reported to reduce inflammation, delays onset of neuronal dysfunction and degeneration (Mattson et al., 2016). Several animal and human studied have revealed that fasting is effective to reduce weight loss, delay aging and optimize metabolism. In humans, fasting (12-24 hours) decline the serum glucose level by 20% or more and depletes hepatic glycogen. This is accompanied by "metabolic switch" to a mode where non hepatic glucose, ketone bodies and free fatty acids are used as energy source. Ketone bodies are derived from acetyl CoA generated from β-oxidation of fatty acids released into the blood stream from adipocyte and also by

the conversion of ketogenic amino acids (Longo and Mattson, 2014). Impairment of this metabolic switch is a key dysfunction of metabolic syndromes like obesity and diabetes (Storlien et al., 2004).

2.4.5.1. Effect of IF on Body composition

Several animal and human studied have suggested that IF is effective for weight loss and beneficial changes in body composition. Weight loss with Intermittent fasting and alternate day fasting have been reported in several human studies. (Klempel et al., 2012, Harvie et al., 2011) (Heilbronn et al., 2005b, Eshghinia and Mohammadzadeh, 2013, Johnson et al., 2007, Varady et al., 2009, Klempel et al., 2013, Bhutani et al., 2013, Varady et al., 2013). These studies suggest that, IF resulted in 3-7% of body weight reduction where as ADF resulted in 4-8% body weight reduction. Slightly higher decrease in ADF than IF could be attributed to more fasting days in ADF as compared to IF. On an average, IF and ADF resulted in 0.25 and 0.75 kg weekly reduction in body weight. Among animal studies, only one study has reported 3% weight loss (Varady et al., 2007) in mice after four weeks of ADF. However, most studies have reported reduced weight gain by 0-40% in comparison to ad libitum control group (Higashida et al., 2013, Karbowska and Kochan, 2012, Li et al., 2013, Beigy et al., 2013, Varady et al., 2010).

Weight loss due to IF or ADF is primarily attributed to decline in fat mass. In humans, IF for 8-24 weeks has been reported to decrease body fat, measured as change in waist circumference, by 1.4-2.3% (Klempel et al., 2012, Harvie et al., 2011). Alternate day fasting also showed similar decline in fat mass, with 3-12 weeks of ADF resulting in 1.3 to 2.4% decline in fat mass (Eshghinia and Mohammadzadeh, 2013, Varady et al., 2009, Bhutani et al., 2013, Klempel et al., 2013, Varady et al., 2013). Among animal studies, change in total fat mass following intermittent or alternate day fasting was not

reported by any study, however, decline in intra-abdominal fat mass by 40% (Higashida et al., 2013), white adipose tissue by 46% (Karbowska and Kochan, 2012), visceral fat by 18% (Varady et al., 2010) and no change in inguinal and epididymal fat mass (Varady et al., 2007).

Weight loss interventions are aimed to reduce body weight by loss in fat mass and conserving lean muscle mass. In addition to changes in fat mass, lean muscle mass change has also been reported by various studies on IF. Intermittent fasting in overweight humans for 24 weeks resulted in 1.3% decline in muscle mass (Harvie et al., 2011). Alternate day fasting for 3-12 weeks has also been reported to decrease muscle mass by 1-2.1% (Heilbronn et al., 2005b, Eshghinia and Mohammadzadeh, 2013, Bhutani et al., 2013, Klempel et al., 2013, Varady et al., 2013, Varady et al., 2009). In comparison to calorie restriction, IF has been reported to cause less muscle mass loss (Harvie and Howell, 2017). Weight loss in form of muscle mass has been reported to be higher in lean non-obese individuals in comparison to obese individuals (Varady et al., 2009, Varady et al., 2013). Contrary to this, some studies comparing IF with calorie restriction, also reported equivalent loss of lean muscle mass (Harvie et al., 2011, Hill et al., 1989).

2.4.5.2. Effect of IF on glucose homeostasis

In humans, intermittent fasting has been consistently reported to improve glucose homeostasis (Harvie and Howell, 2017, Barnosky et al., 2014). Intermittent fasting for 8-24 weeks in overweight and obese individuals have been reported to cause 2-3% decline in fasting blood glucose (Klempel et al., 2012, Harvie et al., 2011). Alternate day fasting for 3-12 weeks resulted in 1-6% decline in fasting blood glucose (Heilbronn et al., 2005a, Eshghinia and Mohammadzadeh, 2013, Johnson et al., 2007, Varady et al., 2009, Varady et al., 2013, Klempel et al., 2013, Bhutani et al., 2013). In particular, three studies have

reported the effect of these fasting interventions in prediabetic individuals (Klempel et al., 2012, Varady et al., 2009, Varady et al., 2013) with a decline of 3-6% in fasting glucose. The extent of decline was found to be directly proportional to the duration of the study with highest decline of 6% reported after 12 weeks of ADF (Varady et al., 2013).

In animal models, decline in fasting blood glucose by 17% has been reported in NMRI mice after six weeks of intermittent fasting (Beigy et al., 2013). However, few studies have reported no significant change in fasting blood glucose after 7-10 weeks of IF in CD-1 wild type mice (Li et al., 2013) and male Wistar rats (Higashida et al., 2013).

In humans, although several studies have reported the effect of IF and ADF on plasma insulin levels but only three studies have reported the effect on prediabetic individuals (Klempel et al., 2012, Varady et al., 2009, Varady et al., 2013). These studies reported 20 – 31% decline in plasma insulin after 8-12 weeks of IF/ADF. Remarkably, two separate studies reported similar decline in insulin level by 20 and 21% after 8 weeks of IF (Klempel et al., 2012) and ADF (Varady et al., 2009) respectively. The decline was comparable despite more number of fasting days implemented in ADF study (3-4 fast days) in comparison to IF (1 fast day). However, interestingly, both interventions prescribed the same level of energy restriction (35-40%), indicating that degree of restriction is a stronger predictor of insulin lowering in comparison to the number of fasting days. In addition to this, duration of the intervention also determines the extent of decrease, for instance, 31% decline in plasma insulin was reported after 12 weeks of (Varady et al., 2013), indicating that longer duration of fasting intervention may be more beneficial (Barnosky et al., 2014).

In male Wistar rats, ADF for 6 weeks resulted in 46% decline in plasma insulin (Higashida et al., 2013). However, contradictory to this and most human studies, 48 days

of ADF in male Wistar rats have been reported to elevate plasma insulin levels by 712% (Karbowska and Kochan, 2012). This indicates that long term effects of IF/ADF on insulin regulation needs further investigation.

Intermittent fasting and ADF studies in humans have constantly reported improvements in insulin sensitivity with these interventions (Barnosky et al., 2014). Consistent improvements in insulin sensitivity (measured in terms of homeostatic model assessment (HOMA-IR)) after 3-4 weeks of IF/ADF have been reported in normo-glycemic and prediabetic individuals. The improvement in insulin sensitivity has been found to be directly proportional to the amount of weight loss. For example, 8% weight loss after 8 weeks of ADF showed 33% improvement in insulin sensitivity (Johnson et al., 2007) however, 4% weight loss after 12 weeks of ADF resulted in 9% improvement in insulin sensitivity (Bhutani et al., 2013). Intermittent fasting studies also reported in improvement in insulin sensitivity by 23% and 27% with weight loss of 4% and 7% after 8 weeks (Klempel et al., 2012) and 24 weeks (Harvie et al., 2011) respectively.

2.4.5.3. Effect of IF on lipid profiles

Few studies have reported the impact IF/ADF on plasma lipids. Weight loss have been found to be not always accompanied by beneficial changes in plasma lipids. A recent 8 weeks ADF study reported 9% weight loss accompanied by significant reduction in LDL cholesterol and fasting TAG (Catenacci et al., 2016). Likewise, significant reduction in plasma LDL and TAG after 1.6% reduction in body weight have been reported after 8 weeks of ADF (Varady et al., 2009). However, another 8 weeks ADF study reported significant decline in TAG but no significant change in LDL despite 1.8% decline in body weight (Johnson et al., 2007). Few studies also reported no change in lipid profile despite weight loss after ADF in humans (Bhutani et al., 2013, Eshghinia and Mohammadzadeh,

2013). Among animal studies, two separate studies reported decline in blood lipid levels following 6 weeks (Higashida et al., 2013) and 11 months (Li et al., 2013) of ADF in CD-1 wild type mice and male Wistar rats respectively.

2.4.5.4. Molecular changes induced by IF

Several studies have investigated the impact of IF on body composition and physiological parameters but very few studies have investigated the molecular adaptations in metabolically important tissues following IF regime. In a study on male Wistar rats, IF 48 days (3 days fasting, 3 days feeding) resulted in increased mRNA expression of FSP27 in white adipose tissue, indicating that despite low calorie intake under IF, fat deposition in adipose tissue is promoted by increased expression of genes involved on lipid deposition (Karbowska and Kochan, 2012). Even though the expression of these genes is higher in IF group, animals in this group still weighed less and had less adipose tissue mass in comparison to ad libitum control group as well as the calorie restricted animals (food intake reduced to 80% of ad libitum control), indicating that molecular adaptation to IF is different from calorie restriction. Increase in FSP27 was also accompanied by increased mRNA expression of PPARg2, C/EBPa and plasma insulin and are speculated to stimulate the expression of FSP27 in white adipose tissue.

Another recent study in mice reported that IF is able to induce favourable health adaptations despite consuming equivalent amount of calories as the ad libitum group indicating that changing feeding pattern without decreasing the calorie intake can induce metabolic adaptations and prevent metabolic syndromes (Kim et al., 2017). According to this study, IF leads to increased expression of beige/brown adipose markers (i.e., *Ppargc1a, Cidea and Ucp1*) and browning of adipose tissue. In addition to this, IF induced elevated expression of M2 macrophage marker genes (e.g., *CLEC10A and IL10*),

indicating the activation of adipose tissue macrophages. Most importantly, IF induced vascular endothelial growth factor (VEGF) specifically in adipose tissue and is responsible for metabolic benefits induced by IF.

2.4.6 High Intensity Interval Training

World health organisation and American college of sports medicine recommend at least 150-250 minutes per week of moderate intensity physical activity for moderate weight loss. For clinically significant weight loss, more than 250 minutes per week of moderate intensity exercise is recommended (World Health Organization, 2010) (American College of Sports Medicine, 2013). Despite the established therapeutic potential, this type of exercise regimes is less favoured by healthy and clinical population due to lengthier time commitments (Batacan et al., 2017). High intensity intermittent/interval exercise/training (HIE/HIIT) has been concluded as time efficient strategy for inducing adaptations generally associated with long term low or moderate intensity exercise. HIIT refers to brief bouts of vigorous activity (80-90% $VO_{2\ max}$) interspersed by relatively longer bouts of low intensity exercise (Gibala et al., 2012, Trapp et al., 2008). There is also a subcategory of HIIT know as sprint interval training which involves brief bouts of all out sprint (more than 100% $VO_{2\ max}$) interspersed by longer bouts of low intensity exercise (Figure 2.7) (Cassidy et al., 2017). In comparison to moderate intensity exercise, HIIT has been

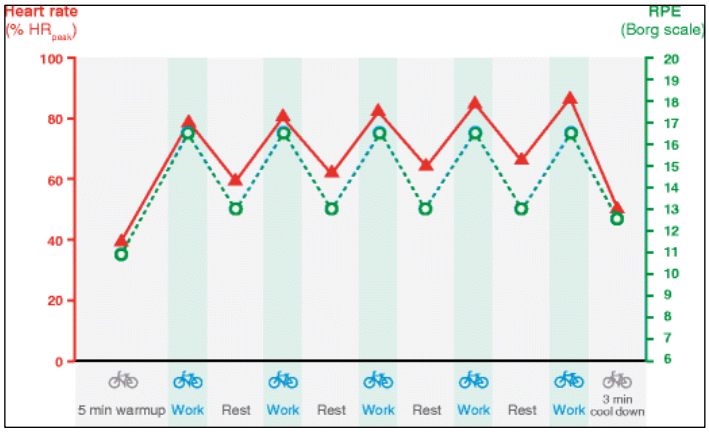

Figure 2.7. An example of a HIIT protocol (Cassidy et al., 2017)

reported to significantly improve aerobic capacity (Karstoft et al., 2014, Schrauwen-Hinderling et al., 2007, Ritov et al., 2010, Mootha et al., 2003), reduce metabolic syndrome associated risks including blood pressure (Mootha et al., 2003), insulin action and lipogenesis (Schrauwen-Hinderling et al., 2007) in wide range of clinical population (Batacan et al., 2017).

2.4.6.1. Effect of HIIT on Body composition

Although HIIT has been extensively studied in context to muscle physiology, limited studies have been carried out to evaluate the effect of HIIT in obese / overweight individuals. Weight loss with HIIT has been reported by several studies in overweight and obese humans (Earnest et al., 2013, Racil et al., 2013, Sartor et al., 2010, Tjonna et al., 2013, Whyte et al., 2013, Zhang et al., 2015, Fisher et al., 2015, Kemmler et al., 2014, Nybo et al., 2010, Shepherd et al., 2015, Martins et al., 2016, Sawyer et al., 2016, Sijie et al., 2012, Sim et al., 2015). Although several studies also reported non-significant change in body weight with HIIT after 5-12 weeks of training (Gillen et al., 2013, Keating et al., 2014, Kong et al., 2016, Sawyer et al., 2016), there are studies which reported weight loss ranging from 1% following 2 weeks of HIIT to 8.4% after 12 weeks of training (Sijie et al., 2012) in humans. There has been a huge variation among the data even within the studies reporting same study duration in overweight and obese individuals, for instance, seven studies have reported 12 weeks HIIT with change in body mass ranging from no change (Gillen et al., 2013, Keating et al., 2014) to up-to 8.4% weight loss (Earnest et al., 2013, Nybo et al., 2010, Sijie et al., 2012, Sim et al., 2015, Zhang et al., 2015). The difference in the results could be attributed to the difference in the HIIT protocol and also the ethnicity of the participants. Among animal studies very few studies have reported the effect of HIIT in obese animals. Two studies have reported 16% and 5% decline in weight

gain in comparison to sedentary control mice after 4 (Cho et al., 2015) and 6 weeks (Lund et al., 2015) of HIIT 5 days per week.

High intensity interval training has been reported to reduce fat mass (Wewege et al., 2017). Similar to change in body weight, the reduction to fat mass ranges from non-significant change reported after five weeks of HIIT (Kong et al., 2016) to 9.7% after 12 weeks of training (Zhang et al., 2015). Consistent with the weight loss, change in fat mass did not correlate with duration of study and studies with similar time duration reported wide range of difference in fat mass reduction. Several studies reporting 12-week intervention period reported fat mass decline ranging from 0.5% to 9.7% (Earnest et al., 2013, Nybo et al., 2010, Racil et al., 2013, Sijie et al., 2012, Sim et al., 2015, Zhang et al., 2015).

Even in the absence of change in body mass, HIIT has been reported to reduce waist circumference (Wewege et al., 2017). Reduction in waist circumference reported ranges from a 2.2% after 16 weeks of HIIT (Kemmler et al., 2014) to 5.8% after 12 weeks of training (Zhang et al., 2015). In addition to waist, reduction in hip circumference by 1.4cm (Martins et al., 2016) and 4.5cm (Zhang et al., 2015) has also been reported after 12 weeks of training. High intensity exercise has also been reported to reduce body mass index (BMI) (Fisher et al., 2015, Racil et al., 2013, Shepherd et al., 2015, Sijie et al., 2012, Sim et al., 2015, Zhang et al., 2015). Reduction in BMI has been reported by several but not all studies and it varies from no change (Kong et al., 2016, Sawyer et al., 2016) after 5-8 weeks of HIIT to a minimum of 0.86% (Fisher et al., 2015) to a maximum of 7.8% decline following 12 weeks of training .

2.4.6.2. Effect of HIIT on glucose homeostasis

Single session of HIIT. In comparison to no exercise has been reported to improve glucose homeostasis (Little et al., 2014). Some studies looking at the long term effect of HIIT reported no change (Kemmler et al., 2014, Racil et al., 2013, Shepherd et al., 2015, Tjonna et al., 2013), while others reported reduced fasting glucose (Tjonna et al., 2008, Fex et al., 2015, Jeppesen and Madsen, 2015, Tjonna et al., 2011, Earnest et al., 2013). Fasting glucose is primarily a marker of hepatic insulin sensitivity (Cassidy et al., 2017). The decline in fasting glucose following HIIT training is generally less than 14% (Fex et al., 2015, Mitranun et al., 2014, Tjonna et al., 2011, Jeppesen and Madsen, 2015, Tjonna et al., 2008), indicating that HIIT by itself, is not potent enough to trigger a significant change on fasting glucose. Although it has been shown that HIIT reduces liver fat but without any significant change in fasting glucose (Cassidy et al., 2016). High intensity interval training for 8 weeks has also been reported to reduce fasting glucose by 29% in high fat diet induced obese mice (Cho et al., 2015), however similar study on HIIT for 10 weeks reported non-significant change in fasting glucose in same animal model (Lund et al., 2015), which could be due to difference in the intensity of exercise.

High intensity interval training has also been reported to reduce fasting plasma insulin in both humans and animals. In humans, huge variation has been found between studies in context to change in plasma insulin. Some studies reported no change (Nybo et al., 2010, Sawyer et al., 2016) where as others have reported change varying from 8.1% to 27% (Karstoft et al., 2013). In addition to fasting insulin, some studies have also reported approximately 20% improvement in insulin sensitivity measures as HOMA-IR following HIIT (Mitranun et al., 2014, Jeppesen and Madsen,

2015, Tjonna et al., 2008, Earnest et al., 2013), whereas some studies have shown no improvement (Shaban et al., 2014, Hollekim-Strand et al., 2014). Likewise, other indicators of glucose homeostasis such as oral glucose tolerance test and glucose area under the curve also showed similar inconsistences among the literature. The major reasons behind these inconsistences are study populations, exercise protocols and the degree of volunteer supervision during exercise but the most plausible is the variation in time of post-intervention measures relative to the last bout of exercise (Cassidy et al., 2017).

2.4.6.3. Effect of HIIT on lipid profiles

High intensity interval training has reported to reduce blood lipids. There are several studies reporting no change in blood lipid profile after HIIT (Kong et al., 2016, Tjonna et al., 2013). Some studies have reported decline in TAG ranging from 2.2% to 11.8% after 6-16 weeks of HIIT (Fisher et al., 2015, Keating et al., 2014, Kemmler et al., 2014, Racil et al., 2013, Sawyer et al., 2016, Shepherd et al., 2015, Zhang et al., 2015). Besides TAG, HIIT has also been reported to reduce total cholesterol in humans (Fisher et al., 2015, Nybo et al., 2010, Racil et al., 2013, Shepherd et al., 2015, Zhang et al., 2015). Reduction in TAG, free fatty acids and total cholesterol following 8 weeks of HIIT has also been reported in high fat diet induced obese mice (Cho et al., 2015).

2.4.6.4. Molecular changes induced by HIIT

Skeletal muscle plays a major role in regulating metabolism and manifest number of changes following acute or chronic HIIT. GLUT-4 content and translocation to plasma membrane is important for glucose homeostasis (Huang and Czech, 2007). In humans, six sessions of HIIT resulted in 369% increase in GLUT-4 content in vastus lateralis muscle of type-2 diabetes patients (Little et al., 2011). Although low expression of

GLUT-4 is not related to insulin resistance, its higher expression is related to improved glucose uptake in the muscle (Ren et al., 1994). Consistent with this study, another 16 week HIIT study reported higher mRNA content of GLUT-4 and higher membrane bound GLUT-4 in type-2 diabetes patients, however there was no overall increase in total GLUT-4 protein content (Karstoft et al., 2014).

In addition to GLUT-4 content, HIIT has been found to increase mitochondrial content, function and markers of mitochondrial biogenesis, all of which have been found to be reduced in individuals with metabolic syndromes (Cassidy et al., 2017). Significant increase in mitochondrial capacity evidenced by an increase in citrate synthase activity and raised content of electron transport chain complexes have been found after 2 weeks of HIIT (90% maximum heart rate) in type-2 diabetes individuals (Little et al., 2011). However, another 16-weeks study reported only increase in citrate synthase mRNA content but not in citrate synthase activity after HIIT (70% VO_2 max) in type-2 diabetes individuals (Karstoft et al., 2014). This discrepancy in results could be due to lower intensity of intervals in this study and more over the muscle biopsies were taken 5–6 days post-training.

High intensity interval training has also been reported to increase both mRNA and protein content of PGC-1α in muscle, which regulates mitochondrial biogenesis (Wu et al., 1999, Little et al., 2010). It has been suggested that ATP turnover during HIIT, differs from usual steady state condition of ATP production and could be responsible for activating the signalling cascade that leads to increase in PGC-1α after HIIT (Daussin et al., 2008).

Calcium ion (Ca^{2+}) handling by sarcoplasmic reticulum plays an important role in muscle fatigue (Cassidy et al., 2017). Enhanced ca2+ reuptake has been reported

following HIIT but not moderate intensity interval training in individuals with metabolic syndrome (Tjonna et al., 2008). Increased ca2+ reuptake by 56-60% after HIIT significantly improve the work capacity of muscle and contributes to the improvement in fitness due to this type of exercise training. Although this study (Tjonna et al., 2008) concluded that HIIT provide more potent stimulus than moderate intensity exercise for skeletal muscle adaptations, definite conclusion cannot be drawn on the basis of single study with sample size of 32 participants and therefore needs further investigations.

CHAPTER 3

MATERIAL AND METHODS

3.1. OVERVIEW

This chapter describes general methods used in three studies presented in this thesis. The central aim of the thesis was to investigate the effect of combined IF and HIIT on changes in body composition, metabolic health and molecular changes in metabolically important tissue namely adipose and skeletal muscle in high fat and sugar diet induced obese mice. The first study is designed to investigate the anti-obesity effect of IF and HIIT and its combination and their effect on glucose homeostasis and blood lipid markers. The second and third study uses various molecular tools to investigate the changes at molecular level (mRNA and miRNA) in adipose and muscle tissue induced by IF, HIIT and combination of these two interventions. General layout of the study is given in figure 3.1. Details of methods specific to the three studies are described in the respective chapters.

3.2. ANIMALS

3.2.1 Procurements and Housing:

One hundred, 8-weeks old, C57BL/6 mice (Wt: 22.2±2.5g Males, 17.6±1.7g Females) were acquired from the Animal Resource Centre (Murdoch, WA, Australia) and housed in animal holding facility at Werribee campus, Victoria University. Mice were housed in groups of 5, at a constant temperature (22^{o}C), under a 12:12-h light-dark photoperiod with *ad libitum* access to food and water (other than fasting periods) in accordance with ethical approval obtained from the Victoria University Animal Ethics

Committee, conforming to the Australian code of practice for the care and use of animal for scientific purposes.

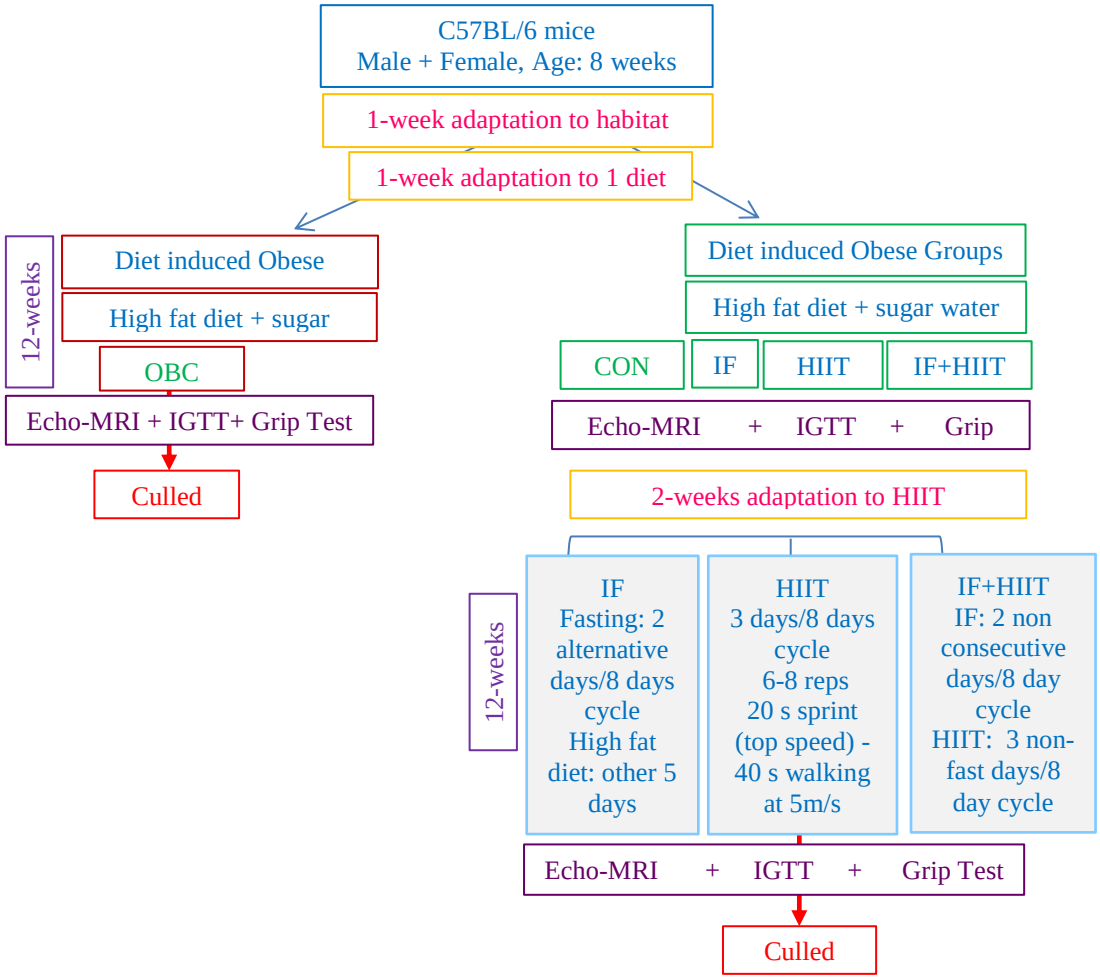

Figure 3.1. General outlay of the study.

3.2.2 Diet

Before starting the high fat diet, animals were given one week of adaptation to animal house and were fed mice chow (Figure 3.2(A)) (meat free rat and mouse diet, Specialty Feeds, Glen Forrest, WA, Australia) and potable water. Animals were gradually introduced to the special diets over a period of one week by increasing the proportion of

special diet mixed with chow, starting with 1:3 (high fat diet: chow diet) for first 2 days, followed by 1:1 for next 2 days and 3:1 for next 3 days and eventually moving to complete high fat diet. Diet induced obese group had *ad libitum* access to high fat diet (HF) (Figure 3.2(B)) (59% energy from fat, SF03-002, speciality feeds, WA, Australia) and high sugar (S) water (30% sugar (weight/volume)) for rest of the study period. Nutritional parameters of both high fat and chow diet are given in table 3.1

Figure 3.2. Animal diets-(A) chow diet and (B) High fat diet.

Table 3.1. Calculated nutritional parameters of diets

Nutritional Parameters	High fat diet (SF03-002)	Chow diet
Protein	19.4%	20%
Total Fat	36.0%	4.8%
Total Digestible Carbohydrate	-	59.4%
Crude Fibre	4.7%	4.8%
AD Fibre	4.7%	7.6%
Digestible energy	22.8 MJ/Kg	14.0 MJ/Kg
% Total calculated digestible energy from lipids	59.0%	12%
% Total calculated digestible energy from protein	15.0%	23%

After 12-weeks of HF/S feeding, mice were divided into five groups: obese baseline control (OBC; Males=8, Females=10), no intervention (CON; Males=7, Females=9); intermittent fasting (IF; Males=8, Females=10), high intensity intermittent exercise (HIIT; Males=8, Females=10) and a combination of the dietary and exercise intervention (IF+HIIT; Males=8, Females=10). Mice from OBC group were killed after 12 weeks of (HF/S) feeding period to establish baseline measures for some variables

compared to the remaining groups. The remaining mice were maintained on the HF/S diet for an additional 12 weeks. After 24 weeks of HF/S feeding and intervention, all remaining mice were culled and tested as described below. Eleven male mice died during the dietary and exercise intervention period, presumably due to the complications of diabetes as a result of the HF/S diet. One female mouse did not gain weight in the control group and was excluded from the study.

3.2.3 Assessment of Caloric Intake

Food and sugar water intake of animals from each cage was measured weekly and average caloric intake per animal per day was calculated. Due to housing constrains of ethical approval and to avoid added stress, animals from the non-fasting groups (control and HIIT) were housed together and animals from fasting groups (IF and IF+HIIT) were housed together. The average caloric intake of the non-fasting groups (control and HIIT animals) was compared to fasting groups (IF and IF + HIIT animals) to determine if intermittent fasting resulted in over compensation of food intake on non-fasting days over the 12-weeks intervention period.

3.3. INTERVENTIONS

All intervention groups underwent an 8-day cycle instead of the 7-day weekly cycle. This was designed to give at least one day break between fasting day and exercise day for combination (IF+HIIT) group and ensure animal wellbeing. To ensure uniformity between the groups, IF and HIIT groups underwent the same 8-day cycle. However, the total period of interventions was restricted to 12 calendar weeks.

3.3.1 Intermittent Fasting Protocol

Mice from IF and IF+HIIT group were deprived of food on two alternate days of an eight day cycle for 12-weeks. During the fasting period, mice were moved to clean

cages with potable water available *ad libitum* and returned to their original cages 24 hours later. On non-fasting days, mice had *ad libitum* access to the HF/S diet (Castello et al., 2010, Varady et al., 2007). Mice were always fasted on day 2 and 7 of the 8-day cycle.

3.3.2 High Intensity Interval Training Protocol

3.3.2.1. Acclimatization to treadmill exercise

All groups were acclimatized to treadmill running through five gradual treadmill training familiarization sessions over a period of 2 weeks prior to intervention commencement according to standard operating procedures for treadmill exercise and Victoria university animal ethics guidelines. For the first two acclimatization sessions, mouse was placed on the treadmill (Panlab, Barcelona, Spain) (Figure 3.3) for 5 min. The treadmill was then turned on at 3cm/sec for 5 min followed by gradual increase in speed over next 5 minutes from 3cm/sec to 12cm/sec. For third and fourth acclimatization sessions, treadmill speed was gradually increased over 5 minutes from 3cm/sec to 12cm/sec, followed by gradual increase in speed over next 10 minutes from 12cm/sec to 20cm/sec. During the final session, speed was gradually increased over 15 minutes from 3cm/sec to 25cm/sec. To minimise the effects of the training adaptation during the acclimatization sessions, all mice, irrespective of their group, underwent the 2-week familiarisation training.

Figure 3.3. Mice treadmill (A), Treadmill control (B), mouse on treadmill (C).

3.3.2.2. Top Speed Test

The protocol for top speed determination was as follows: mice performed an incremental warm up period for 5 min reaching 8 cm/sec on mice treadmill. The speed was slowly increased from 8 to 35 cm/sec and was held at 35 cm/sec for 20 sec and then returned back to 8 cm/sec for 40 sec. Following this, the speed was slowly increased to 40 cm/sec and maintained for 20 sec followed by another 40 sec of active rest at 8 cm/sec. The 20 sec running and 40 sec active rest protocol was repeated with increasing running speed by 5 cm/sec until the mouse was unable to continue running. The running speed prior to this was recorded as the top speed. The average top speed for all mice was 59±2 cm/sec.

3.3.2.3. HIIT Training

The HIIT protocol was a slightly modified protocol used by Tuazon et al. (2015) with the relatively low volume and high intensity shown to have positive effects on body composition and metabolic health (Lund et al., 2015). Briefly, the HIIT training sessions consisted of a 5 min warm up period at 8 cm/sec, followed by 6-8, 20 sec bouts of treadmill running at the previously determined top speed of the individual animal, interspersed by 40 sec active rest at 8 cm/s. To implement overload, the number of intervals was increased from 6 at the beginning of the training intervention to 8 at the end of the eighth HIIT session. HIIT was carried out on three non-consecutive days per 8-day cycle. To encourage the mice to run on the treadmill at their maximum speed, an air puff device was used if the mice appeared to slow down.

3.4. Body Weight and Composition Assessment

Body weight was measured weekly on laboratory weighing balance. Body composition (fat and lean mass) was determined using EchoMRI (EchoMRI- body composition analyser, Echo Medical Systems, Houston, USA) (Figure 3.4) before and after 10-weeks of diet and/or exercise intervention. Briefly, machine was calibrated with canola oil. Once calibrated, mouse was inserted into the mouse tunnel (Figure 3.4 (B)) which was then placed into the EchoMRI machine. The measurement was then started through a computer software attached to the machine. The body composition (Fat mass, lean mass and water) appears on the screen in the form of a table. Once the measurement is complete, mouse was placed back into the cage.

Figure 3.4. EchoMRI machine (A) and mouse tunnel (B).

3.5. CULLING AND TISSUE COLLECTION

To minimise any acute transient effects from either fasting and exercise treatment (Joslin et al., 2017), a period of 48 h was given between the last fasting period and exercise session and any physiological measurement or sample collection for all animals. Mice were anaesthetised with intraperitoneal injection of pentobarbitone (60 mg/kg). Once the mouse was fast asleep, extensor digitorum longus (EDL) muscle from the left leg and both soleus and EDL muscle from right leg were removed surgically. Both muscles from right leg were preserved in optimal cutting temperature (OCT) compound for histological analysis and EDL muscles from left leg were snap frozen in liquid nitrogen and stored in

-80°C freezer until further analysis. After muscle collection, the blood was collected by cardiac puncture and plasma was prepared using EDTA as anticoagulant and centrifugation at 2000g for 15 min. The abdominal cavity was opened and epididymal and periovarian adipose tissue was collected and snap frozen in liquid nitrogen and stored in -80°C freezer until further analysis. Liver, pancreas, heart, kidneys and spleen were removed and weighed on a laboratory scale.

3.6. ISOLATION OF RNA

Total RNA for mRNA and miRNA expression from adipose and muscle tissue was extracted using miRNeasy mini kit (Qiagen). Sample (adipose (100mg)/muscle (10mg)) was homogenised with 700µl of Qiazol lysis reagent in TissueLyser (Qiagen) for 5 min at 50Hz and incubated at room temperature for 5 min. To this homogenate 140µl of chloroform was added and vortexed for 15 sec followed by incubation at room temperature for 2-3 min. The homogenates were centrifuged at 12000g for 15 min at 4°C. Following centrifugation, the upper aqueous phase was transferred to a new collection tube and 525µl of ethanol was added to it and mixed thoroughly. Up to 700µl of this mixture was transferred to RNeasy mini column in a 2ml collection tube followed by centrifugation at 8000g for 15sec at room temperature and flow-through was discarded. This step was repeated with the rest of the mixture and flow- through was again discarded. To the column, 700µl of RWT buffer was added followed by centrifugation at 8000g for 15s at room temperature and flow-through was discarded. After this, 500µl of RPE buffer was added to the column and centrifuged at 8000g for 15s at room temperature and flow-through was discarded. This step was repeated once more. The column was then placed in a fresh 1.5ml collection tube and 30µl of RNase free water was added to the column followed by centrifugation at 8000g for 1 minute at room temperature. This step was

repeated with same collection tube. The RNA content was measured using nano-drop spectrophotometer (DeNovix DS-11, DeNovix, USA). RNA extract was aliquoted and stored in -80°C freezer until further analysis.

3.6.1 Assessment of RNA quality

Agarose gel (1%) was prepared in Tris-acetate-EDTA (TAE) buffer (1X), mixed with SYBR™ Green II RNA gel stain (Invitrogen) and casted in a horizontal gel casting tray with a 15 well comb. RNA (400ng from adipose tissue or 300ng from muscle), mixed with loading dye in 3:1 ratio, were heated at 70°C for 10 min and cooled down on ice for 3 min. Agarose gel was placed in a horizontal gel running tank and filled with TAE buffer (1X). Samples (RNA + loading dye) were loaded into wells in duplicate and gel was run at 80 volts for 45 min. After this, gel was taken out of the gel tray and imaged in gel image and analysis system (ChemiDoc XRS system, Biorad). The 28s and 18s rRNA band ratio was calculated for each sample and ratio between 1.3 to 2 was taken as measure for good RNA quality. RNA extraction was repeated for samples with 28s:18s band ratio less than 1.3. A representative image of RNA agarose gel is shown in Figure 3.5.

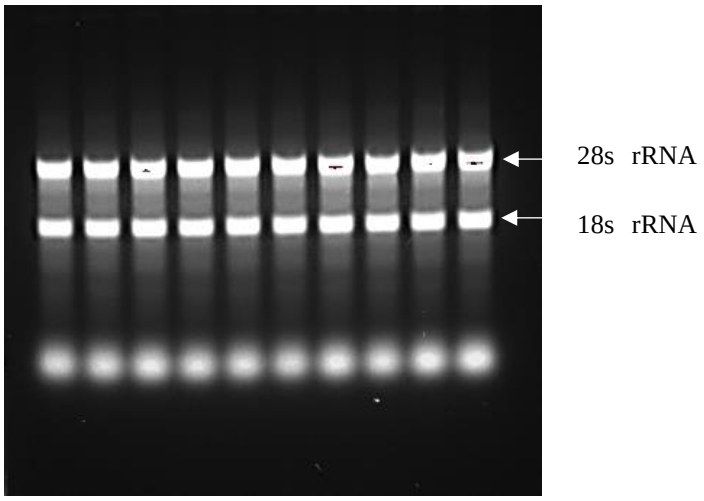

Figure 3.5. Representative image of agarose gel showing two rRNA bands.

3.7. MRNA REVERSE TRANSCRIPTION AND ASSESSMENT OF GENE EXPRESSION

3.7.1 Synthesis of cDNA-reverse transcription

RT^2 first strand kit (Qiagen) was used to synthesize complementary deoxyribonucleic acid (cDNA). RNA sample (400ng), mixed with genomic DNA elimination buffer (2µl) and water (to make up the volume to 10µl) in a 1.5ml tube, was incubated at 42°C for 5 min and immediately placed on ice for 1 minute. To each tube, 10µl of reverse transcription mix (5X buffer BC3, control P2, RE3 reverse transcriptase mix and nuclease free water in a ratio of 4:1:2:3µl) was added and mixed with pipetting up and down. The tubes were incubated at 42°C for exactly 15 min and the reaction was stopped immediately by incubating at 95°C for 5 min. The tubes were then transferred to ice and 91µl of water was added to each tube and mixed properly. The cDNA samples were aliquoted and stored in -20°C freezer until further analysis.

3.7.2 Gene expression

Gene expression was determined using Custom RT^2 PCR array (Qiagen). Each column of 384- well plate consist of 11 genes of interest, 2 housekeeping genes, one genomic DNA control, one positive PCR control and one reverse transcription control. Primers for both genes of interest and housekeeping genes were synthesized by the manufacturer (Qiagen) based on the list and gene symbols supplied by us. Details of genes and primers are provided separately for adipose and muscle tissue in chapter 5 and 6 respectively. Primers were absorbed on the bottom of 384 well optical micro-plate with one primer pair per well. To each well, 10µl PCR mix (RT^2 SYBR green master mix with ROX (passive normalization dye), cDNA and water in a ratio of 5:0.8:4.2µl) was added. The plate was sealed with optical adhesive film and centrifuged at 1000g for 1 min to

remove bubbles. The plate was placed in real time thermal cycler (QuantStudio 7 Flex Real-Time PCR System) with cycling conditions consisting of 1 cycle of 10 min at 95°C to activate *Taq* polymerase and 40 cycles of 15 sec at 95°C for denaturation and 1 minute at 60°C for annealing. The ramp rate was adjusted at 1°C/sec. The fluorescence was measured after each cycle and baseline was defined using automated baseline option in thermal cycler. Cycle threshold (C_T) values generated by the thermal cycler were exported to excel file and change in gene expression expressed as fold change was calculated as:

$\Delta C_T = C_{T(\text{gene of interest})} - C_{T(\text{Housekeeping gene})}$

$\Delta\Delta C_T = \Delta C_{T(\text{intervention})} - \Delta C_{T(\text{control})}$

Fold change = $2^{-\Delta\Delta CT}$

3.8. MiRNA REVERSE TRANSCRIPTION AND ASSESSMENT OF MiRNA EXPRESSION

3.8.1 Synthesis of cDNA-reverse transcription

MiScript II RT kit (Qiagen) was used to synthesize cDNA. RNA sample (250ng) was mixed with 4µl of 5x miScript HiSpec Buffer, 2µl of 10x miScript Nucleics mix, 2µl of miScript reverse transcriptase mix and water (to make up the volume to 20µl) in a 1.5ml tube. The tubes were incubated at 37°C for exactly 60 min and the reaction was stopped immediately by incubating at 95°C for 5 min. The tubes were then transferred to ice and 200µl of water was added to each tube and mixed properly. The cDNA samples were aliquoted and stored in -20°C freezer until further analysis.

3.8.2 MiRNA expression

MiRNA expression was determined using Custom RT^2 PCR array (Qiagen). Each column of 384- well plate consist of 5 adipose tissue miRNAs of interest, 3 skeletal muscle miRNAs of interest, 2 housekeeping miRNAs for each tissue, one positive PCR

control and one reverse transcription control for each tissue. Primers for both, miRNAs of interest and housekeeping miRNAs were synthesized by the manufacturer (Qiagen) based on the list and gene symbols supplied by us. Details of miRNAs and primers are provided separately for adipose and muscle tissue in chapter 5 and 6 respectively. Primers were absorbed on the bottom of 384 well optical micro-plate with one primer pair per well. To each well, 10µl PCR mix (2x QuantiTech SYBR Green PCR master mix, 10x miScript Universal Primer, RNAse free water and template cDNA, in a ratio of 5.5:1.1:3.4:1.0µl) was added. The plate was sealed with optical adhesive film and centrifuged at 1000g for 2 min to remove bubbles. The plate was placed in real time thermal cycler (QuantStudio 7 Flex Real-Time PCR System) with cycling conditions consisting of 1 cycle of 15 min at 95°C to activate HotStarTaq DNA polymerase and 40 cycles of 15 sec at 94°C for denaturation, 30 sec at 55°C for annealing and 30 sec at 70°C for extension. The ramp rate was adjusted at 1°C/sec. The fluorescence was measured after each cycle and baseline was defined using automated baseline option in thermal cycler. Cycle threshold (C_T) values generated by the thermal cycler were exported to excel file and change in miRNA expression expressed as fold change was calculated as:

$$\Delta C_T = C_{T(\text{miRNA of interest})} - C_{T(\text{Housekeeping miRNA})}$$

$$\Delta\Delta C_T = \Delta C_{T(\text{intervention})} - \Delta C_{T(\text{control})}$$

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

3.9. STATISTICAL ANALYSIS

Data is presented as mean ± standard deviation (SD) in tables and figures presented. Details of specific statistical analysis are given in individual chapters.

CHAPTER 4

INTERMITTENT FASTING WITH OR WITHOUT EXERCISE PREVENTS WEIGHT GAIN AND IMPROVES LIPIDS IN DIET-INDUCED OBESE MICE

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4.1. INTRODUCTION

Obesity is a complex multifaceted disease resulting from the interplay between genetics and lifestyle, including economic growth, modernization, and urbanization (van Vliet-Ostaptchouk et al., 2012). The rapid rise in obesity prevalence appears to be a reflection of the changes in dietary and behavioural patterns, with eating habits shifting to greater consumption of energy-dense foods that are high in fats and sugars, while at the same time, levels of physical activity are decreasing. These differences in energy intake and expenditure, often referred to as energy balance, have direct implications for weight regulation, with even small deviations in daily energy balance resulting in large body weight changes over the long term (Romieu et al., 2017). Interventions that target and reverse these small deviations in energy balance may be an effective tool in reducing body weight and adiposity, but also help maintain a stable body weight over the longer period.

Diet and exercise interventions aiming to shift the energy balance towards negative by either decreasing calorie intake and/or increasing physical activity have shown to be effective for weight loss (Strasser et al., 2007). Many iterations of such dietary and physical activity interventions have been proposed, but intermittent fasting (IF) and high intensity interval training (HIIT) have recently been purported as effective strategies (Türk et al., 2017, Tinsley and La Bounty, 2015). Intermittent fasting or alternate day fasting (ADF) is a dietary regime that refers to short periods of intense energy restriction (75-100% reduced caloric intake on fasting day) followed by 'normal' eating on non-fasting days (Barnosky et al., 2014, Heilbronn et al., 2005b). Studies have shown that short to long term intermittent fasting can reduce body weight and fat mass, fasting glucose and insulin levels, and improve insulin sensitivity and lipid profiles (Barnosky et al., 2014, Azevedo et al., 2013). Although calorie restriction (CR) and IF demonstrate comparable benefits on body composition and other health markers, adherence to IF is typically greater compared to the conventional daily CR diets and thus could be more effective over the longer term (Azevedo et al., 2013).

High intensity interval training is promoted as a superior and time efficient method for reducing body and fat mass and other biomarkers of chronic diseases compared to moderate-intensity continuous training. HIIT refers to brief bouts of vigorous intensities (80-90% VO2 max) interspersed by relatively longer bouts of low intensity active recovery or passive recovery (Gibala et al., 2012, Trapp et al., 2008). The effectiveness of HIIT is its ability to produce rapid physiological adaptations that may promote greater energy deficit post-exercise, independent of the changes in total physical activity energy expenditure. Our lab previously reported higher plasma hypoxanthine accumulation and urinary purine base excretion (indirect markers of energy loss from the

muscle) following high intensity exercise compared to continuous exercise (Gerber et al., 2014). These losses could indicate the need for greater energy input for subsequent restorative processes (via intramuscular purine *de novo* replacement) during the recovery period and may in part contribute to the negative energy balance that leads to fat loss following long term high intensity training programs (Racil et al., 2013, Earnest et al., 2013). Our findings and others (Gillen et al., 2013, Sartor et al., 2010) have suggested that training programs that include high intensity and low volume exercise could be effective at creating metabolic disturbances that can result in enhanced weight loss in the form of reduced adipose tissue, but also promote health and fitness benefits normally seen over the longer term.

Manipulating the energy balance equation through exercise and diet are effective strategies to improve body composition and manage lifestyle-related metabolic diseases. However, protocols that are characterised by severe energy restriction or large energy expenditure such as IF and HIIT, respectively, may induce greater physiological changes and/or further stimulate already maximal adaptation rates, independent of changes in energy balance. The current study was designed to investigate the effect of IF and HIIT, alone and in combination, on anthropometric and metabolic health parameters in a model of diet-induced obese mice. We hypothesised that, the combined interventions will result greater prevention of weight gain in the form of fat mass, preserving lean muscle mass despite IF, reductions in fasting glucose levels and markers of insulin sensitivity and glucose tolerance, and improvement in lipid profile compared to either dietary and exercise intervention alone.

4.2. MATERIALS AND METHODS

4.2.1 Animals

Details of animal procurement, housing and diet are mentioned in chapter 3 section 3.2. This study compared five different groups namely: obese baseline control (OBC; Males=8, Females=10), no intervention control (CON; Males=7, Females=9); intermittent fasting (IF; Males=8, Females=10), high intensity intermittent exercise (HIIT; Males=8, Females=10) and a combination of the dietary and exercise intervention (IF+HIIT; Males=8, Females=10).

4.2.2 Assessment of Caloric Intake

Food and sugar water intake of animals from each cage was measured weekly using a laboratory scale and average caloric intake per animal per day was calculated. Due to ethical approval, animals from the control and HIIT groups were housed together and animals from IF and IF+HIIT groups were housed together. The average calorie intake from the CON + HIIT animal ("non-fasting" group) was compared to IF and IF+HIIT animals ("fasting" group) to determine if IF resulted in over compensation of food intake on non-fasting days over the 12-week intervention period.

4.2.3 Interventions

Details of interventions are given in chapter 3, section 3.4.

4.2.4 Body Weight and Composition Assessment

Details of body composition measurement using Echo-MRI are given in chapter 3, section 3.5

4.2.5 Assessment of Glucose Tolerance (AUC)

Fasting blood glucose and glucose tolerance was analysed before and after 10-weeks of diet and/or exercise intervention. Last measurement was done at week-10 instead of week-12 (experimental endpoint), to give a period of 2 weeks between the intraperitoneal glucose tolerance test (IGTT) and culling, since it was found in the preliminary studies that mice show distress after IGTT and take few days to recover. A day before IGTT, body weight and body composition was measured. At 7pm animals are transferred to new cages with potable water available *ad libitum*. Next morning, at 7am, Fasting glucose was measured using handheld glucometer (Accuckeck GO glucometer) from blood collected by snipping the tail tip (1-2mm) following an overnight fast (12hrs). Mice were given intraperitoneal injection of glucose (1.5g/kg body weight) using an insulin syringe (BD ultra-fine, 1ml, 29G) and blood was collected by massaging the tail from base towards the tip and reading was taken at 5, 15, 30, 45, 60, 90 and 120 min following the glucose administration. Glucose tolerance was calculated as glucose area under the curve (AUC) using trapezoidal method (Andrikopoulos et al., 2008).

4.2.6 Blood and organ collection

Mice were anaesthetised with intraperitoneal injection of pentobarbitone (60 mg/kg). Once the mouse was fast asleep, extensor digitorum longus (EDL) and soleus muscle from right leg were removed surgically and weighed. Blood for plasma measurements was collected by cardiac puncture. Plasma was prepared using EDTA as anticoagulant and centrifugation at 2000g for 15 min. The abdominal cavity was opened and liver, pancreas, heart, kidneys and spleen were removed and weighed on a laboratory scale. Details are given in chapter 3, section 3.7.

4.2.7 Assessment of Plasma Insulin, TAG, LDL and HDL Levels

4.2.7.1. Plasma insulin

Plasma insulin was estimated using Ultra-sensitive mouse insulin ELISA kit (Crystal Chem, Inc, IL, USA). Frozen plasma samples were thawed on ice. To anti-insulin antibody-coated microplate, 95µl of sample diluent was added followed by 5µl of plasma sample or standard insulin (0.1 to 12.8ng/ml) in duplicate. The plate was covered with the cover and incubated for 2 hours at 4°C. After 2 hours, all the contents of the well were aspirated and plate was washed five times with wash buffer. After washing, 100µl of horse radish peroxidase-conjugated anti-insulin antibody was added. The plate was covered with the cover and incubated at room temperature. After 30 min, all the contents of the well were aspirated and plate was washed seven times with wash buffer. Immediately after washing, 100µl of the 3, 3´, 5, 5´-tetramethylbenzidine substrate solution was added and plate was incubated at room temperature for 40 min in dark. Enzyme reaction was stopped by adding 100µl of enzyme reaction stop solution. The absorbance was measure at 450nm and subtracted from absorbance at 630nm using a plate reader (SpectraMax i3x). Insulin concentration was calculated using the standard curve.

4.2.7.2. Triglycerides

Plasma TAG concentration was measured using colorimetric quantification assay kit (ABCAM, Cambridge, Massachusetts). To 96 well microplate, 50µl of TAG standard (2-10nmol) or sample (3µl sample + 47µl assay buffer) was added in duplicate. To each well, 2µl of lipase enzyme was added. Plate was incubated at room temperature for 20 min to convert triglycerides to glycerol and fatty acid. This was followed by addition of 50µl of reaction mixture (buffer, triglyceride probe and enzyme mix in a ratio of 46:2:2) and incubating the plate for 60 min at room temperature in dark. After 60 min, absorbance

was measured at 570nm using a plate reader (SpectraMax i3x). TAG concentration was calculated using the standard curve.

4.2.7.3. Low density lipoproteins

Plasma LDL concentration was measured using mouse LDL-cholesterol kit (Crystal Chem, Inc, IL, USA). To a 96 well microplate, 3µl Plasma sample or calibrator (0.53µg to 3.18µg) or blank was added in duplicate followed by addition of 225µl of reagent 1 to each well. The plate was covered and incubated at room temperature for 5 min followed by absorbance at 600nm. After this, 75µl of reagent 2 was added to each well. The plate was covered and incubated at room temperature for 5 min followed by absorbance at 600nm. The LDL concentration was calculated using the standard curve.

4.2.7.4. High density lipoproteins

Plasma HDL concentration was measured using mouse LDL-cholesterol kit (Crystal Chem, Inc, IL, USA). To a 96 well microplate, 3µl Plasma sample or calibrator (0.29µg to 1.78µg) or blank was added in duplicate followed by addition of 225µl of reagent 1 to each well. The plate was covered and incubated at room temperature for 5 min followed by absorbance at 600nm. After this, 75µl of reagent 2 was added to each well. The plate was covered and incubated at room temperature for 5 min followed by absorbance at 600nm. The HDL concentration was calculated using the standard curve.

4.2.8 Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

The homeostatic model assessment of insulin resistance (HOMA-IR) was determined as fasting insulin [mU/L] x fasting glucose [mmol/L])/22.5 (Seimon et al., 2016). The plasma insulin (ng/ml) was measured as stated above and the units were converted to mU/L by dividing the values by 0.0347. Plasma glucose was measured by handheld glucometer (Abbott Freestyle Optium).

4.2.9 Assessment of Muscle Strength

Muscle strength was measured before and after 10-weeks of diet and/or exercise intervention using a grip strength test as previously described by Deacon (2013). Briefly, a grip test apparatus was used which consisted of a stainless steel wire ball connected to a series of steel chain links (Figure 4.1), varying from one (15g) to six (74g). Mouse was held by the base of its tail and allowed to grasp the grip test apparatus lying on a lab bench with its forepaws. Mouse was lifted until the apparatus was raised from the bench. Holding the apparatus at a specific weight for 3 sec on 5 consecutive occasions was deemed a successful lift and the mice would proceed to next heavier weight. If the mice failed to achieve the 3 sec target for 5 consecutive times, the trial was stopped and mouse was assigned the weight/time achieved. A 30 sec rest between lifts was used. The final score was calculated by the number of links (1 to 6) in the heaviest weight held for 3 sec multiplied by time i.e. 3, plus the number of sec heaviest weight was held for less than 3 sec. To calculate absolute muscle strength, the total score was divided by the respective muscle mass. This method was adopted to measure the changes in muscle strength rather than the inverted screen test often used for testing grip strength which is affected by total body weight.

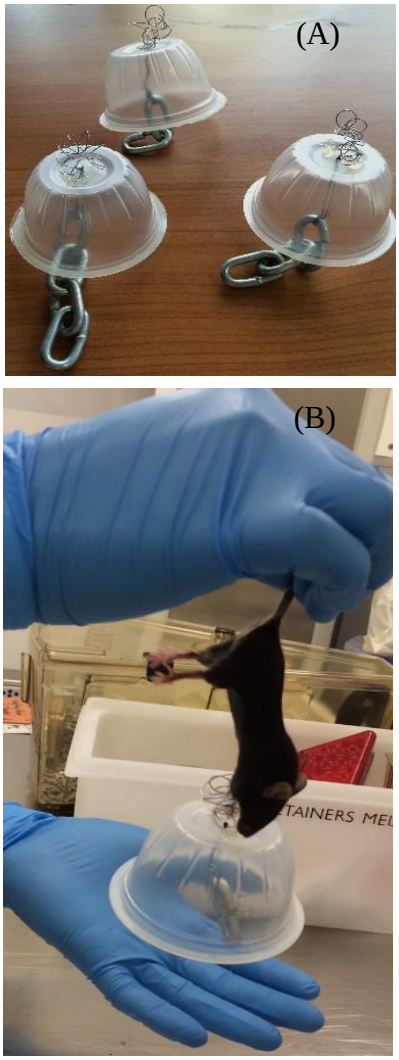

Figure 4.1. Grip strength apparatus (A) mouse performing grip test (B).

4.2.10 Statistical analysis

Data is presented as mean ± standard deviation (SD). Delta changes (pre- and post-intervention) in body weight and body composition (fat mass and lean mass), fasting blood glucose, glucose tolerance and muscle strength were analysed using 4 (CON, IF, HIIT, IF+HIIT) × 2 (0 and 10-weeks) repeated measures analysis of variance (ANOVA) with Tukey's post hoc analysis. Since baseline measurements of plasma insulin, glucose and LDL, HDL and TAG levels were not taken for each intervention group, analysis of delta changes for plasma insulin, HOMA-IR and lipid panels were performed using the mean average of group 1 (HF/S 'obese' control values) as the "baseline" minus the post-intervention values of the respective intervention groups. These delta values were analysed using 4 (CON, IF, HIIT, IF+HIIT) × 2 (0 and 12-weeks) repeated measures ANOVA with Tukey's post hoc analysis. One way ANOVA with Tukey's post hoc analysis was also performed on plasma insulin, HOMA-IR, lipid panels and organ weights at 12-weeks. Baseline variables and average caloric intake between groups was analysed using an independent students' t-test. An alpha level of 0.05 was adopted throughout to reduce Type I statistical errors.

4.3. RESULTS

4.3.1 Effects of 12-weeks of the HF/S diet on body weight of all animals

A significant increase in body weight was observed in male and female mice following the initial 12-weeks consumption of the HF/S diet [Male – Baseline: 22.2±2.5g, 12-week: 36.2±2.1g (~63%↑); Female- Baseline: 17.6±1.7g, 12-week: 28.5±1.5g (~62%↑)] (Figure 4.2).

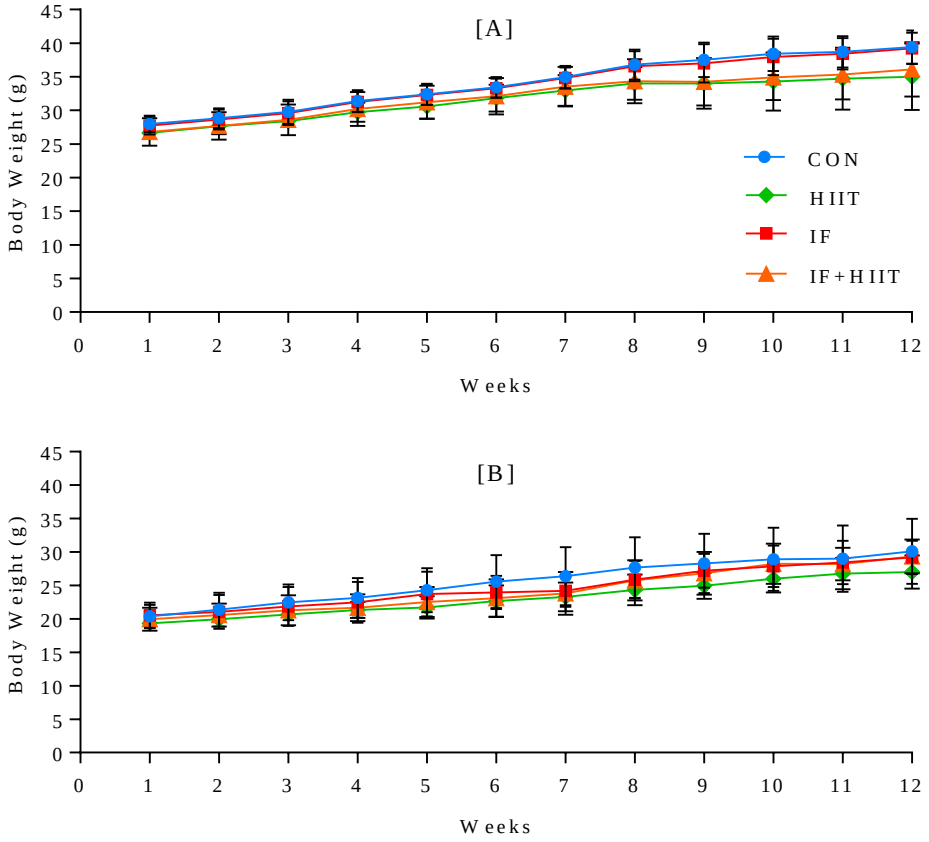

Figure 4.2. Weight gain (g) over the 12-weeks high fat/sugar diet feeding period in (A) male and (B) females. Data is presented as mean of each group and error bars represent standard deviation.

4.3.2 Effects of IF and/or HIIT on body weight and composition

Body weight and composition measured before and after 10-weeks of diet and/or exercise intervention are presented in Figure 4.3 and 4.4.

4.3.2.1. Body weight and fat mass

Following the initial 12-weeks of HF/S diet, body weight and fat mass continued to significantly increase over time in the CON and HIIT groups for both male and female mice ($p<0.05$), and IF female group only ($p<0.05$). After 10-weeks of the diet and/or exercise intervention, male mice displayed significantly lower body weights and reduced fat mass in the IF and IF+HIIT groups compared to the CON ($p<0.05$) and HIIT groups ($p<0.05$) (Figure 4.3(A) and 4.4(A)). In the female mice, the IF + HIIT group displayed significantly lower body weights and reduced fat mass compared to CON ($p<0.05$), IF ($p<0.05$) and HIIT groups ($p<0.05$) (Figure 4.3(B) and 4.4(B)).

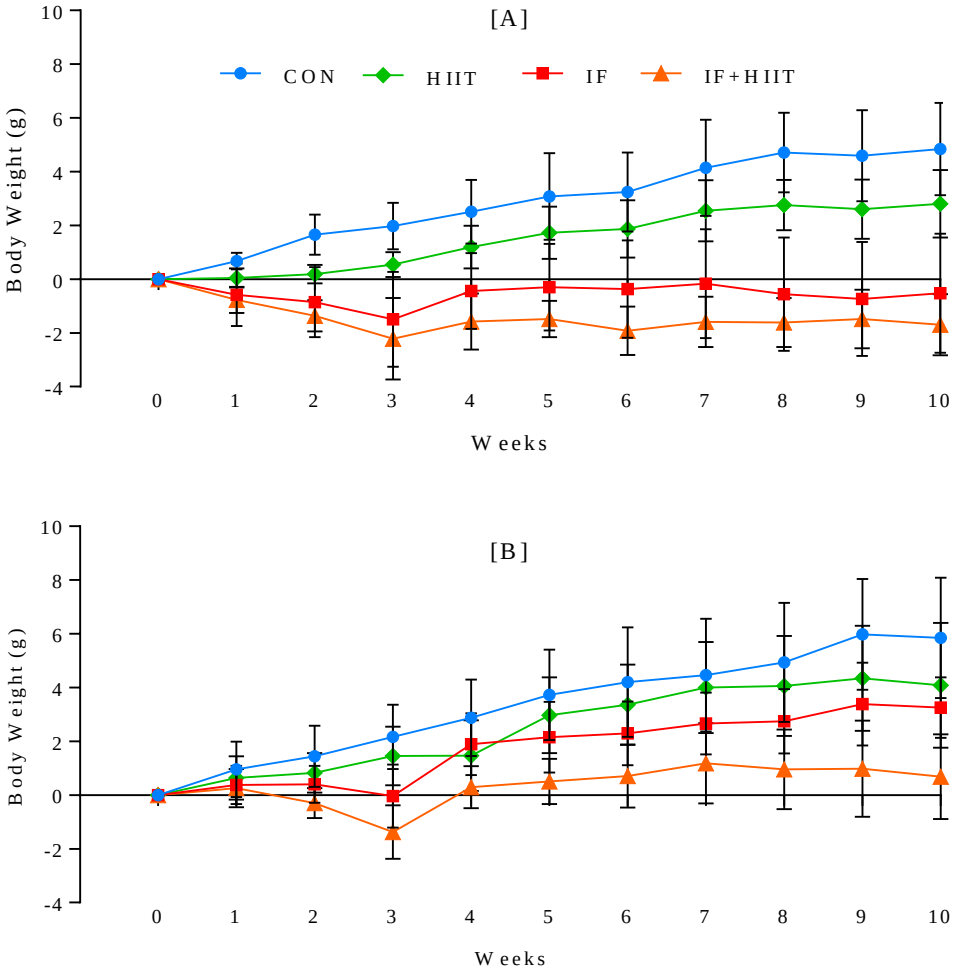

Figure 4.3. Change in body weight over the 10-weeks intervention period in (A) male and (B) females. Data is presented as mean of each group and error bars represent standard deviation.

4.3.2.2. Lean mass

Lean mass significantly increased over the 10-weeks diet and/or exercise intervention period in the CON group for both male and female mice ($p<0.05$) and in the HIIT and IF+HIIT female groups only ($p<0.05$) (Figure 4.4A and 4.4B). No significant differences between intervention groups were observed at the end of the diet and/or exercise intervention period.

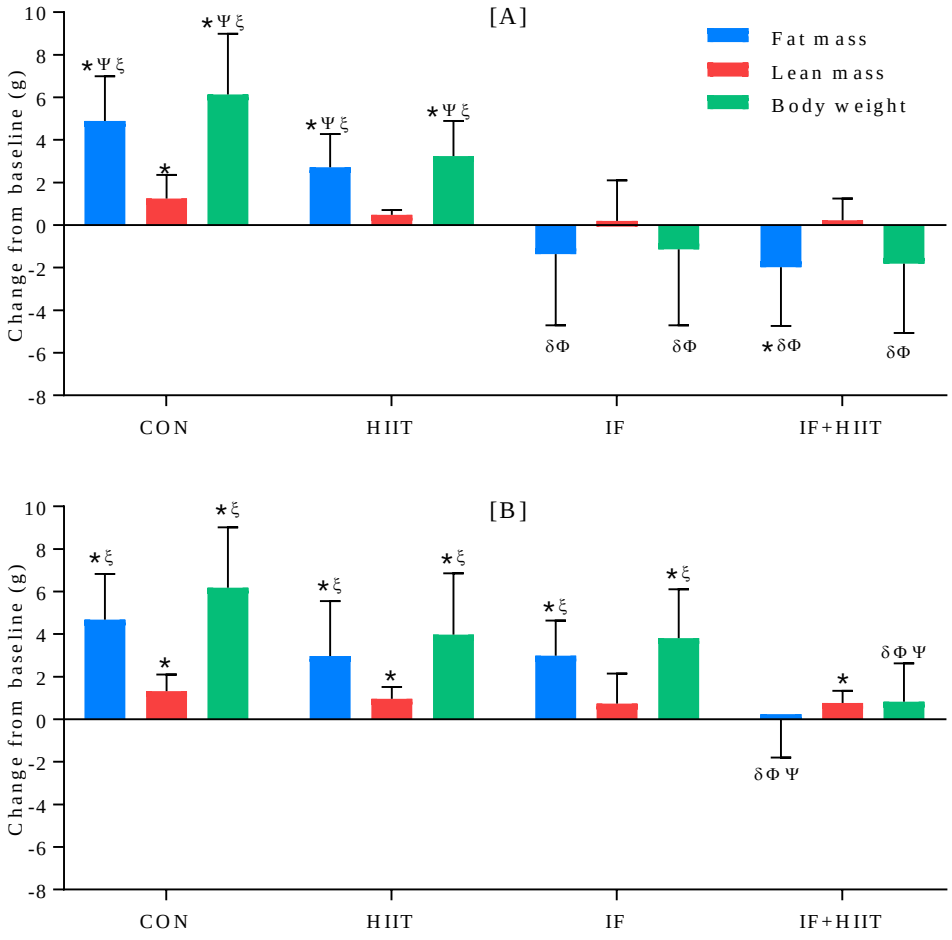

Figure 4.4. Change in body composition (g) in males (A), Females (B), after 10-weeks of intervention. Bars indicate means and error bars represent standard deviation. * significant change over time, δ significantly different from CON, Φ significantly different from HIIT, Ψ significantly different from IF, ξ significantly different from IF+HIIT.

4.3.3 Effects of IF on caloric intake

No significant differences were observed in average caloric intake during the intervention period between the fasting groups (CON and HIIT) and non-fasting groups (IF and IF+HIIT) in both male (14.63±1.47 kcal/mouse/day versus 13.23±1.62 kcal/mouse/day, $p<0.05$) and female mice (11.93±1.42 kcal/mouse/day n versus 11.43±1.58 kcal/mouse/day, $p<0.05$).

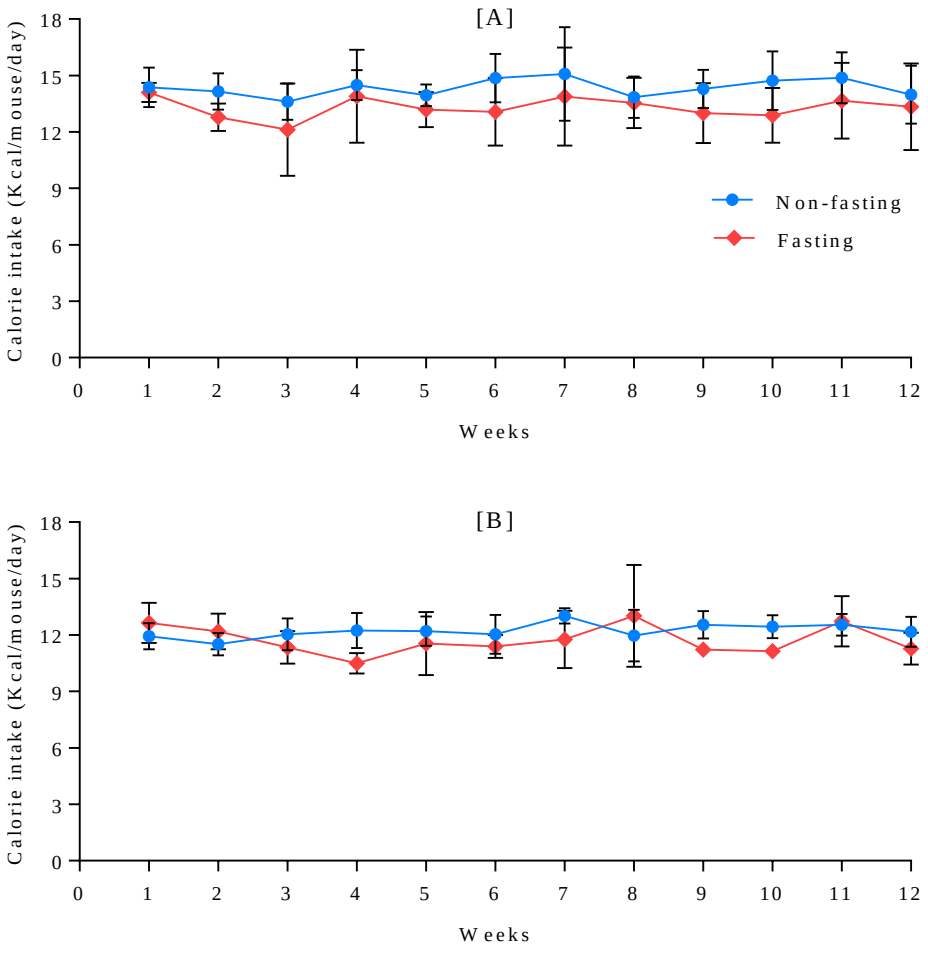

Figure 4.5. Caloric intake over the 12-weeks intervention period in (A) males and (B) females. Data is presented as mean of each group and error bars represent standard deviation.

4.3.4 Effect of IF and/or HIIT on blood glucose, plasma insulin, HOMA-IR and AUC

4.3.4.1. Fasting blood glucose

Fasting blood glucose levels significantly increased in the CON and IF male groups over the 10-weeks diet and/or exercise intervention period ($p<0.05$, Figure 4.6 (A)). At the end of the 10-weeks diet and/or exercise intervention period, the IF group for both male and female mice displayed significantly higher fasting glucose levels compared to the other 3 groups ($p<0.05$).

4.3.4.2. Plasma Insulin

Plasma insulin levels significantly increased over the 12-weeks diet and/or exercise intervention period in both male and female IF groups ($p<0.05$) and female CON ($p<0.05$) and IF+HIIT ($p<0.01$) groups only (Figure 4.6 (B)). No significant differences between groups were observed at the end of the diet and/or exercise intervention period.

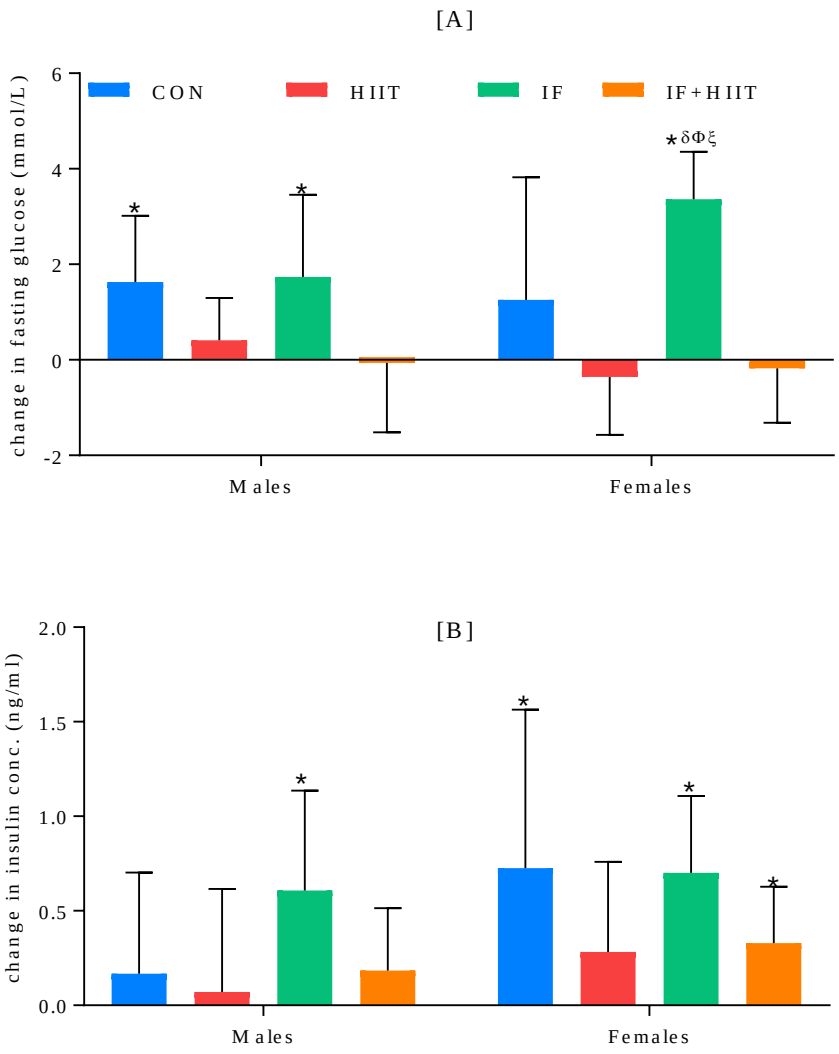

Figure 4.6. Change in (A) fasting glucose and (B) insulin over the 10 or 12-weeks period in males and females. Bars represent means, error bars represent standard deviation. * significant change over time, δ significantly different from CON, Φ significantly different from HIIT, Ψ significantly different from IF, ξ significantly different from IF+HIIT.

4.3.5 Insulin Resistance (HOMA-IR) and Glucose Tolerance (AUC)

HOMA-IR, a marker of insulin resistance significantly increased over the 12-weeks diet and/or exercise intervention period in the male and female IF groups ($p<0.05$, Figure 4.7). However, similar to changes in plasma insulin, female mice also showed a significant increase in HOMA-IR in the CON ($p<0.05$) and IF+HIIT groups ($p<0.05$). In addition, female mice demonstrated a significant increase in AUC (Figure 4.8, 4.9 and 4.10), a marker of glucose tolerance in IF and IF+HIIT groups over a 10-weeks diet and/or exercise interventions period ($p<0.05$, Figure 4.8, 4.9 and 4.10). No significant differences were observed at the end of the diet and/or exercise intervention period (Figure 4.10).

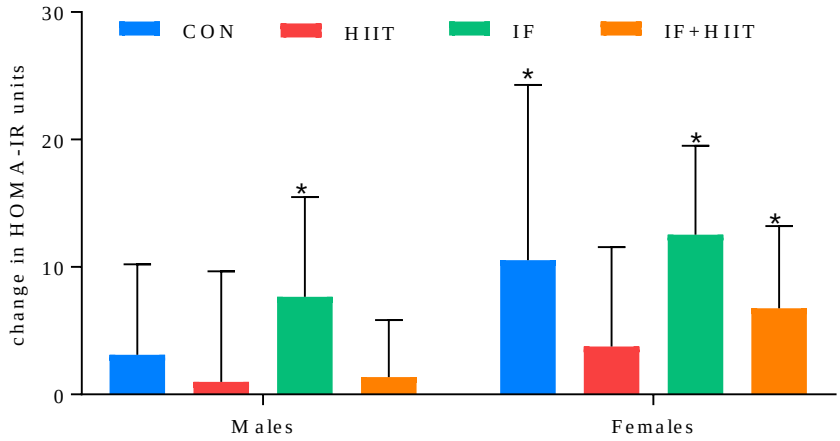

Figure 4.7. HOMA-IR changes from 'obese' baseline in males and females after 12-weeks of diet and/or exercise intervention. Bars represent means, error bars represent standard deviation and * indicates significant change over time.

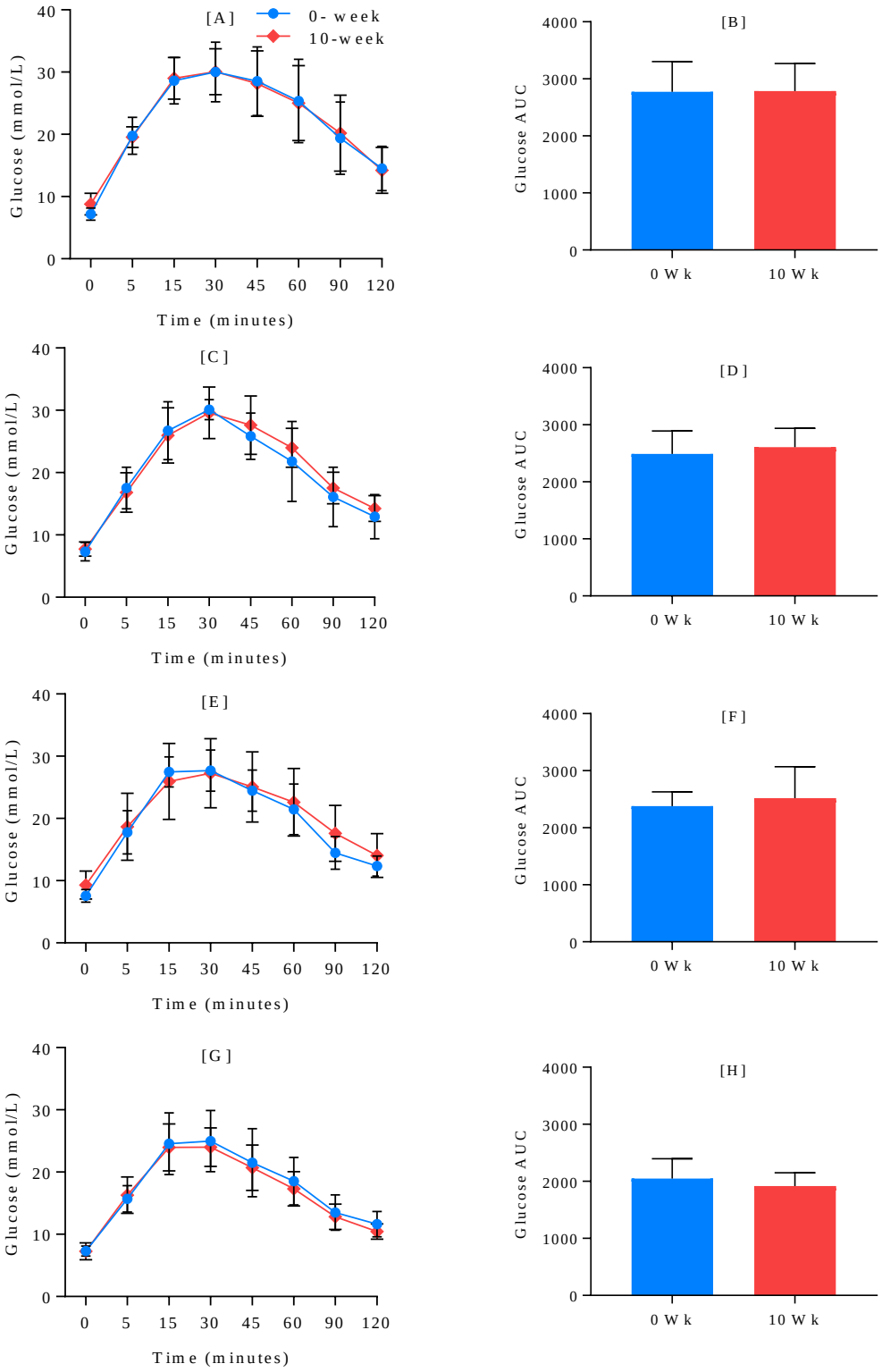

Figure 4.8. Glucose tolerance curves of (A) CON, (C) HIIT, (E) IF, (G) IF+HIIT group and glucose area under the curve of (B) CON, (D) HIIT, (F) IF, (H) IF+HIIT group in males before and after 10-weeks of intervention. Bars represent means, error bars represent standard deviation.

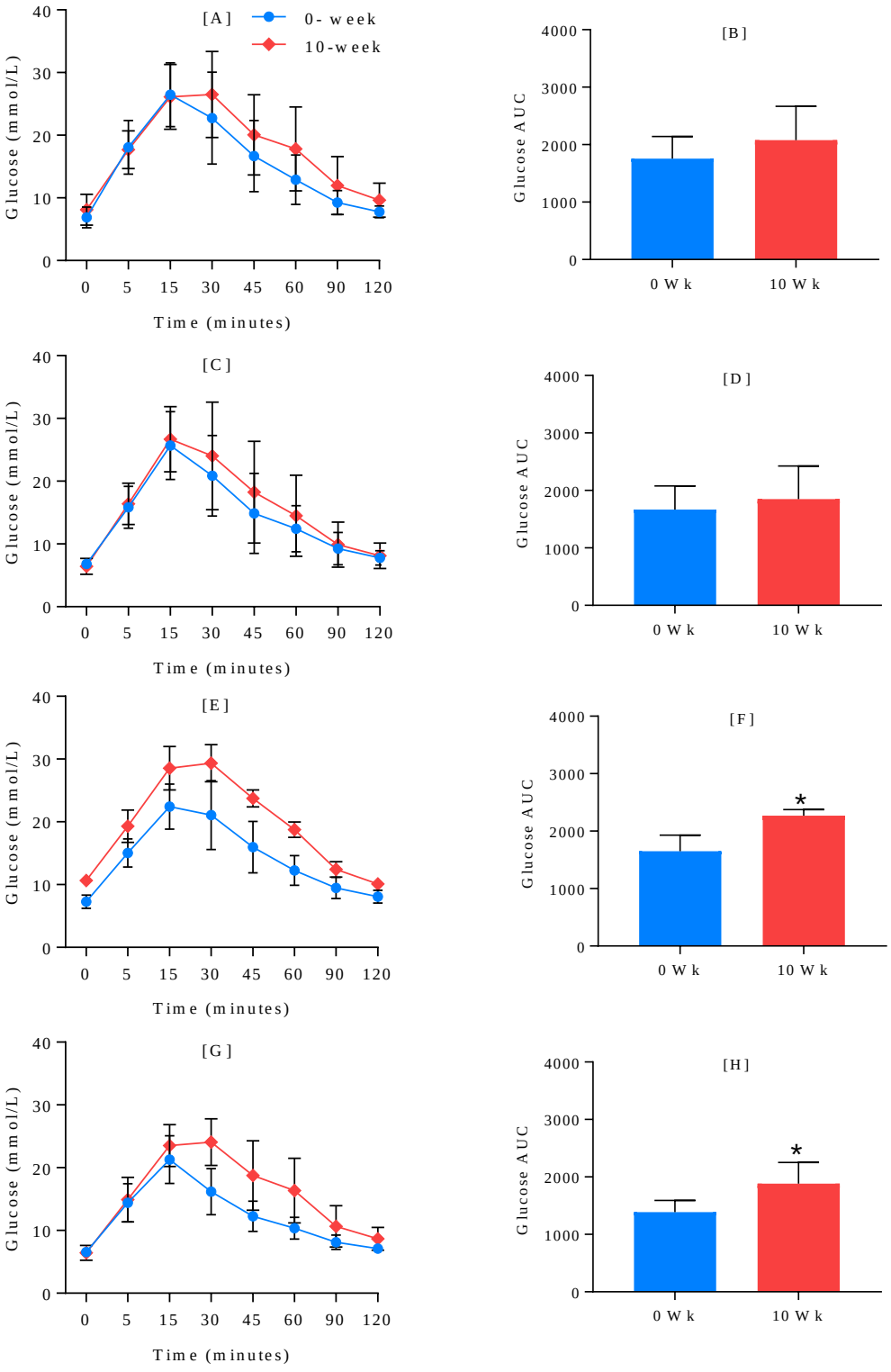

Figure 4.9. Glucose tolerance curves of (A) CON, (C) HIIT, (E) IF, (G) IF+HIIT group and glucose area under the curve of (B) CON, (D) HIIT, (F) IF, (H) IF+HIIT group in females, before and after 10-weeks of intervention. Bars represent means, error bars represent standard deviation and * indicates significant change over time.

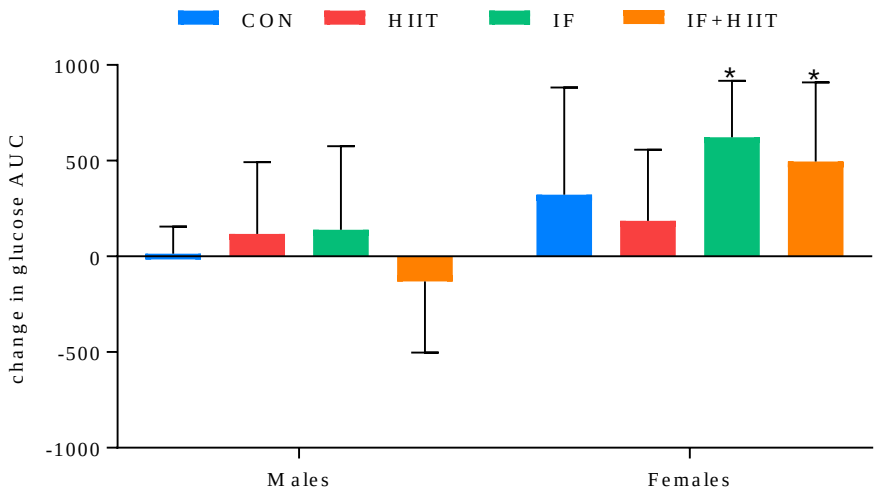

Figure 4.10. Change in glucose area under the curve (AUC) in males and females after 10-weeks of diet and/or exercise intervention. Bars represent means, error bars represent standard deviation and * indicates significant change over time.

4.3.6 Effect of IF and/or HIIT on lipid profiles

Plasma LDL, HDL and TAG levels measured before and after 12-weeks of diet and/or exercise intervention is presented in Table 4.1.

In female mice, plasma LDL levels significantly decreased over the 12-weeks diet and/or exercise intervention period in all groups ($p<0.05$). Conversely, HDL significantly increased over 12-weeks period, but only in the HIIT and IF groups ($p<0.05$). In male mice, LDL levels significantly decreased over the 12-weeks diet and/or exercise intervention period in the IF and IF+HIIT groups ($p<0.05$), whereas CON and HIIT groups significantly increased over that period ($p<0.05$). At the end of the intervention period, plasma LDL levels were significantly lower in the male IF and IF+HIIT groups compared to CON ($p<0.05$) and HIIT ($p<0.05$) groups. No significant differences were observed for plasma HDL and TAG levels in males (Table 4.1). In the female mice, plasma TAG levels were significantly lower in the IF+HIIT group compared to HIIT

groups at the end of the 12-weeks period ($p<0.05$). No significant differences between groups were observed for plasma LDL and HDL levels in females (Table 4.1).

Table 4.1. The effect of 12-weeks of intermittent fasting and high intensity exercise combined or individual on plasma TAG, HDL and LDL in male and female mice.

Variables		OBC	CON	HIIT	IF	IF + HIIT
TAG (mg/dL)	**Males**	60.1 ± 14.6	58.9 ± 10.7	72.1 ± 16.5	62.7 ± 10.6	57.0 ± 27.7
	Females	65.9 ± 20.5	70.9 ± 13.1	75.6 ± 14.8 $^{\xi}$	66.5 ± 15.7	51.3 ± 24.7 $^{\Phi}$
HDL (mg/dL)	**Males**	69.8 ± 12.2	76.2 ± 11.6	68.7 ± 11.3	82.1 ± 32.1	72.1 ± 17.3
	Females	51.1 ± 9.0	65.1 ± 23.0	61.1 ± 9.5 *	58.6 ± 9.1 *	50.8 ± 7.7
LDL (mg/dL)	**Males**	40.5 ± 17.7	82.3 ± 36.0 *$^{\Psi\xi}$	74.8 ± 15.6 *$^{\Psi\xi}$	40.4 ± 16.9 $^{\delta\Phi}$	29.9 ± 8.9 *$^{\delta\Phi}$
	Females	40.5 ± 17.7	24.8 ± 11.8 *	17.8 ± 4.8 *	19.5 ± 6.0 *	27.0 ± 8.9 *

Note: Values are presented as means ±SD. * significant change over time, δ significantly different from CON, Φ significantly different from HIIT, Ψ significantly different from IF, ξ significantly different from IF+HIIT.

4.3.7 Effect of IF and/or HIIT on muscle strength

Absolute and relative muscle strength measured before and after 10-weeks of diet and/or exercise intervention is presented in Table 4.2. Absolute muscle strength was significantly increased in the CON group over the 10-weeks period in the male mice ($p<0.05$). In the female mice, absolute muscle strength was decreased in the IF group over the 10-weeks period ($p<0.05$). Similarly, relative muscle strength (muscle strength expressed per lean muscle mass), also declined over the 10-weeks period in IF females group only. No other significant differences were observed for absolute muscle strength or relative muscle strength (Table 4.2).

Table 4.2. The effect of 10-weeks of intermittent fasting and high intensity exercise combined or individual on absolute and relative muscle strength in both male and female mice.

		CON		HIIT		IF		IF + HIIT	
		0 Wk	10 Wk	0 Wk	10 Wk	0 Wk	10 Wk	0 Wk	10 Wk
Absolute muscle strength (Score) [#]	**Males**	9.4 ± 1.8	10.5 ± 1.7 *	10.1 ± 1.2	10.6 ± 1.4	10.7 ± 1.6	11.2 ± 0.9	10.5 ± 1.1	11.1 ± 1.3
	Females	9.7 ± 2.7	9.8 ± 2.6	10.8 ± 1.9	11.3 ± 2.3	12.1 ± 1.9	9.4 ± 1.6 *	11.8 ± 1.9	12.2 ± 1.6
Relative muscle strength (Score/LM) [$]	**Males**	0.33 ± 0.05	0.35 ± 0.05	0.37 ± 0.04	0.38 ± 0.05	0.37 ± 0.05	0.39 ± 0.04	0.37 ± 0.05	0.39 ± 0.05
	Females	0.44 ± 0.12	0.42 ± 0.10	0.50 ± 0.10	0.50 ± 0.11	0.52 ± 0.11	0.40 ± 0.09 *	0.54 ± 0.09	0.53 ± 0.08

Note: Values are presented as means ± SD. [#] Absolute muscle strength score represents maximum muscle strength produced independent of muscle or body size; [$] Relative muscle strength represents absolute muscle strength divided by lean mass (LM); * indicates significant changes over time.

4.3.8 Effect of IF and/or HIIT on organ weight

Liver weights of male mice in IF+HIIT group was significantly ($p<0.05$) lower than control mice (Table 4.3). No significant difference in liver weight was observed between any other group in males and between any groups in females. Pancreas weight of IF+HIIT and IF group was significantly ($p<0.05$) lower than control in both sexes (Table 4.3). Pancreas weight of females in HIIT group was significantly ($p<0.05$) lower than control. However, pancreas weights of three intervention groups were non-significantly different from each other in both genders. Skeletal muscle (soleus and EDL), heart, kidneys, spleen weights were non-significantly different between different groups in both sexes (data not shown).

Table 4.3. The effect of 12-weeks of intermittent fasting and high intensity exercise combined or individual on liver and pancreas weight in both male and female mice.

	Liver		Pancreas	
	Males	**Females**	**Males**	**Females**
CON	$1.76 \pm 0.49^{\xi}$	1.55 ± 0.46	$0.13 \pm 0.03^{\Psi\xi}$	$0.14 \pm 0.04^{\Phi\Psi\xi}$
HIIT	1.48 ± 0.40	1.44 ± 0.24	0.12 ± 0.04	$0.10 \pm 0.02^{\epsilon}$
IF	1.40 ± 0.12	1.44 ± 0.16	$0.10 \pm 0.02^{\epsilon}$	$0.11 \pm 0.01^{\epsilon}$
IF+HIIT	$1.20 \pm 0.14^{\epsilon}$	1.34 ± 0.11	$0.09 \pm 0.01^{\epsilon}$	$0.09 \pm 0.02^{\epsilon}$

Note: Values are presented as means ±SD. * significant change over time, δ significantly different from CON, Φ significantly different from HIIT, Ψ significantly different from IF, ξ significantly different from IF+HIIT.

4.4. DISCUSSION

Intermittent fasting with or without high intensity interval training resulted in significant prevention in weight gain in male mice despite concurrently consuming a high fat and sugar diet. The reduction in weight was predominantly due to a significant decrease in fat mass, with no loss in lean muscle mass. At the end of the diet and/or exercise intervention period, LDL levels were significantly lower in the male IF and IF + HIIT groups compared to controls, whereas female mice demonstrated significantly lower TAG levels in the IF+HIIT group compared to HIIT group. High intensity interval training alone was also able to reduce weight and fat mass gain, albeit not significantly. Interestingly, intermittent fasting appeared to have a negative impact on markers of glycaemic control, especially within the female mice, with significant elevations in fasting blood glucose, glucose tolerance and HOMA-IR observed at the end of the intervention period. The changes in body composition, glycaemic control and lipid panels seem to be gender specific with male mice demonstrating greater changes in most variables compared to the female mice, independent of the intervention. The results from

the present study suggest that IF with or without HIIT exercise can be an effective strategy to induce weight loss despite concurrently consuming a high fat and sugar diet.

4.4.1 Effect of IF and/or HIIT on body composition

Following an initial 12-weeks of HF/S feeding to induce obesity, a further 10-weeks of HF/S diet caused a significant increase in body mass and fat mass by ~16% and ~47%, respectively in CON male mice and by ~19% and ~46%, respectively in CON female mice. These changes are comparable to those reported elsewhere using a similar diet (Gotthardt et al., 2016, Davis et al., 2017). Combining IF for 2 days per week and HIIT for 3 days per week produced the most effective changes in body weight and fat mass compared to either IF or HIIT intervention groups individually. The IF+HIIT group weighed ~9g and ~6g less and displayed ~66% and 24% lower fat mass in the males and females, respectively, compared to the CON mice. The weight and fat loss was also greater compared to the HIIT and IF groups; although not significantly different between the male IF groups. The body compositional changes observed in the combined diet and exercise group supports the primary hypothesis of the study and provides evidence of an additive affect when implemented concurrently. Studies using combined CR with exercise have reported reduced weight gain in comparison to control animals (no diet and exercise) (Huang et al., 2010, Huffman et al., 2008). Huffman and colleagues (2008) administered 24-weeks of CR (18% restriction of high fat diet) combined with five days per week of treadmill running at speeds approximately 13-15 m/min which resulted in weight gain prevention (~15g) and fat mass reductions (~45%) compared to CR only. Similarly, weight gain prevention (~15g) was also observed following 30% CR when combined with voluntary wheel running (distance not measured) for 8-weeks (Huang et al., 2010). These changes, especially in weight, were greater than the body compositional

changes observed in the current study and are likely due to a larger energy deficiency created by the greater energy restriction and/or higher exercise-induced energy expenditure of the protocols used (Huang et al., 2010, Huffman et al., 2008).

Despite the differences in total energy restriction and energy expenditure, the aforementioned studies using the combination of prolonged energy restriction and lower intensity exercise did not report weight loss, but rather prevention of weight gain following the intervention period while consuming a high fat diet (Huang et al., 2010, Huffman et al., 2008). Conversely, the current study demonstrated weight and fat mass loss in male mice at the end of the intervention period, despite concurrently consuming a high fat and sugar diet. Moreover, with no significant differences in caloric intake between the "fasting" and "non-fasting" groups, it is possible that the fasting groups with 2 days less access to food increased their food intake on non-fasting days. In light of this, it could be suggested that the changes in body weight and fat mass are due to mechanisms other than energy balance (i.e. hormonal, inflammation, genetic etc,) and requires more research to understand the additive effects of both IF and HIIT interventions at a systemic and tissue-specific cellular level.

The intermittent fasting intervention also resulted in significant weight loss (~18% or ~7.3g) and fat mass reduction (~58% or ~6.9g) compared to CON group; albeit only in male mice. Previous studies using ADF regimes have also shown improvements in body composition, with reduced gains in body mass over a 4- to 6-weeks period compared to mice consuming a standard chow diet (Varady et al., 2007, Karbowska and Kochan, 2012). Using a similar model of diet-induced obesity to that of the current study, 4-weeks of ADF led to lower body mass and fat mass gains by approximately 13% and 50%, respectively, compared to high fat fed controls (Gotthardt et al., 2016). Similarly,

Higashida and colleagues (2013) also showed significantly less weight gain (~27%) and intra-abdominal fat accumulation (~39%) following 6-weeks of ADF compared to the high fat fed controls in rats (Higashida et al., 2013). The greater difference in reduced weight gain reported in the Higashida et al. (2013) study versus the present study (~27% vs. ~16%) could be due to the frequency of fasting days, as the Higashida et al. (2013) implemented their 21 days of fasting within a 6-weeks period, whereas the current study implemented 24 days of fasting within a 10-weeks period. The discrepancy in body fat changes is most likely a reflection of the location of fat mass measured. Notwithstanding, it is evident from the current study and others that IF is an effective method of reducing body weight and fat mass in mice consuming either standard chow or a high fat and sugar diet. The results are most likely a reflection of a negative energy balance created by the reduced caloric intake on fasting days.

Another strategy to create a negative energy balance is to increase energy expenditure greater than energy intake (Keating et al., 2017). In the current study, mice completing 10-weeks of HIIT, 3 times a week resulted in reduced gains in body mass and fat mass by approximately 5% and 8% and 6% and 10% in male and female mice, respectively compared to CON mice. Greater prevention of weight and fat mass gain have been observed in other studies with up to 16% and 45% prevention in weight and fat gain, respectively (Cho et al., 2015, Lund et al., 2015). However, such differences are most likely due to the training volume, with these studies undertaking 10-12 bouts of HIIT, 5 days a week for 8-10-weeks (Cho et al., 2015, Lund et al., 2015) compared to the present study of 8 bouts, 3 days per week. Furthermore, the HIIT group in the present study concurrently consumed a diet high in fat and sugar during the intervention period and thus, any change in appetite (i.e. increase) because of the exercise training would have

resulted in increased consumption. Although the caloric intake of HIIT group could not be measured separately, it can be speculated that mice in HIIT had high caloric intake in comparison to non- fasting exercise group. Consuming a standard chow diet may have attenuated weight gain or perhaps led to weight loss.

Lean mass was lower in all intervention groups compared to the CON mice in both males and females at 10-weeks. However, this was most likely a reflection of their reduced weight gain rather than loss of lean mass, with reduced fat mass to lean mass ratio demonstrated in the IF and combination groups. The increase in fat mass to lean ratio following the HIIT intervention is contradictory to other studies (Boudou et al., 2003, Trapp et al., 2008). As mentioned previously, any increase in appetite as a result of exercise would result in greater consumption of high fat and sugar, which may lead to greater fat deposition, rather than lean mass growth. We did hypothesise that the combination group would display greater lean mass preservation compared to the IF group due to the added benefit of exercise. However, lean mass was lower in the combination group compared to the IF intervention group in males in males. Previous studies have shown improved lean mass preservation when calorie restriction is combined with treadmill exercise (Huffman et al., 2008, Weiss et al., 2015, Foster-Schubert et al., 2012), indicating the role of exercise in preserving lean mass. Although lean mass was lower in the combination group compared to the IF intervention group, muscle quality may have been greater. Indeed, relative muscle strength was higher in both male and female mice in the combination group compared to IF intervention group. This could be a result of improved muscle quality, rather than muscle mass *per se* as the mice were required to lift an object and hold for as long as they could, rather than hang for a period of time which is highly dependent on the weight of the mouse.

4.4.2 Effect of IF and/or HIIT on glucose homeostasis

Glucose intolerance and insulin resistance have been noted in animal models of diet-induced obesity (Gotthardt et al., 2016, Lund et al., 2015, Montgomery et al., 2013). In the current study, high fat feeding resulted in hyperglycaemia, insulin resistance and glucose intolerance in both male and female mice, with elevated glucose levels before the start of IF and/or HIIT interventions, indicating diabetes onset. Despite previous studies demonstrating improved glycaemic control and reduced diabetes risk following even modest weight reduction, the present study was unable to confirm such findings following the diet and/or exercise interventions. The effect of IF on glucose metabolism and glycaemic control was unexpected. Both male and female IF groups exhibited significant increases in fasting glucose and plasma insulin and thus glucose tolerance and insulin resistance at 10- or 12-weeks. Although these results seem contradictory to the commonly reported view that IF lowers fasting blood glucose levels and improves glycaemic control (Barnosky et al., 2014), it has been reported that long term (32-weeks) IF, but not CR, can lead to redox imbalances, insulin receptor nitration, and thus glucose intolerance in rats (Cerqueira et al., 2011). The intervention period in the current study was only 10- to 12-weeks, however, this period is long in comparison to the total life span of mice and equates to about 20 years of human years (Dutta and Sengupta, 2016). The adverse effects from long term IF could be attributed to the overeating pattern adopted by IF animals on non-fasting days as observed in the current study and others (Thomas et al., 2010, Cerqueira et al., 2011). This frequent feeding/fasting cycle may be a risk factor for age-associated obesity and insulin resistance (Park et al., 2014), which may be exacerbated when consuming a high fat and sugar diet.

4.4.3 Effect of IF and/or HIIT on plasma lipids

Elevated TAG and LDL levels have been reported in animal models of diet-induced obesity (Montgomery et al., 2013, Li et al., 2013). Likewise, in the present study, high fat feeding resulted in hyperlipidaemia. Previous IF studies in animals have reported lower LDL (Li et al., 2013) and TAG levels (Karbowska and Kochan, 2012, Li et al., 2013) in IF mice, although only male animals were included in these studies. In the present study, males and females exhibit different response. In males, no significant difference in TAG levels between groups was observed however there was a significant lower LDL level in IF and IF+HIIT group in comparison to CON and HIIT group. The difference in LDL level among intervention groups aligns well with the changes in body weight and fat mass as mentioned above, indicating that IF and IF+HIIT are effective for maintaining lower plasma LDL in diet induced obese male mice. HIIT by itself did not have any significant effect on plasma lipids. No change in plasma TAG level with HIIT has also been reported by another study on diet induced obese mice (Cho et al., 2015, Davis et al., 2017). In females, IF+HIIT group showed significantly lower TAG level in comparison to HIIT only group, indicating the effect of combining IF and HIIT on maintaining low TAG levels in the blood as reported by other IF studies (Li et al., 2013, Karbowska and Kochan, 2012). LDL levels showed no significant difference between various groups in females and surprisingly all groups (intervention and CON) showed significantly lower LDL level in comparison to obese baseline control group. The reason behind this change is unknown and needs further investigation. Taken together, in line with the previous literature, IF and its combination with HIIT is effective in lowering some plasma lipids, if not all. However, HIIT despite being claimed to be effective in lowering plasma lipids by some human studies (Ouerghi et al., 2017, Fisher et al., 2015),

did not show any beneficial effect on plasma lipids in diet induced obese mice. As mentioned earlier, the intensity and frequency of HIIT employed in this study could be the reason behind no apparent benefit.

4.4.4 Effect of IF and/or HIIT on muscle strength

Obesity has been reported to affect muscle strength in high fat diet induced obese mice (Tam et al., 2015). However, in the present study, diet induced control group showed increase in absolute muscle strength, although when expressed as relative muscle strength, it did not show any change over 10-week intervention period. We assumed that HIIT will improve muscle strength, however muscle strength was unaffected by HIIT and/or IF in males. In females, both relative and absolute muscle strength was reduced in the IF group, which again was most likely a reflection of the decrease in muscle mass observed in that group. No other significant changes in muscle strength was noted in other groups. An observation of interest was that the females in IF+HIIT group demonstrated similar strength values to the HIIT and control group, which indicates that decline in muscle strength observed following the IF regime in the female mice was counteracted by exercise.

4.4.5 Effect of IF and/or HIIT on organ weight

Increase in adipose tissue mass during weight gain is often associated with ectopic accumulation of fats in other tissues like heart, muscle, liver and pancreas (O'Brien et al., 2017, Stern et al., 2016, Ruberg et al., 2010). Accumulation of ectopic fats in pancreas and liver leads to conditions like non-alcoholic fatty pancreas disease and non-alcoholic fatty liver disease respectively (Tariq et al., 2016). Lifestyle interventions like HIIT (Cho et al., 2015), IF (Baumeier et al., 2015, Antoni et al., 2017) and calorie restriction (Larson-Meyer et al., 2006) have been reported to prevent ectopic lipid accumulation. However,

in the present study, neither IF nor HIIT alone showed any significant difference on liver weight in comparison to *ad libitum* control group. Liver weights of males in IF+HIIT was lower in comparison to control males indicating the beneficial effect of this combined intervention in preventing lipid accumulation in liver in diet induced obese male mice. Unlike liver, IF with and without exercise showed significantly lower pancreas weight in comparison to CON group in both sexes. In females, even the HIIT only group showed significantly lower pancreas weight. These observations indicate that despite high fat/sugar diet, both male and female mice in IF with and without HIIT groups and females on HIIT only group maintained lower pancreas weight, demonstrating the effectiveness these interventions in preventing ectopic fat accumulation in pancreas.

4.4.6 Sexually dimorphic response to IF and/or HIIT

An observation of interest was the gender specific responses to the same diet and/or exercise intervention. Changes in body weight and composition, markers of glucose homeostasis, lipid profiles, muscle strength and ectopic lipid accumulation were clearly different between males and females, with males demonstrating greater improvements in most, if not all markers. Previous lifestyle intervention studies and recent systematic reviews also confirm that males tend to lose more weight than females in response to lifestyle interventions (Williams et al., 2015, Baillot et al., 2015, Gallagher et al., 2017), but also acknowledge than females also lost weight. The exact mechanisms behind these reported gender differences have yet to be fully explored in detail. Male and female mice exhibit differences in adipose tissue distribution and functionality (Valencak et al., 2017), with obese female mice having higher fat mass, which was also noted in the present study and a tendency to mobilize fats more slowly in comparison to weight-matched males (Nickelson et al., 2012, Mauvais-Jarvis, 2015). Furthermore, females tend

to store free fatty acids as triglycerides whereas males tend to oxidise free fatty acid under similar resting conditions (Uranga et al., 2005). Finally, hormonal differences between sexes may also influence the responses to diet and exercise with males exhibiting higher levels of circulating leptin and females higher levels of circulating adiponectin (Valencak et al., 2017). In addition, elevated estrogen levels following IF, have been linked to the development of insulin resistance in females (Kumar and Kaur, 2013) (Valencak et al., 2017). Clearly more research is needed in this area to understand the mechanisms behind these gender specific responses but also help guide diet and exercise recommendations in the future.

4.5. CONCLUSION

Current evidence suggests that as little as 10 minutes of high intensity exercise can improve metabolic health and aerobic capacity (Gillen et al., 2016) and alternative day fasting can reduce obesity-associated changes in body composition, fasting insulin and glucose concentrations (Varady et al., 2009, Varady et al., 2013). The current study wanted to confirm the aforementioned findings by mimicking similar protocols in animal models. Secondly, to investigate the combined effects of such regimes and its long-term impact, and thirdly, unlike human studies which typically don't change the research participant's diet, we wanted to observe the effects of these diet and exercise regimes while concurrently consuming a high fat and sugar diet. The present study is the first study to demonstrate superior effects on body composition and lipid profiles following combined IF and HIIT compared to either diet or exercise intervention alone while concurrently consuming a high fat and sugar diet. These observations are likely due to changes in energy balance, with a greater negative energy balance shift compared to the other groups. However, given no significant differences between 'fasting' and 'non-

fasting' intervention groups and observations from previously published CR and exercise studies, it could also be suggested that our observations may be due to more than just energy balance differences. An observation of interest was the gender specific responses to the same diet and/or exercise intervention and could indicate potential hormonal differences influencing metabolic control/adaptation in mice. The results of the present study suggest that the combination of diet and exercise are most effective at attenuating the negative impact that high fat and sugar diet has on body weight, composition and lipid levels.

CHAPTER 5

MOLECULAR BASIS OF REDUCED FAT MASS AND ASSOCIATED BENEFITS IN ADIPOSE TISSUE INDUCED BY INTERMITTENT FASTING WITH OR WITHOUT HIGH INTENSITY INTERVAL TRAINING IN DIET-INDUCED OBESE MICE

5.1. INTRODUCTION

Chapter 4 investigated the effects of IF and HIIT, alone and in combination, on anthropometric and metabolic health parameters in diet-induced obese mice. The main findings of chapter 4 was that intermittent fasting with or without high intensity interval training resulted in significantly less weight gain, predominantly due to less adipose tissue accumulation, in male mice despite concurrently consuming a high fat and sugar diet for 12 weeks. Chapter 5 will investigate diet and/or exercise induced changes in adipose tissue by examining changes in microRNA and mRNA expression related to adipose tissue function, hypoxia, and inflammation.

Adipose tissue plays a central role in lipid metabolism and energy homoeostasis (Valencak et al., 2017). The combination of high energy intake and low energy expenditure results in the storage of excess calories in the form of adipose tissue, leading to adipose tissue expansion and disturbance in the systemic energy homeostasis (Rutkowski et al., 2015). Adipose tissue expansion also disrupts the normal functioning of adipocytes, leading to dysfunction in the lipolytic and secretory functions, increased inflammation, insulin resistance (Greenberg and Obin 2006, Guilherme, Virbasius et al. 2008), and adipose tissue hypoxia (van Beek, van Klinken et al. 2015). These repercussions are underpinned by changes in various signalling molecules and expression of genes involved in fine tuning the structural and functional integrity of adipose tissue

(Manteiga et al., 2013). For instance, high fat intake downregulates gene expression related to fatty acid uptake (Fatty acid binding protein 4), oxidation (Hydroxyacyl-Coenzyme-A dehydrogenase), insulin signalling (insulin receptor substrate 2) and glucose uptake (GLUT3) in mice (Choi et al., 2015). These effects may partially be responsible for the decrease in energy expenditure, fat accumulation and impaired insulin sensitivity typically observed during high fat feeding (Choi et al., 2015). High fat feeding also upregulates pro-inflammatory genes like TNFα (van der Heijden et al., 2015) and hypoxia related gene like hypoxia-inducible factor 1-alpha (He et al., 2011); which contribute to the pro-inflammatory state that is associated with an obesity phenotype.

Intermittent fasting alone is effective in reducing body weight, in the form of adipose tissue mass, while having minimal effects on muscle mass. Researchers have begun to identify potential drivers of such physiological changes at cell and molecular level following intermittent fasting. These include enhance expression of adipose tissue genes related to: adipogenesis such as cell death-inducing DFFA-like effector C (*CIDEC*) and CIDEA (Kim et al., 2017), peroxisome proliferator activated receptor gamma (Karbowska and Kochan, 2012), markers of white adipose tissue to brown adipose tissue conversion such as *PGC1α*, and uncoupling protein 1 (Kim et al., 2017, Li et al., 2017a) following intermittent fasting.

Similar to intermittent fasting, high intensity interval training (HIIT) alone can also enhance body composition and improve metabolic health. However, unlike intermittent fasting which reduces calorie intake, HIIT increases energy expenditure. Notwithstanding, it is clear that manipulations of energy intake and expenditure that can push the energy balance towards negative side is the key driver of these effects. Under high fat fed conditions, previous studies have demonstrated HIIT's ability to improve

glucose handling as evidenced by increased 2-Deoxy-D-glucose uptake within adipose tissue, despite no change in expression of inflammatory genes like *IL6*, *TNFa* or the neutrophil chemoattractant *Kc* in mice (Marcinko et al., 2015). In addition, HIIT has shown to enhance adipose tissue angiogenesis by increasing expression of vascular endothelial growth factor (VEGF) gene expression (Ghafari Homadini et al., 2017), which is otherwise reduced in obesity (Disanzo and You, 2014).

Recent addition to the complexity of the molecular signals regulating adipose tissue and systemic homeostasis is miRNAs. Several studies have reported that miRNAs play a crucial role in maintenance of structural and functional integrity of adipose tissue under both normal and dysfunctional states (Wilson et al., 2017, Deiuliis, 2016, Price and Fernández-Hernando, 2016). For instance, miR-27a has been found to be critical in maintaining adipose tissue mass and its downregulation can lead to adipose tissue dysfunction (Kim et al., 2010). Limited research has examined changes in adipose tissue miRNA expression following either diet and/or exercise intervention. Recently, upregulation of miR-149-3p has been found to promote fasting induced switching of subcutaneous to visceral adipose tissue (Ding et al., 2016), while enhanced expression of miR-10b promotes endurance exercise training induced β-adrenergic lipolysis in adipose tissue (Tsiloulis et al., 2016).

The objective of the study was to evaluate the effect of IF with and without HIIT on adipose tissue expression of genes involved in adipogenesis, lipogenesis, lipolysis, lipid handling, oxidation, hypoxia, inflammation and beiging in diet-induced obese mice. Expression of microRNAs related to the genes targeted in the present study were also investigated to further elucidate their regulatory involvement. MiRNAs were selected on the basis of their relationship to the investigated genes, either reported in the literature

and/or found through miRWALK software (Dweep et al., 2011). As the combination of IF+HIIT was most effective in reducing fat mass, it was hypothesized that combination of IF with HIIT will show additive or synergistic effect in altering the expression of genes and miRNAs in adipose tissue.

5.2. MATERIALS AND METHODS

5.2.1 Animals

Details of animal procurement, housing and diet are mentioned in chapter 3 section 3.2. Briefly, this study included four different groups namely: no intervention (CON; Males=7, Females=9); intermittent fasting (IF; Males=8, Females=10), high intensity intermittent exercise (HIIT; Males=8, Females=10) and a combination of the dietary and exercise intervention (IF+HIIT; Males=8, Females=10). All groups were maintained on high fat/high sugar (HF/S) diet for 12 weeks to induce obesity and then underwent respective interventions for another 12 weeks while still consuming HF/S diet. Details of interventions is given in chapter 3 section 3.4. After 24 weeks of HF/S feeding and intervention, all mice were killed and tested as described below.

5.2.2 Culling and tissue collection

Mice were anaesthetised with intraperitoneal injection of pentobarbitone (60 mg/kg) and killed by cardiac puncture. The abdominal cavity was opened and epididymal and periovarian white adipose tissue (WAT) was collected, snap frozen in liquid nitrogen and stored in -80°C freezer until further analysis.

5.2.3 Adipose tissue gene and miRNA expression

Total RNA was extracted form adipose tissue, reverse transcribed to generate cDNA and subjected to RT-PCR for gene and miRNA expression. Procedures for mRNA and miRNA isolation, cDNA synthesis, RT-PCR analysis and calculation of fold change

are presented in detail in chapter 3, section 3.7. Beta-2 microglobulin (B2M) and RNU6 were used to normalize mRNA and miRNA expression data, respectively. The primers for genes and miRNAs were purchased from and designed by QIAGEN (Chadstone, Victoria, Australia). The list of genes and miRNAs targeted in this study are given in table 5.1 and table 5.2 respectively.

Table 5.1. List of targeted genes in adipose tissue with full official names, NCBI reference sequence numbers and Qiagen catalogue number.

Gene symbol	Official full name	NCBI Reference sequence number	Qiagen Catalogue number
PPARγ	Peroxisome proliferator activated receptor gamma	NM_001127330	PPM05108C
CIDEC	Cell death-inducing DFFA-like effector c	NM_178373	PPM25558B
SIRT1	Sirtuin 1	NM_001159589	PPM05054A
LIPE	Lipase, hormone sensitive	NM_001039507	PPM03313A
FOXO1	Forkhead box O 1	NM_019739	PPM03381C
FABP4	Fatty acid binding protein 4, adipocyte	NM_024406	PPM04517A
HIF1A	Hypoxia inducible factor 1, alpha	NM_010431	PPM03799C
HADH	Hydroxyacyl-Coenzyme A dehydrogenase	NM_008212	PPM35732B
TRPV4	Transient receptor potential cation channel, subfamily V, member 4	NM_022017	PPM36070A
LEP	Leptin	NM_008493	PPM03504B
UCP1	Uncoupling protein 1	NM_009463	PPM05164B
B2M	Beta-2 microglobulin	NM_009735	PPM03562A

Table 5.2. List of miRNAs analysed in adipose tissue, miRbase accession numbers and Qiagen miScript Primer Assay Catalog Numbers.

Mature miRNA ID	miRBase Accession No.	Qiagen miScript Primer Assay Catalog No.
mmu-miR-27a-3p	MIMAT0000537	MS00001351
mmu-miR-24-3p	MIMAT0000219	MS00005922
mmu-miR-222-3p	MIMAT0000670	MS00007959
mmu-miR-143-3p	MIMAT0000247	MS00001617
mmu-miR-145a-5p	MIMAT0000157	MS00001631

5.2.4 Statistical analysis

Data is presented as mean ± standard deviation. To evaluate the effect of interventions on mRNA expression, intervention groups namely HIIT, IF, and IF+HIIT were compared against no intervention CON group. Difference in mRNA expression and miRNA expression between groups were analysed using one-way ANOVA and Tukey's test was used as post hoc analysis. Correlation between mRNA and miRNA expression in each intervention group was calculated with Pearson correlation test for *PPARγ* Vs miR-27a, *FABP4* Vs miR-24, *HIFα* Vs miR-222, Lep Vs miR-143 and *FOXO1* Vs miR-145. An alpha level of 0.05 was adopted throughout to reduce Type I statistical errors.

5.3. RESULTS

5.3.1 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to structure and function

5.3.1.1. CIDEC

White adipose tissue *CIDEC* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.1 (A) and (B) respectively. Gene expression of *CIDEC* was significantly lower in the IF group compared to the HIIT group in both males ($p<0.01$) and females ($p<0.01$). In female mice, the *CIDEC* expression was significantly lower in the IF group compared to the CON group ($p<0.01$) and the HIIT group ($p<0.01$). The combined exercise and IF group also showed significantly lower *CIDEC* expression, however, only compared to HIIT group ($p<0.05$).

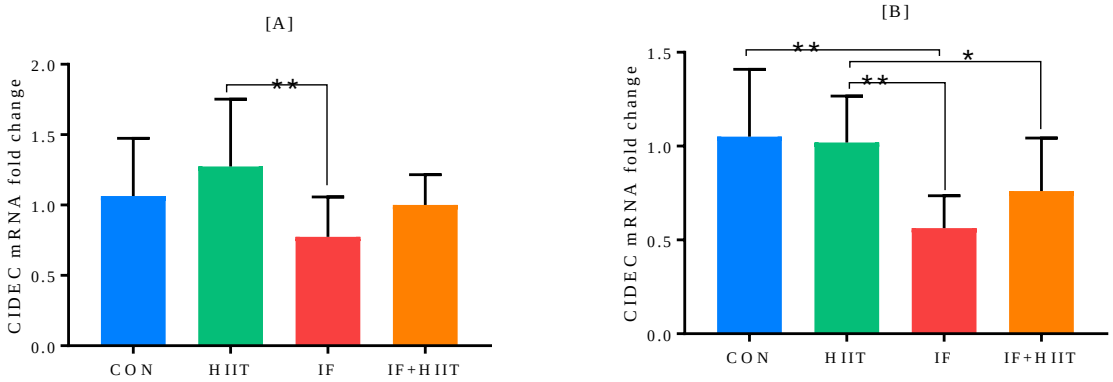

Figure 5.1. Effect of IF, HIIT and IF+HIIT on *CIDEC* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively.

5.3.1.2. Leptin

White adipose tissue leptin gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.2 (A) and (B) respectively. In both males and females, gene expression of leptin in CON group was significantly higher in comparison to IF ($p<0.01$) and IF+HIIT ($p<0.01$) groups. In males only, gene expression of leptin was significantly higher in the HIIT group compared to the IF ($p<0.05$) and IF+HIIT ($p<0.01$) groups.

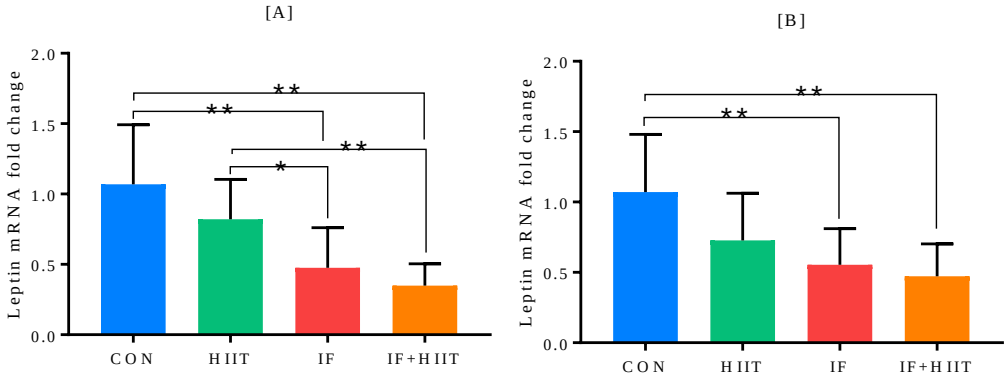

Figure 5.2. Effect of IF, HIIT and IF+HIIT on *LEP* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively.

5.3.1.3. PPARγ

White adipose tissue *PPARγ* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.3 (A) and (B) respectively. In males, IF+HIIT group showed significantly higher expression of *PPARγ* in comparison to CON group ($p<0.05$). In females, no significant differences were observed between control and IF and/or HIIT groups at the end of 12-week intervention period.

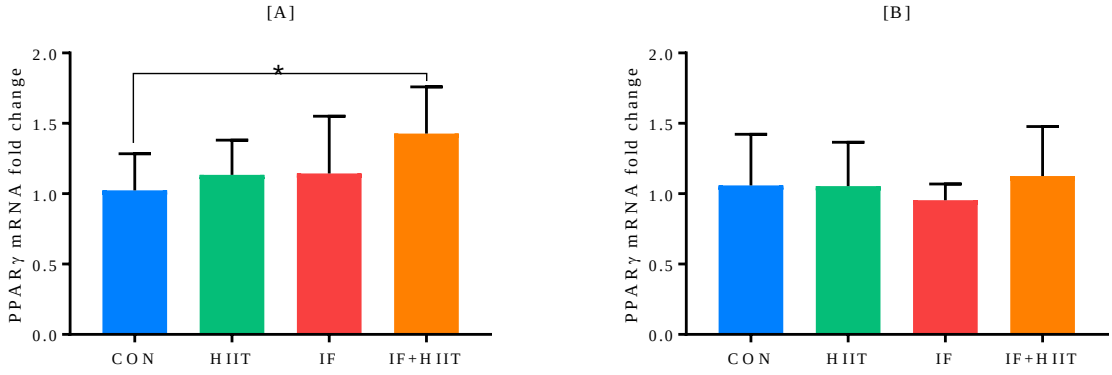

Figure 5.3. Effect of IF, HIIT and IF+HIIT on *PPARγ* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between groups at $p<0.05$ level of significance.

5.3.1.4. FOXO1

White adipose tissue *FOXO1* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.4 (A) and (B) respectively. In females, both IF and IF+HIIT group showed significantly lower expression of *FOXO1* relative to both CON ($p<0.01$) and HIIT ($p<0.01$) groups. In males, no significant differences in *FOXO1* gene expression between control and IF and/or HIIT groups were noted at the end of 12-week intervention period.

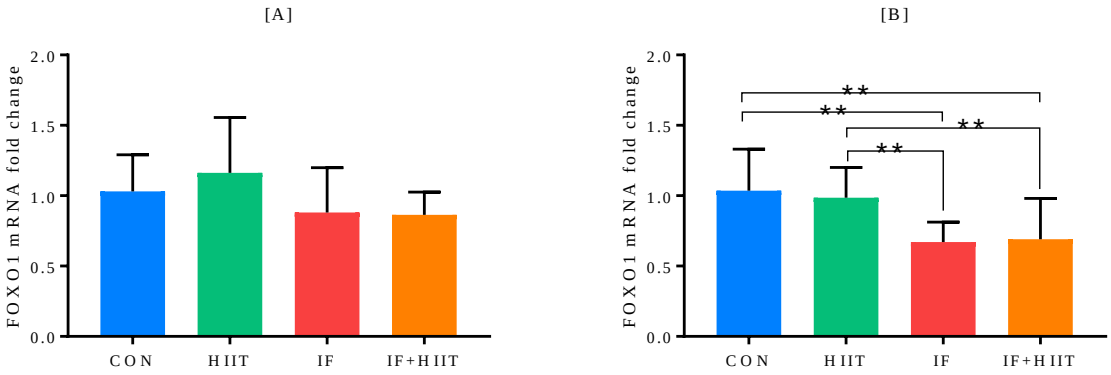

Figure 5.4. Effect of IF, HIIT and IF+HIIT on *FOXO1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at p<0.01 level of significance.

5.3.2 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to Fatty acid oxidation and trafficking

5.3.2.1. HADH

White adipose tissue *HADH* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.5 (A) and (B) respectively. Gene expression of *HADH* in IF group was significantly lower in comparison to IF+HIIT group in both males ($p<0.05$) and females ($p<0.01$).

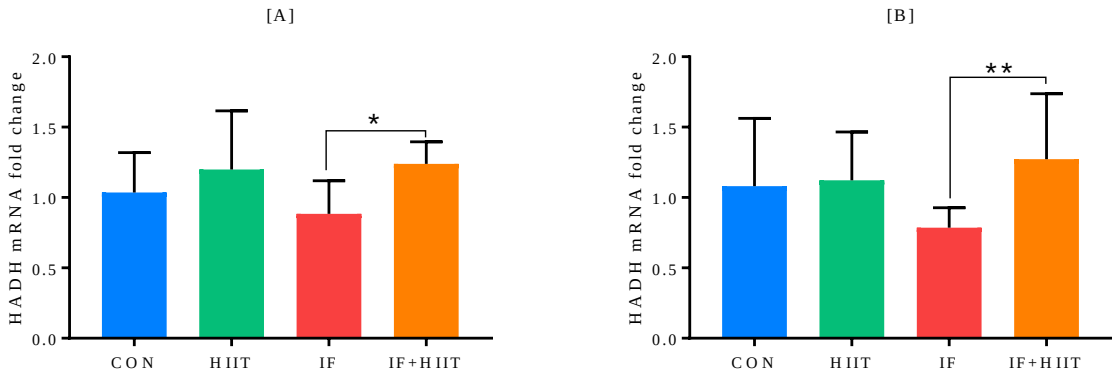

Figure 5.5. Effect of IF, HIIT and IF+HIIT on *HADH* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively.

5.3.2.2. FABP4

White adipose tissue *FABP4* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.6 (A) and (B) respectively. Gene expression of *FABP4* in IF+HIIT group was significantly higher in comparison to CON ($p<0.01$), HIIT ($p<0.01$) and IF ($p<0.01$) groups in males only. In females, IF group showed significantly ($p<0.01$) lower expression of *FABP4* compared to HIIT group at the end of 12-week intervention period.

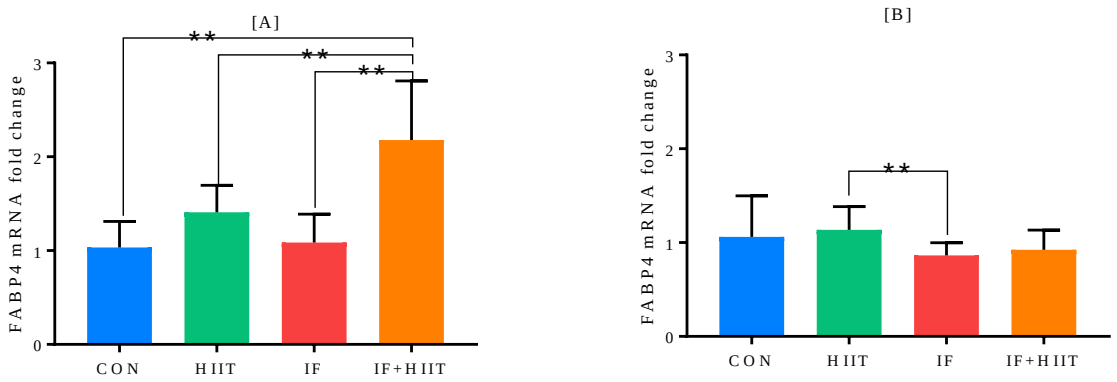

Figure 5.6. Effect of IF, HIIT and IF+HIIT on *FABP4* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance.

5.3.2.3. Hormone sensitive lipase

White adipose tissue hormone sensitive lipase (HSL) gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.7 (A) and (B) respectively. At the end of 12-week intervention period, no significant differences in HSL gene expression were observed between control and IF and/or HIIT groups in both males and females.

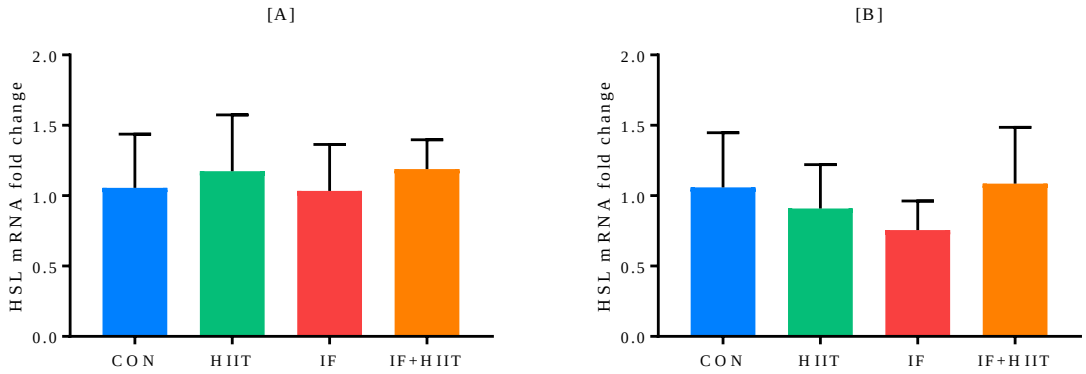

Figure 5.7. Effect of IF, HIIT and IF+HIIT on *HSL* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation.

5.3.3 The effect of 12 weeks of IF and/or HIIT on WAT gene expression of SIRT1

White adipose tissue *SIRT1* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.8 (A) and (B) respectively. In males, IF group showed significantly lower expression of *SIRT1* in comparison to IF+HIIT group ($p<0.01$). In females, both IF and IF+HIIT showed significantly lower expression of *SIRT1* compared to both CON ($p<0.01$) and HIIT ($p<0.01$) groups.

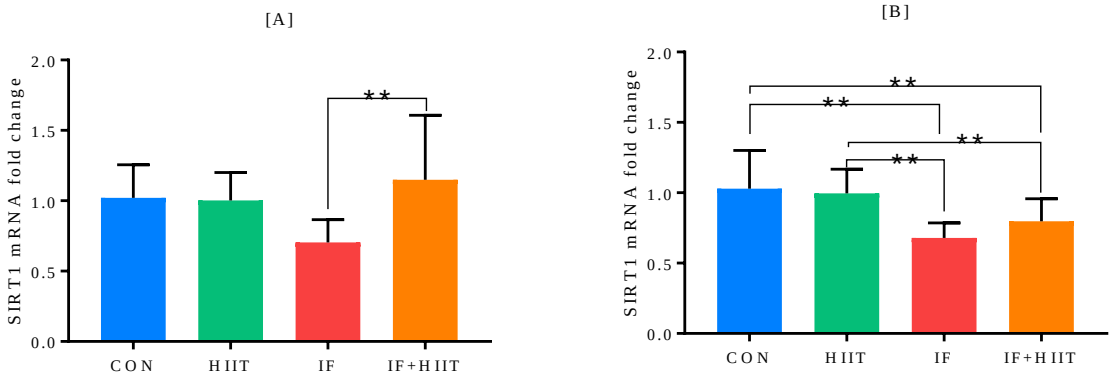

Figure 5.8. Effect of IF, HIIT and IF+HIIT on *SIRT1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at p<0.01 level of significance.

5.3.4 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to inflammation

5.3.4.1. TRPV4

White adipose tissue *TRPV4* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.9 (A) and (B) respectively. Gene expression of *TRPV4* in the IF group was significantly lower in comparison to CON group in both males ($p<0.05$) and females ($p<0.01$) and the HIIT group in females only ($p<0.01$). In females, IF+HIIT group also showed significantly lower expression of *TRPV4* gene in comparison to CON group ($p<0.01$).

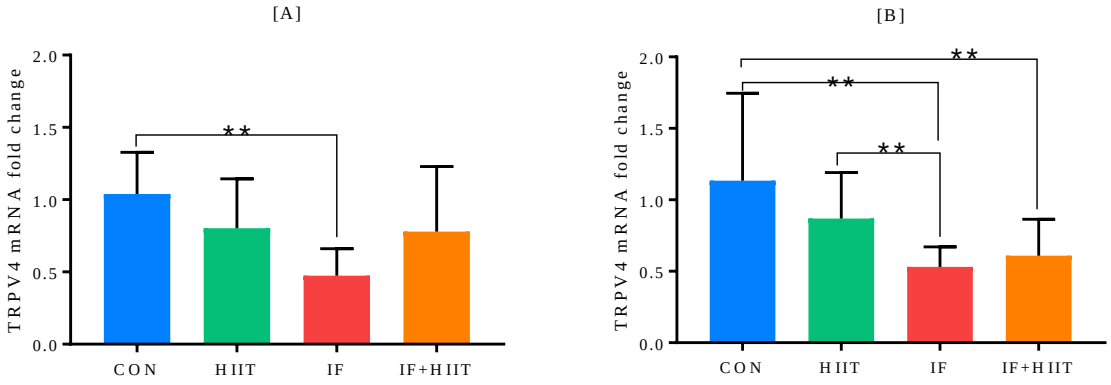

Figure 5.9. Effect of IF, HIIT and IF+HIIT on *TRPV4* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance.

5.3.5 The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to hypoxia

5.3.5.1. HIF1α

White adipose tissue *HIF1α* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.10 (A) and (B) respectively. In males, IF group showed significantly lower *HIF1α* gene expression relative to CON group ($p<0.01$). In females, no significant differences in *HIF1α* gene expression were noted between control and IF and/or HIIT groups at the end of 12-week intervention period. The effect of 12 weeks of IF and/or HIIT on WAT gene expression related to white adipose tissue beiging.

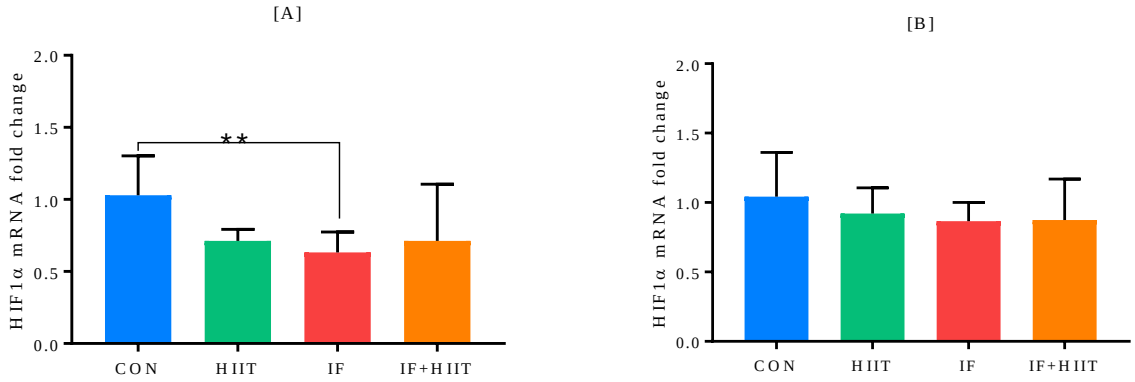

Figure 5.10. Effect of IF, HIIT and IF+HIIT on *HIF1α* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between groups at $p<0.01$ of significance.

5.3.5.2. UCP1

White adipose tissue *UCP1* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.11 (A) and (B) respectively. At the end of 12-week intervention period, no significant differences in *UCP1* gene expression were observed between CON and IF and/or HIIT groups in both males and females.

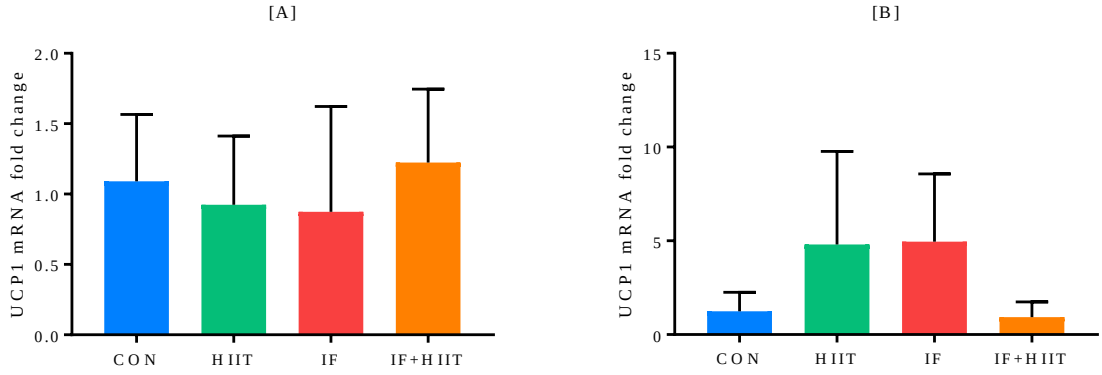

Figure 5.11. Effect of IF, HIIT and IF+HIIT on *UCP1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *B2M* gene expression. Bars represent means, error bars represent standard deviation.

5.3.6 The effect of 12 weeks of IF and/or HIIT on miRNA expression relating to adipogenesis and glucose handling in adipose tissue

5.3.6.1. miR-27a

White adipose tissue miR-27a expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.12 (A) and (B) respectively. At the end of 12-week intervention period, no significant differences in miR-27a expression were observed between CON and IF and/or HIIT groups in both males and females.

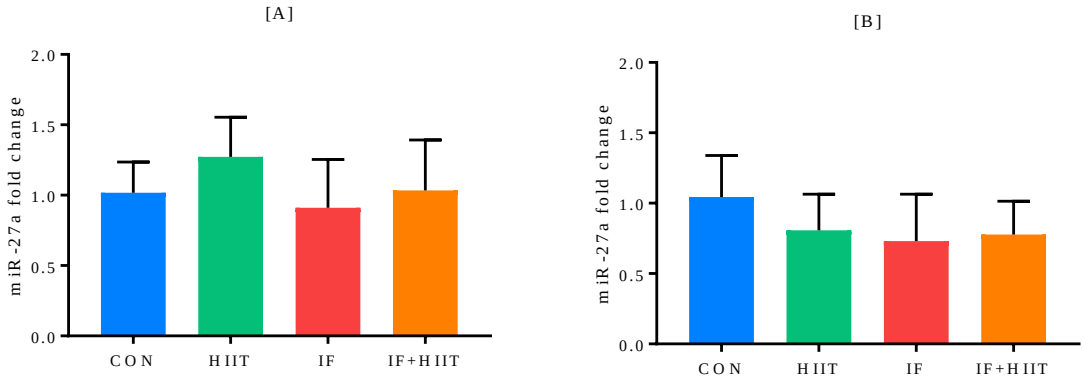

Figure 5.12. Effect of IF, HIIT and IF+HIIT on miR-27a expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation.

5.3.6.2. miR-24

White adipose tissue miR-24 expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.13 (A) and (B) respectively. In males, expression of miR-24 was significantly higher in the HIIT group compared to the CON ($p<0.01$), IF ($p<0.01$) and IF+HIIT ($p<0.01$) groups. In females, no significant difference in miR-24 expression were noted between CON and IF and/or HIIT groups at the end of 12-week intervention period.

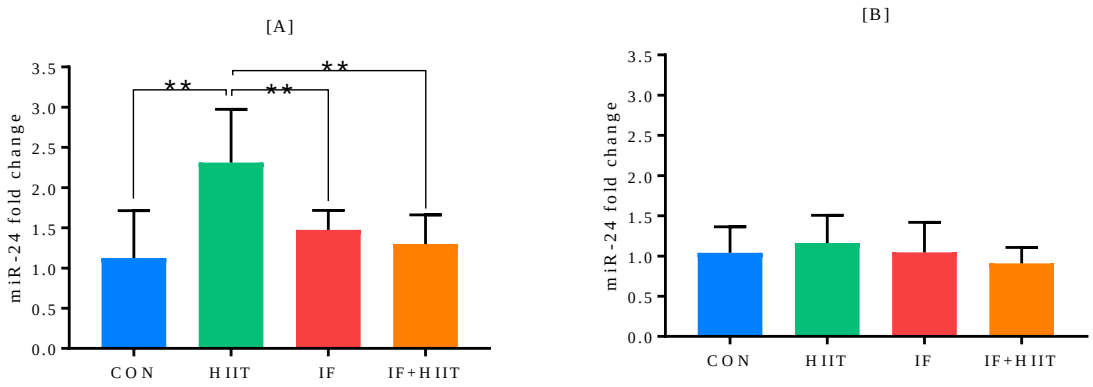

Figure 5.13. Effect of IF, HIIT and IF+HIIT on miR-24 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at p<0.01 level of significance.

5.3.6.3. miR-222

White adipose tissue miR-222 expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.14 (A) and (B) respectively. In males, expression of miR-222 was significantly higher in the HIIT group compared to the IF+HIIT group ($p<0.01$). In females, no significant difference in miR-222 expression were noted between CON and IF and/or HIIT groups at the end of 12-week intervention period.

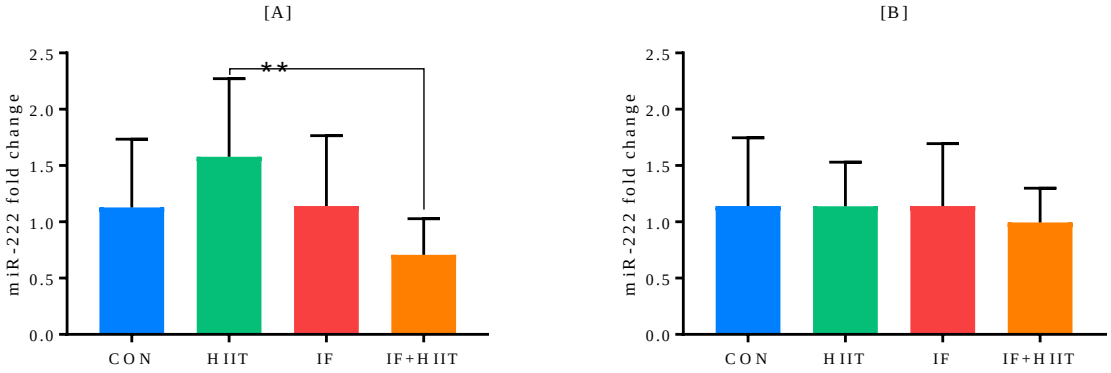

Figure 5.14. Effect of IF, HIIT and IF+HIIT on miR-222 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between groups at p<0.01 level of significance.

5.3.6.4. miR-143

White adipose tissue miR-143 expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.15 (A) and (B) respectively. In males, expression of miR-143 was significantly higher in the HIIT group compared to the CON ($p<0.01$) and IF+HIIT ($p<0.01$) groups. In females, no significant differences in miR-143 expression were noted between CON and IF and/or HIIT groups at the end of 12-week intervention period.

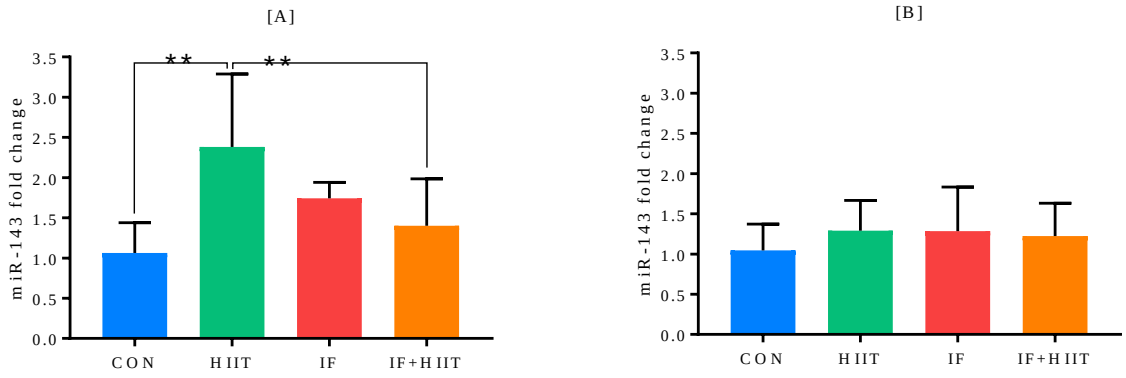

Figure 5.15. Effect of IF, HIIT and IF+HIIT on miR-143 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (**) brackets represent significant difference between groups at $p<0.01$ level of significance.

5.3.6.5. miR-145

White adipose tissue miR-145 expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 5.16 (A) and (B) respectively. In males, HIIT group showed higher expression in comparison to CON ($p<0.01$) and IF+HIIT ($p<0.01$) groups. The IF group showed significantly higher expression compared to CON group ($p<0.05$). In females, expression of miR-145 in CON group was significantly lower than HIIT ($p<0.01$) and IF+HIIT ($p<0.01$) group.

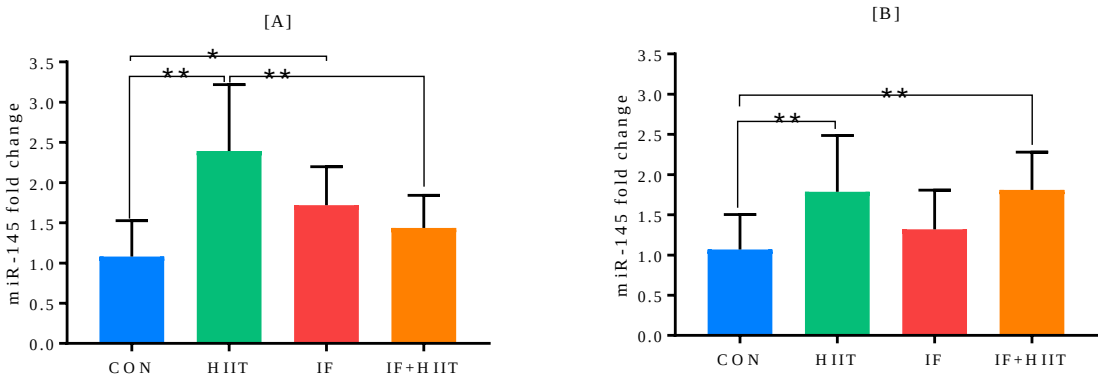

Figure 5.16. Effect of IF, HIIT and IF+HIIT on miR-145 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) brackets represent significant difference between groups at $p<0.05$ and $p<0.01$ level of significance respectively.

5.3.7 Correlation analysis between mRNA and miRNA expression

Pearson correlation coefficient (R) and significance values (p) between gene and miRNA expression in males and females are given in table 5.3 and 5.4 respectively. Significant inverse correlation was found between *HIF1α* gene and miR-222a expression in males HIIT group (R=-0.68, p<0.05) (Figure 5.17 (A)) and between *FOXO1* gene and miR-145 expression in males IF+HIIT group (R=-0.81, p<0.016) (Figure 5.17 (B)).

Table 5.3. Correlation between mRNA and miRNA expression in adipose tissue in males.

	***PPARγ* Vs miR-27**	***FABP4* Vs miR-24**	***HIF1α* Vs miR-222a**	***LEP* Vs miR-143**	***FOXO1* Vs miR-145**
CON	0.91 *(0.01)*	0.68 *(0.07)*	-0.32 *(0.27)*	0.54 *(0.13)*	0.76 *(0.04)*
HIIT	0.70 *(0.03)*	0.29 *(0.26)*	**-0.68** *(0.05)*	-0.52 *(0.11)*	0.55 *(0.10)*
IF	0.52 *(0.09)*	0.15 *(0.36)*	-0.18 *(0.34)*	-0.26 *(0.29)*	0.26 *(0.27)*
IF+HIIT	-0.05 *(0.45)*	-0.49 *(0.13)*	-0.45 *(0.13)*	0.14 *(0.37)*	**-0.81** *(0.01)*

Note-Values represent Pearson correlation coefficient (R) and values in brackets represent p values.

Table 5.4. Correlation between mRNA and miRNA expression in adipose tissue in females.

	***PPARγ* Vs miR-27**	***FABP4* Vs miR-24**	***HIF1α* Vs miR-222**	***Lep* Vs miR-143**	***FOXO1* Vs miR-145**
CON	0.33 *(0.21)*	0.27 *(0.26)*	0.19 *(0.32)*	-0.21 *(0.31)*	-0.45 *(0.13)*
HIIT	0.72 *(0.01)*	0.51 *(0.05)*	0.29 *(0.21)*	-0.54 *(0.07)*	0.49 *(0.08)*
IF	-0.40 *(0.16)*	0.28 *(0.25)*	-0.49 *(0.11)*	0.39 *(0.17)*	0.45 *(0.15)*
IF+HIIT	0.19 *(0.31)*	-0.11 *(0.39)*	-0.17 *(0.33)*	-0.39 *(0.15)*	0.17 *(0.33)*

Note-Values represent Pearson correlation coefficient (R) and values in brackets represent p values.

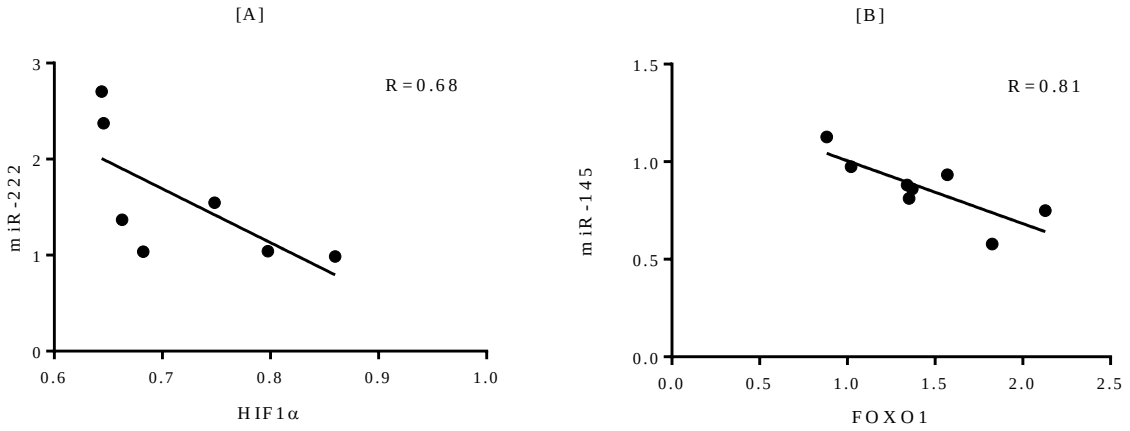

Figure 5.17. Correlation between (A) HIF1α and miR-222a in HIIT group (R=-0.68, p<0.05) and (B) FOXO1 and miR-145a in males from IF+HIIT group (R=-0.81, p<0.016) in males.

5.4. DISCUSSION

The major finding of chapter 5 is that IF with or without HIIT demonstrated greater changes in expression of WAT genes relating to fragmentation of unilocular lipid droplets, lipolysis, fatty acid oxidation and efflux compared to either HIIT and/or CON group. Increased expression of such genes within WAT supports the enhanced fat loss observed in chapter 4. Lower expression levels of leptin, pro-inflammatory markers such as TRPV4 and markers of hypoxia were also observed in the combined group providing further support to the anthropometric findings in chapter 4. Interestingly, the insulin resistant phenotype observed in the IF groups for both male and female mice also displayed higher levels of SIRT1 gene expression within WAT which have recently been reported to be related to systemic insulin resistance (Stefanowicz et al., 2018). Similar to Chapter 4 findings, the changes at the gene level were also gender specific.

5.4.1 Adipose tissue structure and function

White adipose tissue functions as an energy reservoir where excess circulating fatty acids are transported to WAT, converted to triglycerides, and stored as unilocular lipid droplets. Accumulation of fats as unilocular lipid droplets is said to be regulated by CIDEC, also known as fat specific protein 27(Fsp27) (Karbowska and Kochan, 2012, Duncan et al., 2007). In the present study, IF resulted in significantly lower *CIDEC* expression compared to the HIIT and CON group in females and only compared to the HIIT group in males. Expression of *CIDEC* was also lower in the combined IF and HIIT group compared to CON, albeit not significantly. Lower expression of *CIDEC* indicates fragmentation of unilocular lipid droplets into smaller lipid droplets, by promoting phosphorylation of perilipin1 and translocation of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) (Tsiloulis and Watt, 2015), which results in enhanced

lipolysis (Marcinkiewicz et al., 2006). Lower expression of CIDEC following calorie restriction has been reported by previous studies in humans after 12 weeks (Magnusson et al., 2008) and mice after 4 weeks of high fat diet (Wang et al., 2016). Contrary to this, IF (3 days feeding: 3 days fasting) for ~48 days showed increased expression of CIDEC (Karbowska and Kochan, 2012), suggesting that IF might be stimulating lipid deposition in adipose tissue. The Karbowska and Kochan (2012) study used lean rats and thus the effects of IF on CIDEC expression could be opposing, as the majority of studies demonstrating lower expression following reduced caloric intake are on models of diet-induced obesity. Indeed protein expression has been reported to be lower, while gene expression higher following high fat diet (Reynolds et al., 2015). In line with lower levels of CIDEC expression, leptin expression, which is increased with weight gain, was significantly lower in IF and IF +HIIT group compared to the CON group in both males and females. Lower expression of leptin could be reflection of the reduced fat mass accumulation observed in the diet and exercise intervention groups. It could also be driven by *PPARγ*. Leptin expression is negatively regulated by *PPARγ*, with higher expression of *PPARγ* reducing leptin expression (Mousavi et al., 2015, Kersten, 2000). In the present, higher expression levels of *PPARγ* were observed in all diet and/or exercise intervention groups in males only, though only statistically significant in the IF +HIIT group. Furthermore, a significant negative correlation between expression of *PPARγ* and the leptin gene was observed ($R=-0.68$, $p<0.05$) in males. Increased *PPARγ* expression has been linked to amelioration of obesity-related metabolic dysfunction and decreased markers of inflammation within white adipose tissue (Yang et al., 2013). Conversely, *PPARγ* has also been considered a thrifty gene and can mediate high fat diet induced adipocyte hypertrophy (Hara et al., 2000, Auwerx et al., 2003). Though protein

expression of *PPARγ* was not measured, based on the improved body composition observed in chapter 4 and other studies reporting enhanced expression with IF and exercise (Karbowska and Kochan, 2012, Stanford et al., 2015), we can speculate that increased expression of *PPARγ* is having a positive benefit within the adipose tissue, by reducing inflammatory induced dysfunction or affecting the expression of other functional genes.

FOXO1 is a regulator of cellular metabolism and survival in response to nutrient availability and environmental stress by acting as a direct target of insulin action (Kim et al., 2009). Exposure of preadipocytes to insulin along with other adipogenic hormones simultaneously activates cell proliferation and differentiation. It is suggested that role of FOXO1 role in preadipocytes is to help with transition from clonal expansion (i.e. cell cycle) to terminal differentiation, possibly by cooperating with PPARγ to induce a post-mitotic growth arrest that is a prerequisite for the subsequent differentiation (Morrison and Farmer, 1999). In the current study, both IF and IF+HIIT groups in the female mice demonstrated significantly lower expression of FOXO1 compared to the CON group. Although this trend was similar in males, it was not deemed statistically significantly. Indeed, expression of *FOXO1* was found to be correlated ($R=-0.81$, $p<0.01$) with miR-145 in the male IF+HIIT group. MiR-145 has been shown to target *FOXO1* in adipose tissue and its enhanced expression attenuates lipolysis (Lin et al., 2014). Reduced FOXO1 expression would suggest a decrease in transition from clonal expansion (i.e. cell cycle) to terminal differentiation in preadipocytes.

5.4.2 Fatty acid oxidation and trafficking

High fat diet disrupts molecular machinery involved in triacylglycerol (TAG) breakdown and fatty acids release from adipose tissue (Gaidhu et al., 2010). Elevation in

both oxidation and mobilization of fats is necessary for normal functioning of adipose tissue (Gaidhu et al., 2010). In the present study, IF + HIIT in both males and females showed higher *HADH* gene expression relative to IF group, suggesting a higher β-oxidation capacity, as *HADH* is an important enzyme that catalyses the penultimate and rate limiting step in β-oxidation (van Hove et al., 2006, Verhoef et al., 2013). Similarly, IF+HIIT group, but only in male mice, demonstrated an increased expression of *FABP4* compared to all other groups. FABP4 is responsible for intracellular fatty acid trafficking and is an indicator of lipid handling capacity of adipocytes (Verhoef et al., 2013). Superior weight loss in the IF+HIIT group compared to either diet or exercise intervention alone could be a reflection of higher lipid oxidation and efflux. This was further supported by positive correlation found between *HADH* and *FABP4* gene expression in IF+HIIT groups in both males ($R=0.66$, $P<0.05$) and females ($R=0.87$, $P<0.001$) in the present study. Similar correlation between *HADH* and *FABP4* was also reported by another study in humans after 8-weeks of calorie restriction induced weight loss, which reported improved metabolic homeostasis, higher trafficking and β-oxidation of fatty acids (Camps et al., 2015). Further, enhanced oxidation and trafficking has been shown after caloric restriction for 5 weeks (500Kcal/day) with increased expression of *HADH* and *FABP4* in adipose tissue of obese men and women (Bouwman et al., 2014). In contract, IF alone in the present study, rather showed lower *HADH* and *FABP4* gene expression. Low expression indicates low fatty acid mobilisation and efflux from adipose tissue (Hertzel et al., 2002, Furuhashi et al., 2014). In females, HIIT only group also showed higher expression of *FABP4* relative to IF only group. This was in line with the previous study where aerobic exercise training for 10 weeks has been reported to increase the *FABP4* gene expression in adipose tissue of obese Zucker rats (Krskova et al., 2012).

5.4.3 Adipose tissue SIRT1 expression and systemic insulin sensitivity

Obesity is often associated with insulin resistance and type 2 diabetes (Wu and Ballantyne, 2017). Though, IF has been reported to improve insulin sensitivity (Barnosky et al., 2014), IF groups in both males and females and IF+HIIT group in females showed poor insulin sensitivity, even worse than CON group. A recent study has reported that insulin sensitivity in young obese men decreased with obesity and was positively related to *SIRT1* expression in adipose tissue but not muscle (Stefanowicz et al., 2018). *SIRT1*, an NAD+-dependent deacetylase enzyme, is an important nutrient sensor in various metabolic tissues (Chalkiadaki and Guarente, 2012). In the present study, expression of *SIRT1* was found to be suppressed in IF groups in both males and females and IF+HIIT group in females. This pattern was very similar to the insulin resistant state exhibited by these groups. Although the exact explanation behind deterioration of insulin sensitivity in IF groups needs extensive investigation, *SIRT1* suppression in adipose tissue is a contributing factor towards the insulin resistant states of these groups as hypothesised by Stefanowicz et al. (2018).

Treadmill exercise training (30m/min, 45 min/day, 5 days/week) for 6-weeks has been reported to enhance *SIRT1* activity in adipose tissue of rats (Ferrara et al., 2008). In the present study, females in HIIT group showed higher *SIRT1* expression in comparison to both IF and IF+HIIT group. Also in males, combining HIIT with IF resulted in higher expression of *SIRT1*, in comparison to IF only, suggesting that HIIT rescued *SIRT1* expression, which was otherwise downregulated by IF in males. These groups (HIIT in females and IF+HIIT in males) also showed lower insulin resistance in comparison to respective IF only group.

Also, it has been shown that *SIRT1* induces FOXO1 deacetylation which promotes nuclear retention of FOXO1 leading to maintenance of FOXO1 induced signalling pathway and decreased adipose tissue mass (Li et al., 2017b). This is in accordance with the present study where enhanced expression of *SIRT1* gene in males from IF+HIIT group was accompanied by lower fat mass, which could be attributed to *SIRT1* induced deacetylation of FOXO1 suggested by Li et al. (2017b).

5.4.4 Inflammation

Aerobic exercise training for 60min/day, five time/week has been reported to decrease the expression of pro-inflammatory marker *TRPV4* gene in adipose tissue of chow fed mice with lower epididymal fat mass than sedentary group (Castellani et al., 2014) and high fat diet fed obese mice with similar epididymal fat mass as sedentary group (Chen et al., 2015). *TRPV4* is a non-specific calcium ion channel that is highly expressed in adipose tissue (Chen et al., 2015) and is an important regulator of pro-inflammatory cytokines (Ye et al., 2012). In contrast to the previous reports (Castellani et al., 2014, Chen et al., 2015), HIIT group in both males and females in the present study did not show any significant changes in *TRPV4* gene expression, which could be attributed to lower exercise volume and this stimulus used in the present study. Similar changes in T*PV4* expression was noted between males and females from all groups. In males, *TRPV4* was significantly lower in the IF group compared to CON. In females, *TRPV4* was significantly lower in the IF group compared to CON and HIIT. The IF+HIIT was also significantly lower than the CON group.

To the authors knowledge, no reports related to adipose tissue *TRPV4* expression following dietary restriction such as calorie restriction or IF have been published. The lower expression of *TRPV4* in all intervention groups, albeit only significantly in IF group

and combined group (female only) suggests reduced inflammation and potentially enhanced oxidative metabolism and energy homeostasis within adipose tissue (Ye et al., 2012).

5.4.5 Hypoxia

High fat diet induced obesity leads to adipose tissue hypoxia with increased gene expression of *HIF1α* (Fujisaka et al., 2013) an adipose tissue hypoxia marker gene (He et al., 2011). *HIF1α* gene expression of IF only group in males showed significantly lower expression relative to CON group, indicating that reduction in fat mass due to IF was also accompanied by concomitant decrease in adipose tissue hypoxia. Previously, both short term (4 weeks) (Ye et al., 2007) and long term (8 months) (Higami et al., 2006) caloric restriction has been reported to decrease the expression of *HIF1α* gene and protein content in epididymal adipose tissue of mice. Similarly, moderate intensity exercise training for 5 days per week has been reported to decrease the mRNA level of *HIF1α* in epididymal adipose tissue of mice after 8 weeks (Ko and Kim, 2013, Ahn and Kim, 2014) and Wistar rats after 9 weeks (Sakurai et al., 2010). The HIIT only group in the present study showed a declining trend ($p>0.07$) relative to CON group, suggesting that HIIT was capable of minimizing hypoxia in adipose tissue despite lower intensity and frequency in comparison to the previous studies mentioned above. Also, *HIF1α* is a direct target of miR-222 (Chartoumpekis et al., 2012). Expression of miR-222 was also higher in HIIT group relative to IF+HIIT group in males, indicating that lowering of *HIF1α* gene expression in HIIT group could be due to inhibition by miR-222. This was further supported by significant ($p<0.05$) negative correlation ($R=-0.68$) found between *HIF1α* and miR-222 expression in HIIT group in males. Gene expression of HIF1α in IF+HIIT groups in males also showed a lower trend ($p<0.07$) relative to CON, indicating lower

level of hypoxia. Although individually IF and HIIT showed lower hypoxia, combination of IF with HIIT did not show any synergistic effect. Also, this effect of IF and/or HIIT was very gender specific and was only observed in males.

5.4.6 Adipose tissue browning

Interestingly, alternate day fasting (15 cycles of every other day fasting) in mice has recently been reported to induce white adipose tissue browning (Li et al., 2017a), however in the present study, IF with or without HIIT showed no change in adipose tissue browning, as no significant change in *UCP1* gene expression was observed in both males and females. The reason could be the pattern of fasting which was 5:2 (fast:feed day) in the present study and was less extensive (fasting period of 25% of total study period) in comparison to every other day (fasting period-50% of total study period) in the study by Li et al. (2017a). Another reason could be the difference in the WAT depot investigated which was epididymal in the present study and inguinal in the Li et al. (2017a) study. No browning of adipose tissue with exercise was not a big surprise as no change in *UCP1* expression has previously been reported by other studies after 11 weeks (Davis et al., 2017) and 5 weeks of HIIT training (Marcinko et al., 2015).

5.4.7 Expression of miRNAs

The effect of IF with or without HIIT appeared to have minimal impact on the expression of miRNA's investigated in the present study. In contrast, HIIT group demonstrated significantly higher expression in comparison to most groups, especially the control group. It is not readily apparent to the authors as to why the HIIT group displayed significantly higher levels of miR-24, 222, 143 and 145, which are involved in promoting adipogenesis and reducing glucose uptake and insulin sensitivity (Jin et al., 2016, Kang et al., 2013, Deiuliis, 2016). Interestingly, the body composition data in the

present study showed that the HIIT group actually increased their fat mass over time (not significantly), which was opposite to the both the IF and IF+HIIT groups. A confounding factor could be the continuation of high fat and sugar feeding while undertaking HIIT. Increase in appetite because of exercise could lead to higher intakes of fat and sugar. Although difference in food intake between exercise and non-exercise groups could not be determined in the present study due to ethical housing limitations, previous studies have reported higher food intake following acute HIIT in humans (Pomerleau et al., 2004) and eight weeks of HIIT in mice (Wang et al., 2017).

Another unexpected finding was the lower expression of these miRNAs in the control group compared to most other groups, albeit not significantly in most cases. This was opposite to what we would expect, given increase in fat mass within the CON group. This unexpected finding could be explained by pleiotropic effects of this class of molecules. For example, several miRNAs, including miR-143 and 222, exhibit inverse patterns of regulation during adipogenesis compared with later stages of obesity (Xie et al., 2009a), indicating that obesity leads to loss of miRNAs that characterize fully differentiated and metabolically active adipocytes. A major limitation in the current thesis is that we didn't measure the baseline expression values for each intervention group, therefore we are unable to determine the changes over time for each group. We can only determine the expression levels of each miRNA at one time point and compare to each group. The direction of change for miRNA expression may be different depending on the levels at baseline and these miRNAs are probably losing control over their stipulated function due to advancement in obesity state as reported by Xie et al. (2009a).

5.5. CONCLUSION

Body compositional analysis reported in chapter 4 suggested that IF with or without HIIT prevented weight gain by reducing fat mass accumulation in mice despite consuming high fat/sugar diet. Molecular investigation targeted in the present study on adipose tissue revealed that, lower fat mass accumulation observed in this group was associated with fragmentation of unilocular lipid droplets, enhanced lipolysis, fatty acid oxidation and efflux from adipose tissue. The results of the present study, suggest that combination of IF and HIIT, not only prevents fat accumulation by increasing fat oxidation and efflux but also promotes other cellular benefits like preventing inflammation and ameliorates the negative impact of high fat/high sugar diet on adipose tissue structure and functioning. This study also noticed that the insulin resistant state induced by IF in mice reported in chapter 4 was related to suppressed *SIRT1* gene expression in adipose tissue of IF groups, providing a slight hint to the underpinning mechanism behind negative impact of IF on insulin sensitivity and needs an elaborate investigation. Intermittent fasting with or without HIIT does not seems to impact the expression of miRNA investigated in this study, however, HIIT induced the expression of most of them especially in males. This portion of the study needs more in-depth experimental investigation with true baseline and post intervention controls to understand the behaviour and involvement of these miRNAs on exercise induced adaptations in adipose tissue under high caloric intake.

CHAPTER 6

INTERMITTENT FASTING AND HIGH INTENSITY INTERVAL TRAINING DISPLAY OPPOSING EFFECTS ON MARKERS OF SKELETAL MUSCLE OXIDATIVE METABOLISM, INSULIN SIGNALLING, INFLAMMATION AND ATROPHY IN DIET-INDUCED OBESE MICE

6.1. INTRODUCTION

Both chapter 4 and 5 have described superior effects of IF when combined with HIIT at a whole body level, with significantly less weight gain, particularly in the form of adipose tissue, and at a molecular level, with greater changes in expression of genes relating to fatty acid oxidation, efflux, and fat accumulation compared to either IF or HIIT alone. Given the detrimental effects of HFD on skeletal muscle function such as development of insulin resistance and the reports that fasting may induce lean muscle mass loss, which is counterproductive to long term ability to maintain weight loss, we wanted to continue examine changes at whole muscle level and molecular level with gene and miRNA targets related to oxidative metabolism, mitochondrial function, inflammation, insulin signalling and atrophy.

High fat/high sucrose feeding for 28 days has been reported to increase adipogenesis, muscle atrophy and inflammation in muscle indicated by increased expression peroxisome proliferator activated receptor gamma (PPARγ), muscle ring finger 1 (MURF1) and macrophage chemo attractant protein-1 (MCP1) (Collins et al., 2016). Intermittent fasting and HIIT have the capacity to reverse the negative effects of high calorie intake at body composition level and also in adipose tissue as mentioned in the previous chapters. In addition to this, few studies have also reported that IF in skeletal

muscle favours glycogen replenishment by increasing phosphorylation of glycogen synthase, and decreased muscle protein synthesis indicated by decreased phosphorylation of mammalian target of rapamycin (mTOR) (Soeters et al., 2009) and increased lipid oxidation indicated by increased expression of carnitine palmitoyltransferase I (CPT1) (Heilbronn et al., 2005a). Likewise, HIIT has been linked to enhanced skeletal muscle glucose uptake, mitochondrial biogenesis, improved insulin signalling indicated by enhanced expression of GLUT4 in obese mice (Davis et al., 2017), and diabetic humans (Little et al., 2011), increased peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) expression (Little et al., 2010), and increased insulin-stimulated superoxide dismutase (SOD) activity, c-Jun N-terminal kinase (JNK), P38 mitogen-activated protein kinases (p38MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) phosphorylation in obese men (Parker et al., 2016). However, the effects of IF and HIIT alone or in combination on energy sensing pathways such AMPK/SIRT1, oxidative metabolism, insulin signalling and fibre type shifts require further investigation.

Relatively recently, microRNAs (miRNAs) have been reported to regulate various cellular pathways governing skeletal muscle structure and function in normal and obese state (Wilson et al., 2017). Muscle specific miRNAs like miR-1, miR-133a and miR-133b, known as myomirs are downregulated in skeletal muscle of high fat diet induced obese mice (Chen et al., 2012). In contrast, acute endurance training results in upregulation of miR-133a and miR-133b in skeletal muscle (Russell et al., 2013). Exercise induced increase in expression of miR-133a has been related to mitochondrial biogenesis (Nie et al., 2016). However, the fate of these and other muscle specific

miRNAs in response to IF and HIIT in diet induced obese mice is unknown and warrants investigation.

Thus, the objective of this study was to evaluate the effect of IF with and without HIIT on skeletal muscle expression of genes related to mitochondrial function, metabolism, muscle atrophy, insulin signalling, inflammation, expression of muscle specific miRNAs and changes in muscle mass and fibre type. We hypothesised that combination of IF and HIIT will show a synergistic effect on changing the muscle fibre composition from fast to slow-twitch fibre type and increase mitochondrial function, re-establish the co-ordinated upregulation of β-oxidation and downstream targets (TCA cycle, ETC and NADH shuttle system), which is generally not coordinated under obese conditions (Muoio et al., 2008) as well as will reduce inflammation and muscle atrophy in diet induced obese mice.

6.2. MATERIALS AND METHODS

6.2.1 Animals

Details of animal procurement, housing and diet are mentioned in chapter 3 section 3.2. Briefly, this study included four different groups namely: no intervention (CON; Males=7, Females=9); intermittent fasting (IF; Males=8, Females=10), high intensity intermittent exercise (HIIT; Males=8, Females=10) and a combination of the dietary and exercise intervention (IF+HIIT; Males=8, Females=10). All groups were maintained on high fat/high sugar (HF/S) diet for 12 weeks to induce obesity and then underwent respective interventions for another 12 weeks while still consuming HF/S diet. Details of interventions is given in chapter 3 section 3.4. After 12 weeks of HF/S feeding and intervention, all mice were killed and tested as described below.

6.2.2 Culling and tissue collection

Mice were anaesthetised with intraperitoneal injection of pentobarbitone (60 mg/kg). Once anaesthesia is conformed using pupillary and toe pinching reflex, skin is removed from the left and right hind legs. Left extensor digitorum longus (EDL) muscle was removed and snap frozen for RNA extraction. Right EDL and soleus muscles were removed, covered with Optimum Cutting Temperature (OCT) compound and froze in isopentane bath cooled in liquid nitrogen for fibre typing. All muscle samples were stored in -80°C freezer until further analysis.

6.2.3 Myosin ATPase Fibre typing

EDL and soleus muscles were sliced to a thickness of 10μm in cryostat (Leica Biosystems) with chamber temperature set at -19°C and sample holder temperature set at -16°C. Tissue sections were collected on adhesive slides, air dried at room temperature and then kept at 4^{o}C until further processing. Slides were incubated in acid pre-incubation solution (54.3mM sodium acetate and 32.6mM sodium barbital adjusted to pH 4.60) for 7 minutes. Slides were washed with a wash solution (pH 7.8) containing 18mM calcium chloride ($CaCl_{2)}$ and 100mM TRIS-HCl. Slides were then incubated in ATP incubation solution for 45 minutes at room temperature on a rocking platform. Following this slides were incubated in 11mM $CaCl_2$ solution for 3 minutes and this step was repeated three times. Slides were then incubated in 2% cobalt chloride ($CoCl_2$) for 1.5 minutes and this step was repeated one more time. After this slides were incubated for 30 seconds in 10mM Sodium barbital and this step was repeated for another 5 times. Slides were then incubated for 1 minute in 1% ammonium sulphide in a fume cupboard. Slides were then washed with tap water followed by 5 consecutive washings in distilled water. Slides were dehydrated in ethyl alcohol solutions of increasing concentration (70, 80, 90, 95 and

100%) by incubating the slides for 2 minutes in each alcohol solution. Slides were rinsed in xylene for 5 minutes. Coverslips were mounted on glass slides using Dibutylphthalate Polystyrene Xylene (DPX). Images were collected using an Olympus BX53 microscope with Olympus Cellsens imaging software. From each section 100 fibres (both types) were counted and percentage of type I and IIa in soleus and IIa and IIb in EDL was calculated out of them (Ranatunga and Thomas, 1990). Dark fibres were identified as type I in soleus and type IIb in EDL muscles and light fibres were identified as type IIa in both soleus and EDL muscles.

6.2.4 Skeletal muscle gene and miRNA expression

Total RNA was extracted form whole left EDL muscle, reverse transcribed to generate cDNA and subjected to RT-PCR for gene and miRNA expression. Details of mRNA and miRNA isolation, cDNA synthesis, RT-PCR analysis and calculation of fold change are given in chapter 3, section 3.7. Peptidylprolyl isomerase A (*Ppia*) and RNU6 were used to normalize mRNA and miRNA expression data respectively. The primers for genes and miRNAs were purchased from and designed by QIAGEN (Australia). The list of genes and miRNAs targeted in this study are given in table 6.1 and table 6.2 respectively.

Table 6.1. List of targeted genes with full official names, NCBI reference sequence numbers and Qiagen catalogue number.

Gene symbol	Official full name	NCBI Reference sequence number	Qiagen Catalogue number
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	NR_027710	PPM03360I
AMPK	Protein kinase AMP-activated catalytic subunit alpha 2	NM_178143	PPM29410F
SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1	NM_001159589	PPM05054A
CPT1	Carnitine palmitoyltransferase Ib	NM_009948	PPM57688A
CS	Citrate synthase	NM_026444	PPM29621B
COX-IV	Cytochrome c oxidase subunit 4 isoform 2	NM_053091	PPM32878E
TNFα	Tumor necrosis factor-alpha	NM_013693	PPM03113G
AS160	Akt substrate of 160 kDa	NM_001081278	PPM40317A
UCP3	Uncoupling protein 3	NM_009464	PPM25342F
MAFbx	F-box protein 32	NM_026346	PPM38061A
MuRF1	Muscle RING finger 1	NM_001039048	PPM61645B
Ppia	Peptidylprolyl isomerase A	NM_008907	PPM03717B

Table 6.2. List of miRNAs, miRbase accession numbers and Qiagen miScript Primer Assay Catalog Numbers.

Mature miRNA ID	miRBase Accession No.	Qiagen miScript Primer Assay Catalog No.
mmu-miR-696	MIMAT0003483	MS00002870
mmu-miR-133a-3p	MIMAT0000145	MS00007294
mmu-miR-133b-3p	MIMAT0000769	MS00007301

6.2.5 Statistical analysis

Data is presented as mean ± standard deviation. To evaluate the effect of interventions, intervention groups namely HIIT, IF, and IF+HIIT were compared against no intervention CON group. Difference in mRNA expression and miRNA expression between groups were analysed using one-way ANOVA and Tukey's test was used as post hoc analysis. Correlation between mRNA and miRNA expression in each intervention group was calculated with Pearson correlation test for *PGC1α* Vs miR-696, *TNFα* Vs

miR-133a and *MURF1* Vs miR-133b. An alpha level of 0.05 was adopted throughout to reduce Type I statistical errors.

6.3. RESULTS

6.3.1 The effect of IF and/or HIIT on skeletal muscle mass and morphology

6.3.1.1. Muscle weight

Weight of soleus and EDL muscles following 12 weeks of IF and/or HIIT in both male and female mice are presented in Figure 6.1 (A&C) and (B&D) respectively. Muscle weight for both soleus and EDL muscles were similar between control and IF and/or HIIT groups at the end of the 12-week intervention period in both males and females.

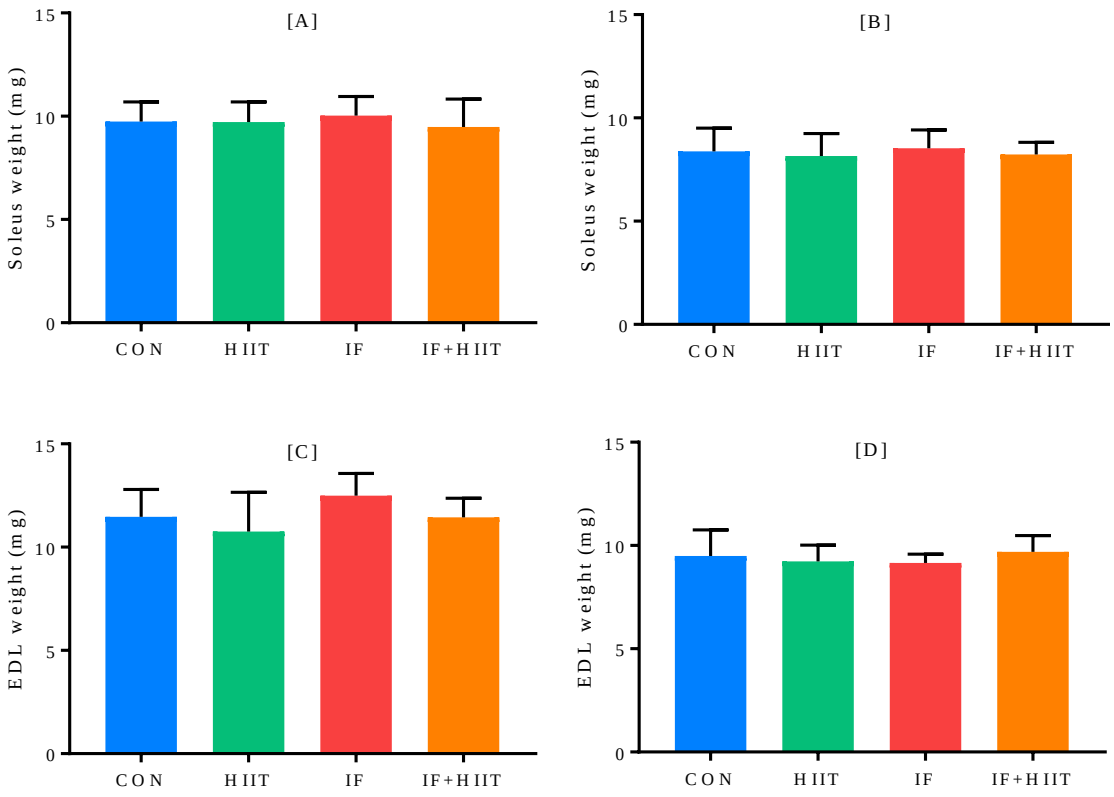

Figure 6.1. Effect of IF, HIIT and IF+HIIT on weight (mg) of soleus muscle in (A) males and (B) females and EDL muscle in (C) males and (D) females. Bars represent means, error bars represent standard deviation.

6.3.1.2. Muscle fibre type

Muscle fibre type I and IIa in soleus muscle and IIa and IIb in EDL muscle following 12 weeks of IF and/or HIIT are presented as percentage (bars) in Figure 6.2 and 6.3 respectively. Representative images showing fibre type distribution in (A) soleus and (B) EDL muscle are presented in figure 6.3A and B respectively. No significant differences in fibre type distribution for both soleus and EDL muscle were noted between control and IF and/or HIIT groups at the end of the 12-week intervention period in both males and females.

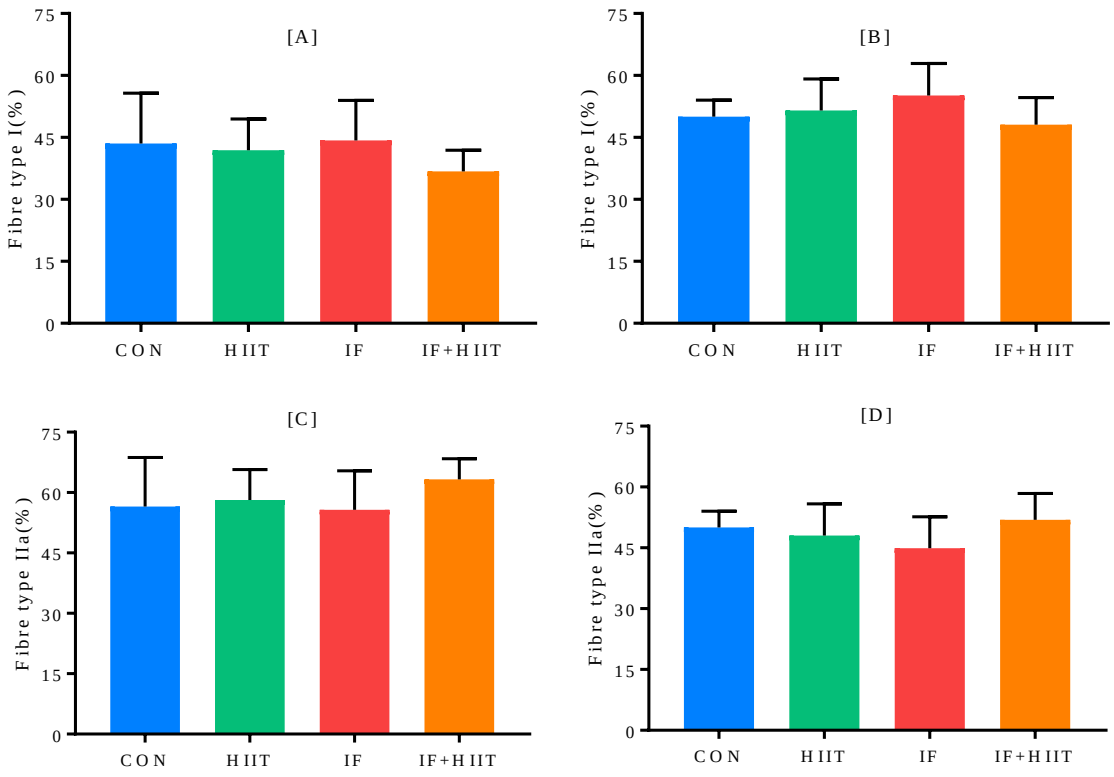

Figure 6.2. Effect of IF, HIIT and IF+HIIT on soleus muscle type I fibre in (A) males and (B) females and type IIa fibre in (C) males and (D) females. Bars represent means, error bars represent standard deviation.

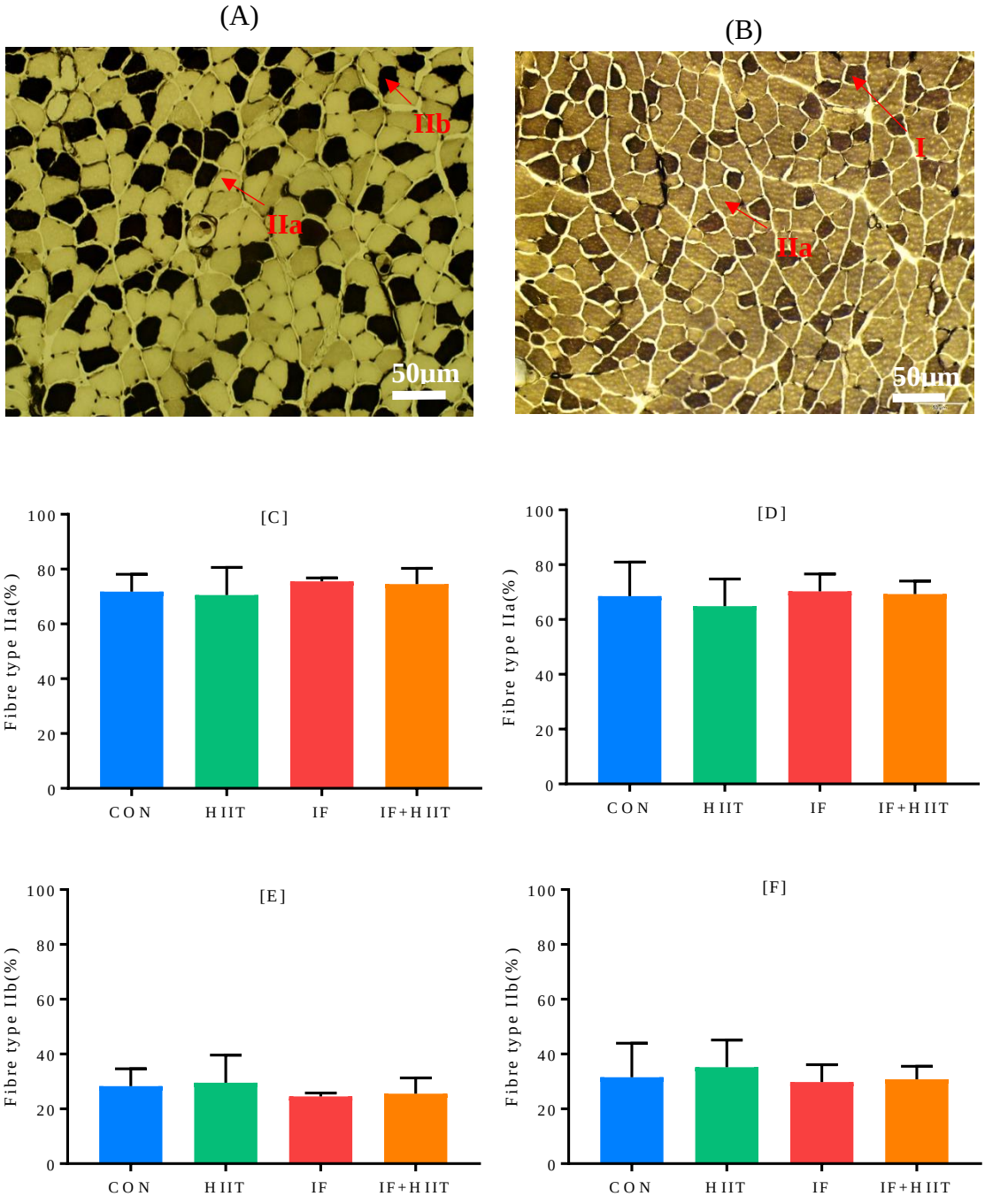

Figure 6.3. Representative images of (A) soleus and (B) EDL muscle showing fibre type distribution. Effect of IF, HIIT and IF+HIIT on ED muscle type IIa fibre in (C) males and (D) females and type IIb fibre in (E) males and (F) females. Bars represent means, error bars represent standard deviation.

6.3.2 The effect of IF and/or HIIT on gene expression of AMPK-SIRT1-PGC1α axis

6.3.2.1. AMPK

Muscle *AMPK* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.4 A and B respectively. In males, *AMPK* gene expression in IF group was significantly lower ($p<0.01$) than IF+HIIT group. In females, no significant differences were observed in AMPK gene expression between control and IF and/or HIIT groups at the end of 12-week intervention period.

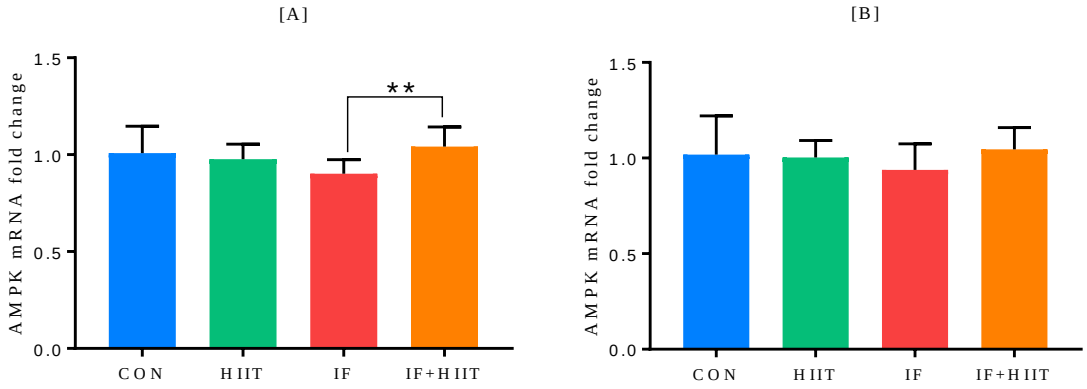

Figure 6.4. Effect of IF, HIIT and IF+HIIT on *AMPK* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.2.2. SIRT1

Muscle *SIRT1* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.5 A and B respectively. *SIRT1* gene expression in IF group was significantly lower than both CON and HIIT groups in both males and females ($p<0.01$), and significantly lower than IF+HIIT group in males only ($p<0.01$). The IF+HIIT group displayed significantly lower expression of *SIRT1* in comparison to HIIT group in females ($p<0.01$) but not in males.

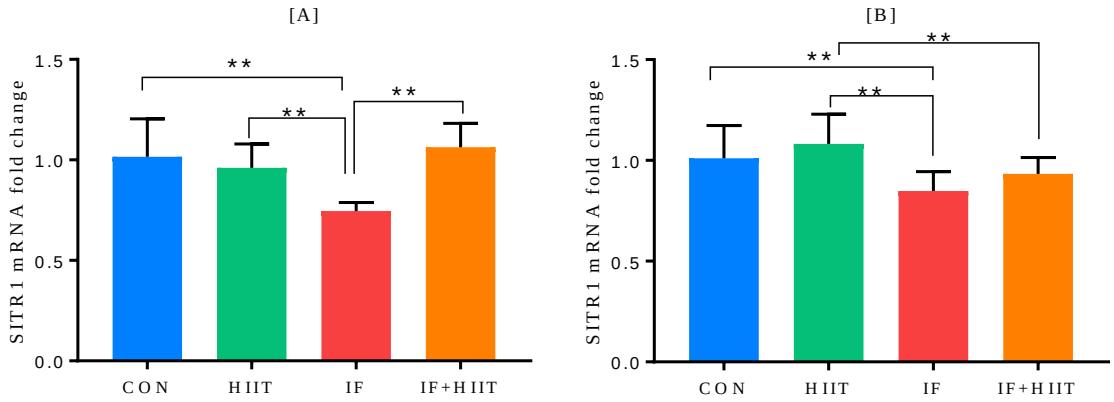

Figure 6.5. Effect of IF, HIIT and IF+HIIT on *SIRT1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at $p<0.01$ level of significance.

6.3.2.3. PGC1α

Muscle *PGC1α* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.6 A and B respectively. Gene expression of *PGC1α* in IF group was significantly lower than CON group ($p<0.05$) in males and significantly lower than HIIT groups ($p<0.05$) in females. Also in females, gene expression of PG*C1α* in IF group showed lower trend ($p<0.064$) than CON group.

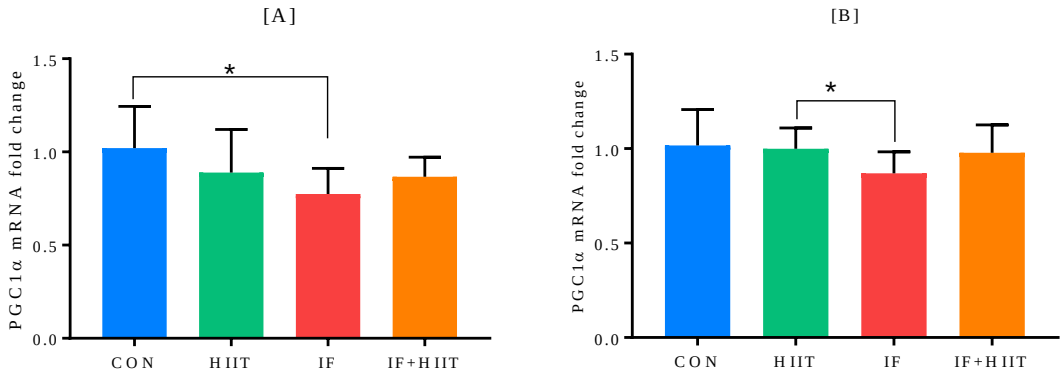

Figure 6.6. Effect of IF, HIIT and IF+HIIT on *PGC1α* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.3 The effect of IF and/or HIIT on gene expression related to oxidative metabolism

6.3.3.1. CPT1

Muscle *CPT1* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.7 A and B respectively. *CPT1* gene expression in IF group was significantly ($p<0.01$) lower than HIIT group in both males and females and both CON ($p<0.01$) and IF+HIIT ($p<0.01$) groups in males only.

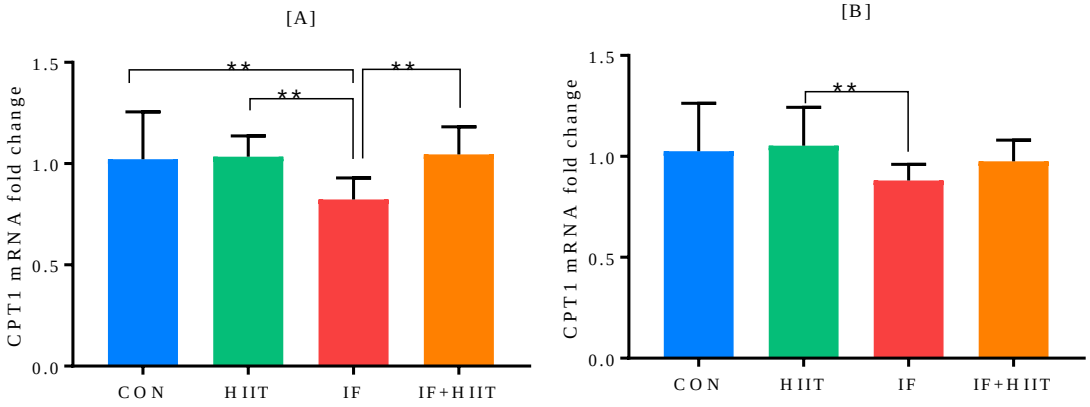

Figure 6.7. Effect of IF, HIIT and IF+HIIT on *CPT1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance.

6.3.3.2. Citrate Synthase

Muscle citrate synthase *(CS)* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.8 A and B respectively. Citrate synthase (*CS*) gene expression in CON group was significantly ($p<0.05$) higher than IF group in males. In females, no significant differences were observed in *CS* gene expression between different groups at the end of 12-week intervention period.

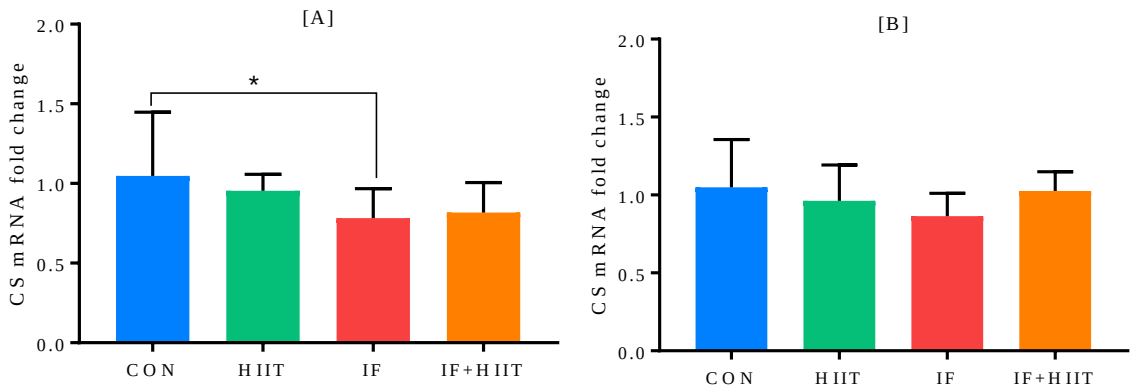

Figure 6.8. Effect of IF, HIIT and IF+HIIT on citrate synthase *(CS)* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.3.3. COX-IV

Muscle *COX-IV* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.9 A and B respectively. *COX-IV* gene expression in IF+HIIT group was significantly ($p<0.05$) higher than HIIT group in females. In females, IF+HIIT group showed a higher trend ($p<0.064$) for *COX-IV* gene expression relative to CON group. In males, no significant differences were observed in *COX-IV* gene expression between different groups at the end of 12-week intervention period.

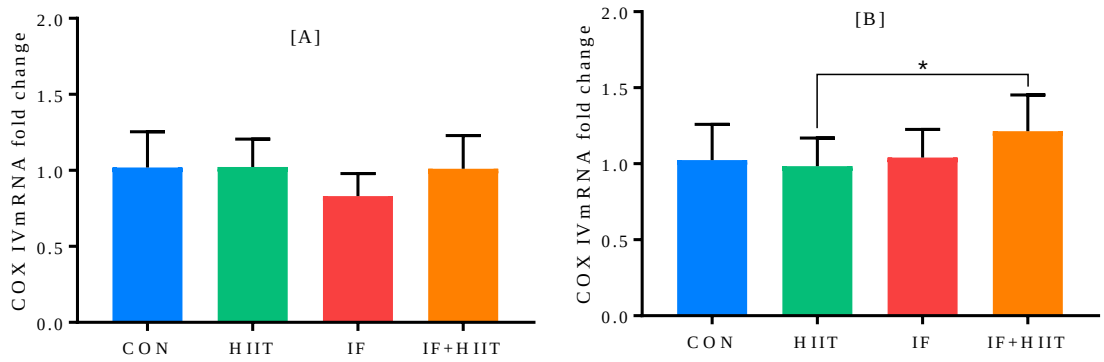

Figure 6.9. Effect of IF, HIIT and IF+HIIT on *COX-IV* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.3.4. UCP3

Muscle *UCP3* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.10 A and B respectively. In both males and females gene expression of *UCP3* in CON group was significantly higher($p<0.01$) than IF group. Also, in both males and females, *UCP3* gene expression in IF group was significantly ($p<0.01$) lower than both HIIT and IF+HIIT groups. *UCP3* gene expression of HIIT group was higher in comparison to CON in males ($p<0.05$).

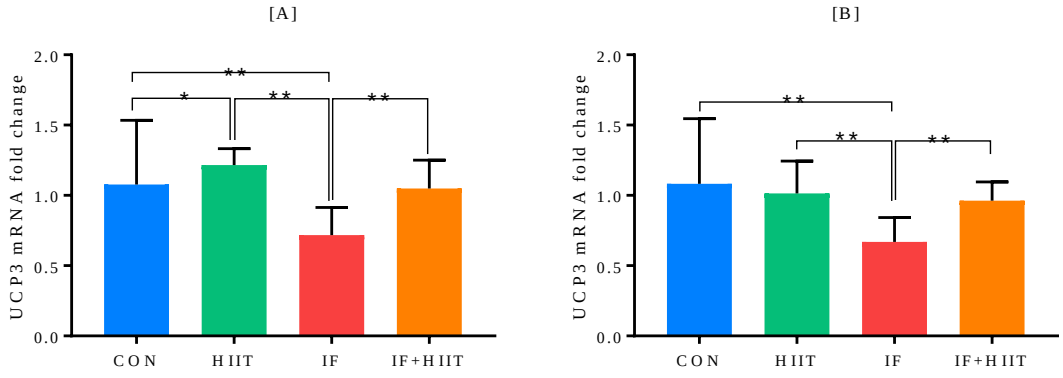

Figure 6.10. Effect of IF, HIIT and IF+HIIT on *UCP3* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) bracket represent significant difference between group at $p<0.05$ and $p<0.01$ level of significance respectively.

6.3.4 The effect of IF and/or HIIT on gene expression related to Inflammation

6.3.4.1. TNF

Muscle TNF*α* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.11 A and B respectively. In males, *TNFα* gene expression in CON group was significantly ($p<0.01$) higher than HIIT, IF and IF+HIIT groups in males but not in females. There were no significant differences in *TNFα* gene expression between IF, HIIT and IF+HIIT intervention groups in both male and females at the end of 12-week intervention period.

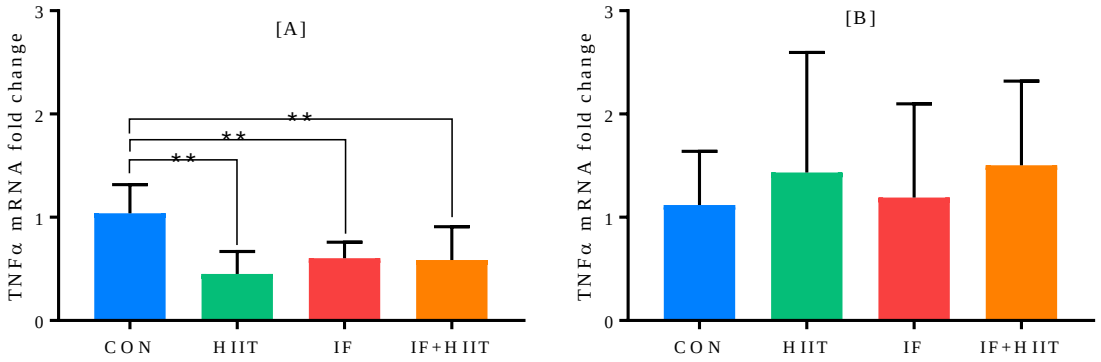

Figure 6.11. Effect of IF, HIIT and IF+HIIT on *TNFα* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance.

6.3.5 The Effect of IF and/or HIIT on gene expression related to Insulin signalling

6.3.5.1. AS160

Muscle *AS160* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.12 A and B respectively. In males, *AS160* gene expression in IF+HIIT group was significantly higher ($p<0.01$) than IF group. In females, no significant differences were observed in *AS160* gene expression between different groups at the end of 12-week intervention period.

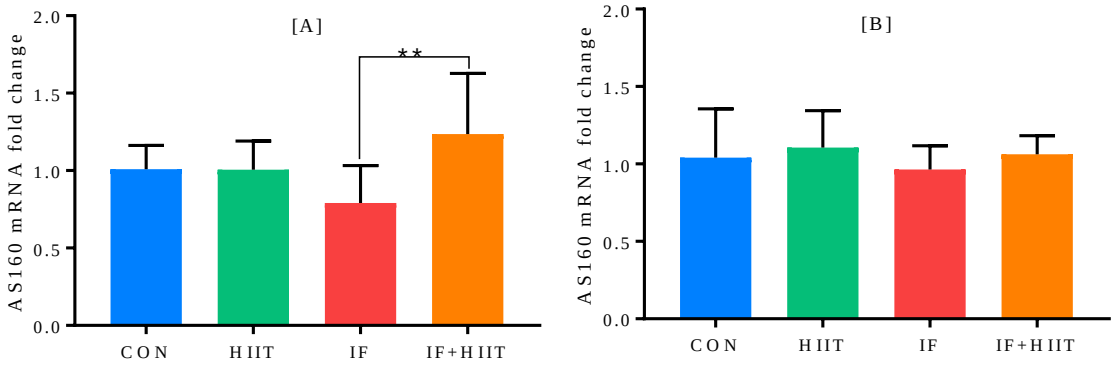

Figure 6.12. Effect of IF, HIIT and IF+HIIT on *AS160* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at $p<0.01$ level of significance.

6.3.6 The effect of IF and/or HIIT on gene expression related to muscle atrophy

6.3.6.1. MAFbx

Muscle *MAFbx* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.13 A and B respectively. Gene expression of *MAFbx* in CON group was significantly lower ($p<0.01$) than HIIT group in males. Also, HIIT group showed significantly higher expression relative to both IF ($p<0.01$) and IF+HIIT ($p<0.01$) groups. Gene expression of *MAFbx* in IF group was significantly lower ($p<0.01$) than IF+HIIT group in males but in females, no significant difference was observed in both these groups. In females, *MAFbx* gene expression was significantly higher ($p<0.01$) in both CON and HIIT groups in comparison to both IF and IF+HIIT groups.

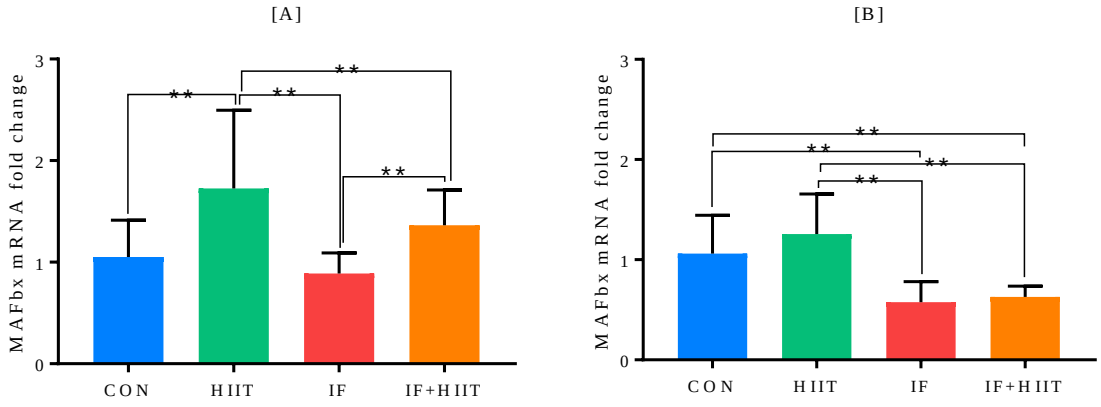

Figure 6.13. Effect of IF, HIIT and IF+HIIT on *MAFbx* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (**) bracket represent significant difference between group at p<0.01 level of significance.

6.3.6.2. MURF1

Muscle *MuRF1* gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.14 A and B respectively. *MuRF1* gene expression in CON group was significantly lower than HIIT group in both males ($p<0.01$) and females ($p<0.05$). In both males and females, HIIT group showed significantly higher expression of *MuRF1* gene in comparison to both IF ($p<0.01$) and IF+HIIT ($p<0.01$) groups. In females, *MuRF1* gene expression was significantly higher in CON group in comparison to IF ($p<0.01$) and IF+HIIT ($p<0.01$) groups.

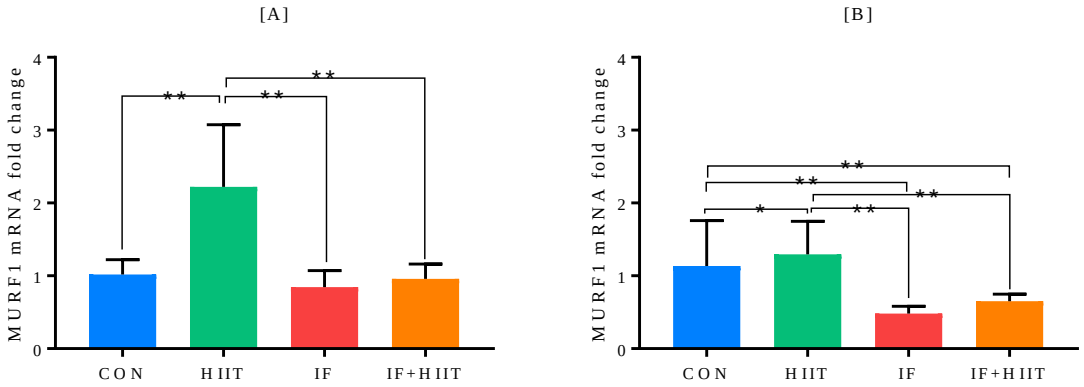

Figure 6.14. Effect of IF, HIIT and IF+HIIT on *MURF1* gene expression (mRNA fold change) relative to CON group in (A) males and (B) females. Gene (mRNA) expression is normalized to *ppia* gene expression. Bars represent means, error bars represent standard deviation. Asterisk (*) and (**) bracket represent significant difference between group at $p<0.05$ and $p<0.01$ level of significance respectively.

6.3.7 The effect of IF and/or HIIT on expression of myomiRs

6.3.7.1. miR-696

Muscle miR-696 gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.15 A and B respectively. In males, miR-696 showed significantly higher expression in IF+HIIT group in comparison to CON ($p<0.05$) and HIIT ($p<0.05$) groups. In females, miR-696 expression was significantly higher in both IF ($p<0.05$) and IF+HIIT ($p<0.05$) groups in comparison to CON group.

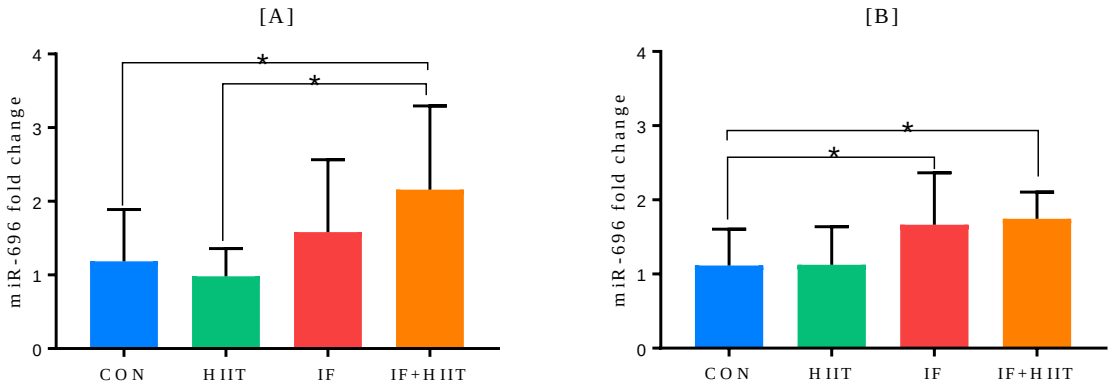

Figure 6.15. Effect of IF, HIIT and IF+HIIT on miR-696 expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.7.2. miR-133a

Muscle miR-133a gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.16 A and B respectively. In females, IF+HIIT group showed significantly lower expression of miR-133a in comparison to CON ($p<0.05$), IF ($p<0.05$) and HIIT ($p<0.05$) groups. In males, no significant differences in miR-133a expression were observed between different groups at the end of 12-week intervention period.

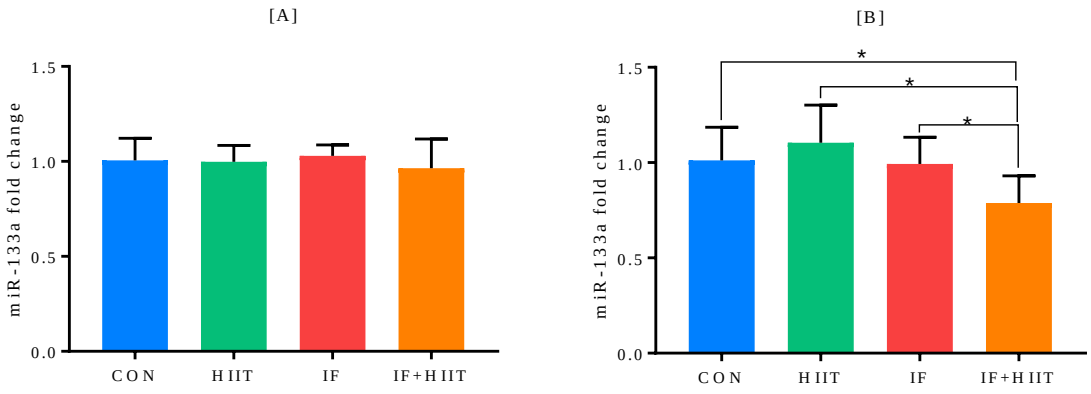

Figure 6.16. Effect of IF, HIIT and IF+HIIT on miR-133a expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.7.3. miR-133b

Muscle miR-133b gene expression following 12 weeks of IF and/or HIIT in both male and female mice are presented as fold changes relative to CON group in Figure 6.17 A and B respectively. In females, IF group showed significantly higher ($p<0.05$) expression of miR-133a in comparison to CON ($p<0.05$) and IF+HIIT ($p<0.05$) groups. No significant differences were observed in miR-133a expression between HIIT group and CON and other intervention groups. In males, no significant differences in miR-133a expression were observed between different groups at the end of 12-week intervention period.

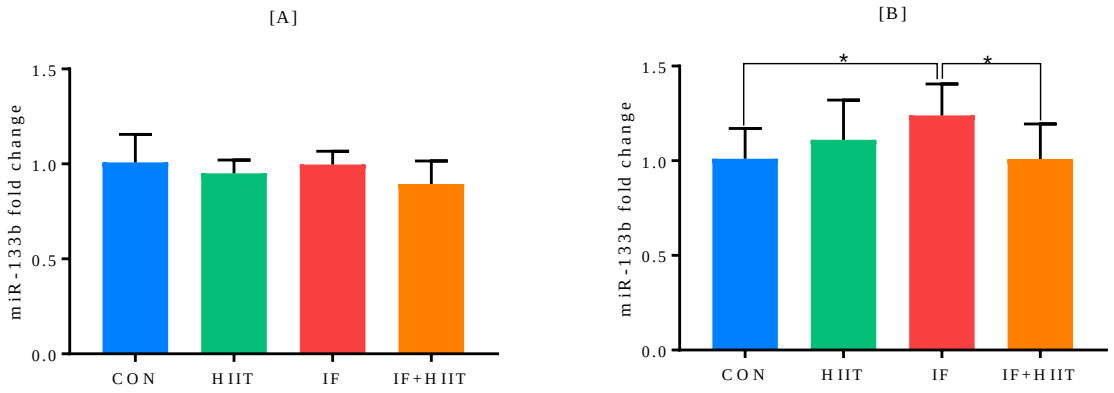

Figure 6.17. Effect of IF, HIIT and IF+HIIT on miR-133b expression (fold change) relative to CON group in (A) males and (B) females. MiRNA expression is normalized to *RNU6* expression. Bars represent means, error bars represent standard deviation. Asterisk (*) bracket represent significant difference between group at $p<0.05$ level of significance.

6.3.8 Correlation analysis between mRNA and miRNA expression

Pearson correlation coefficient (R) and significance values (p) between gene and miRNA expression in males and females are given in table 6.3 and 6.4 respectively. Gene expression and miRNA expression between each group in both males and females did not show any significant correlation in any of the compared mRNA and miRNAs at the end of 12-week intervention period.

Table 6.3. Pearson correlation coefficient (R) between mRNA and miRNA expression in males.

	***PGC1α* vs miR-696**	***TNFα* Vs miR-133a**	***MURF1* Vs miR-133b**
CON	-0.31 *(0.30)*	0.44 *(0.28)*	0.77 *(0.06)*
HIIT	0.21 *(0.32)*	-0.25 *(0.30)*	0.14 *(0.39)*
IF	-0.28 *(0.26)*	-0.52 *(0.12)*	-0.43 *(0.17)*
IF+HIIT	0.31 *(0.23)*	-0.52 *(0.10)*	-0.53 *(0.09)*

Table 6.4. Pearson correlation coefficient (R) between mRNA and miRNA expression in females.

	***PGC1α* vs miR-696**	***TNFα* Vs miR-133a**	***MURF1* Vs miR-133b**
CON	-0.36 *(0.21)*	-0.20 *(0.35)*	-0.29 *(0.29)*
HIIT	0.84 *(0.01)*	-0.42 *(0.21)*	-0.21 *(0.35)*
IF	-0.27 *(0.25)*	-0.20 *(0.31)*	0.61 *(0.06)*
IF+HIIT	-0.42 *(0.16)*	-0.34 *(0.21)*	-0.39 *(0.17)*

6.4. DISCUSSION

In the present study, short burst of high intensity interval training resulted in increased mitochondrial biogenesis, oxidative metabolism, fatty acid oxidation and ATP production in skeletal muscle. However, IF had an opposite effect on skeletal muscle manifested as lower mitochondrial biogenesis, oxidative metabolism, fatty acid oxidation, ATP production and insulin sensitivity suggested by the expression of marker genes related to them. The combination of IF and HIIT did not show any synergistic effect, however various marker genes especially related to oxidative metabolism (*SIRT1*, *AMPK*, *CPT1*, *AS160*) showed the expression level higher than IF only group indicating that HIIT was able to rescue their expression level and mitigating the effect of IF on lowering metabolism. However, this role is reversed in atrophic gene expression as atrophy induced by HIIT was mitigated by IF in IF+HIIT in both males and females. Although the effect was gender specific, both IF and HIIT alone or in combination showed decline in obesity induced inflammation in males. Even though no superior effect of combining IF with HIIT has been observed, combination of these two interventions can induce beneficial adaptations in skeletal muscle with either one of them compensating the unfavourable effects of other and keeping the balance.

6.4.1 AMPK-SIRT1-PGC1α signalling axis

Activation of AMPK and SIRT1 is necessary for induction of PGC1α which is a positive regulator of mitochondrial biogenesis and respiration, adaptive thermogenesis, gluconeogenesis as well as many other metabolic processes (Austin and St-Pierre, 2012). Conditions that create energy deficit like exercise and reduced caloric intake, upregulate AMPK/SIRT1/PGC1α signalling axis (Canto and Auwerx, 2009). For instance, *PGC1α* expression increases in skeletal muscle with acute exercise (running with swimming) in

rats (Terada and Tabata, 2004), chronic exercise training for 10-days in humans (Stepto et al., 2012) and acute HIIT in men (Gibala et al., 2009). In the present study, females in HIIT group showed higher expression of *PGC1α* compared to IF only group. Likewise, HIIT group showed higher expression of SIRT1 in comparison to IF in both males and females and compared to IF+HIIT group in females. Higher expression of SIRT1 has been reported previously after single bout of endurance exercise in skeletal muscle of rats (Suwa et al., 2008) and after six weeks of HIIT in humans (Gurd et al., 2010). Higher *SIRT1* expression in these groups suggests energy deficit which increases NAD^+/NADH ratio, which in turns triggers the activity of SIRT1 and likewise the expression level also (Canto and Auwerx, 2009). Higher expression of SIRT1 leads to deacetylation of phosphorylated PGC1α. Phosphorylated PGC1α activates its own promoter and enhances its expression (Ruderman et al., 2010), which in turn upregulates mitochondrial biogenesis and respiration, adaptive thermogenesis and gluconeogenesis (Austin and St-Pierre, 2012), increased capacity for generation of ATP, increased angiogenesis and increased glucose uptake, likely via increase in *GLUT4* expression (Chan and Arany, 2014, Wende et al., 2007, Michael et al., 2001). In addition to this, expression of *UCP3* in HIIT group was higher in comparison to IF only group in both males and females which may be attributed to higher triglyceride content of skeletal muscle and increased markers of de novo lipogenesis in response to exercise, which is typical following HIIT (Davis et al., 2017). UCP3 has been hypothesised to provides protection against (lipid-induced) mitochondrial dysfunction or oxidative stress (Schrauwen et al., 2001) and its higher expression in HIIT group suggests its beneficial effects on skeletal muscle.

Caloric restriction induced negative energy balance has been shown to enhance the expression of PGC1α in skeletal muscle of overweight males and females after 6-

months (Civitarese et al., 2007) and enhanced expression of SIRT1 in high fat diet fed mice after 3 months (Chen et al., 2008a). Intermittent fasting is considered to induce body compositional changes similar to caloric restriction and transcriptional profile of calorie restriction and intermittent fasting has been found to be similar (Pearson et al., 2008), suggesting that IF should induce AMPK/SIRT1/PGC1α signalling axis. Surprisingly, all the components of this axis were found to be suppressed in IF group in the present study. Intermittent fasting group showed lower *PGC1α* gene expression in comparison to CON in males and HIIT group in females. Along the same line, IF group showed lower expression of *AMPK* in comparison to IF+HIIT in males. Also, SIRT1 expression in IF group was lower in comparison to CON and HIIT groups in both males and females and also compared to IF+HIIT group in males. Downregulation of AMPK/SIRT1/PGC1α observed in IF group could be the reason behind lower glucose tolerance and insulin sensitivity observed in IF mice in the present study, as upregulation of *PGC1α* is related to insulin sensitivity (Gastaldi et al., 2007). Previous IF studies have reported no change in PGC1α expression in response to 22 days of alternate day fasting in human skeletal muscle (Heilbronn et al., 2005a). This also suggests that IF may not simply be operating at the level of energy balance and there may be other signalling pathways through which it is able to induce the beneficial effects acclaimed by several studies. Intermittent fasting in both males and females, also resulted in lower expression of *UCP3* in comparison to CON, HIIT and IF+HIIT groups. Similar decline in *UCP3* expression has been reported by other weight loss associated calorie restriction (Davis et al., 2017, Schrauwen et al., 2000) studies, indicating reduced resting metabolic rate and/or increased metabolic efficiency.

Contrary to our hypothesis, combination of IF and HIIT did not show any synergistic effect on AMPK/SIRT1/PGC1α and UCP3 expression. However, expression of *AMPK*, *SIRT* and UCP3 was higher in IF+HIIT group in males in comparison to IF only group. In females, UCP3 expression was higher and AMPK gene expression showed a higher trend ($p<0.09$) in comparison to IF only group. In addition, PGC1α gene expression in IF+HIIT group for both males and females, did not show any significant decline despite IF regime similar to IF group. This suggested that although combination of IF with HIIT may not show any synergistic effect, HIIT can prevent the suppression of these energy sensing molecules during IF in skeletal muscle.

6.4.2 Oxidative metabolism

Mitochondrial fatty acid oxidation (β-oxidation) is regulated by CPT1, a mitochondrial transmembrane enzyme and is rate limiting for long-chain fatty acid entry into the mitochondria for β-oxidation (Bruce et al., 2009). Inhibition of CPT1 increases lipid deposition and aggravates insulin resistance in diet induced obese mice (Stephens et al., 2007, Kim et al., 2000). Intermittent fasting group in both males and females in the present study showed lower CPT1 expression in comparison to HIIT only group in both males and females and both CON and IF+HIIT groups in males. This suggested that IF supresses β-oxidation of fatty acids in skeletal muscle. Also, Citrate synthase (*CS*) expression was lower in IF group in comparison to CON indicating lowered oxidative capacity of IF males as *CS* is a biomarker for mitochondrial density and oxidative capacity (TCA cycle) of skeletal muscle (Vigelsø et al., 2014, Li et al., 2016). Also, the COX-IV expression, indicator of oxidative phosphorylation capacity in skeletal muscle (Larsen et al., 2012), was lower, albeit non-significantly ($p<0.11$) in males IF group. Indicating that IF lowers skeletal muscle fatty acid oxidation, and downstream TCA cycle and oxidative

phosphorylation. Previous IF study has reported no change in *CS* expression in response to 22 days of alternate day fasting in human skeletal muscle (Heilbronn et al., 2005a). Although no previous reports on *COX-IV* expression after IF were found in the literature, calorie restriction has been reported to cause no decline in electron transport chain (ETC) activity in SM of overweight humans (Civitarese et al., 2007, Toledo et al., 2008). The cause of decline in skeletal muscle oxidative metabolism due to IF observed in present study could be an adaptive response to conserve energy, however more elaborative study is required to understand he underpinning mechanism.

High intensity interval training for 10 weeks and 6 weeks of aerobic exercise training, increases gene expression of CPT1 in skeletal muscle of high fat diet fed rats (Shen et al., 2015) and mice (Niu et al., 2010) respectively. Likewise, in the present study, HIIT group showed higher expression of CPT1 in comparison to IF only groups in both males and females, which suggests higher β-oxidation of fatty acids in skeletal muscle. Also, Skeletal muscle CPT1 overexpression has been reported to protects rats from high fat diet induced insulin resistance (Bruce et al., 2009). Despite higher β-oxidation level, no concomitant increase in CS and COX-IV gene expression in HIIT groups in both males and females were observed. Although previously, increase in CS activity after 6 weeks of voluntary wheel running has been reported in chow fed mice (Palacios et al., 2009, Röckl et al., 2007). Discrepancy in the results could be due to difference in the diet, as the mice in the present study were on HF/S diet before and during the HIIT period in comparison to standard chow diet reported by the aforementioned studies. Moreover, the total distance covered per day reported by one of these studies (Rockl et al., 2007) is 4.5km/day which is far less than the distance covered by mice in the present study (150m/day) although at higher intensity.

Combination of IF and HIIT in the present study showed higher expression of CPT1 in comparison to IF only group in males indicating that the inhibiting effect of IF on skeletal muscle β-oxidation was compensated by HIIT. Although, no enhancement in CPT1 expression in IF+HIIT group in females and CS expression in both males and females was observed, their CPT1 and CS expression did not show significant decline despite IF. This shows that combination of HIIT with IF can prevent lowering of CPT1 and CS expression in the muscle, which tends to fall with IF only. Expression of COX-IV in IF+HIIT group in females was higher in comparison to HIIT only group, indicating enhanced ETC activity and ATP production. Similar results were reported by Menshikova et al. (2005) where calorie restriction when combined with exercise increased ETC activity in skeletal muscle of humans, although exercise control group was not included in the study. One commonality between this study and the present study is lower body mass and fat mass in the combination group which could be the reason behind enhanced ETC expression or activity.

Although the exact mechanism of insulin resistance still remains elusive, in the state of positive energy, β-oxidation is induced, however the downstream TCA cycle and ETC remain inactive, causing accumulation of incomplete fatty acid oxidation products, hypothesised to cause insulin resistance in skeletal muscle (Muoio et al., 2008). However, lifestyle interventions (dietary and exercise) are expected to simultaneously upregulate β-oxidation and downstream TCA cycle and ETC. In females, higher expression of *CPT1* with concomitant higher expression of *CS* (although non-significantly) and *COX-IV* in IF+HIIT group, suggest that there was increase in fat oxidation (β-oxidation) with simultaneous increase in TCA cycle and ETC, which according to the Muoio et al. (2008), should have improved the insulin sensitivity. However, no improvement in insulin

sensitivity at systemic level (indicated by HOMA-IR, (chapter 4, section 4.3.5)) or at skeletal muscle level (indicated by AS160 expression) was observed which suggested that the hypothesis, proposed by Muoio et al. (2008) may not be the only mechanism through which insulin sensitivity is regulated.

6.4.3 Insulin signalling

Skeletal muscle are primarily responsible for systemic insulin mediated glucose uptake and disposal (Ijuin et al., 2015). Relatively recently, Akt substrate of 160 kDa (AS160), a Rab GTPase-activating protein has been reported to regulates GLUT4 translocation in adipocytes and muscle (Kramer et al., 2006, Kane et al., 2002) under both insulin stimulated and exercise induced states (Parker et al., 2016, Parker et al., 2017). Activity of AS160 (phosphorylation) has been reported to increase after acute endurance exercise (Deshmukh et al., 2006), acute HIIT (Parker et al., 2017) and 12 weeks of endurance- or strength-oriented exercise training in humans with improvement in insulin sensitivity (Consitt et al., 2013). Although the expression and activity of AS160 is affected by insulin and exercise, this study was primarily interested in exercise-induced changes. In the present study no significant change in AS160 expression was observed in HIIT only group. However, IF+HIIT group in males showed higher expression of AS160 relative to IF only group. Higher expression of *AS160* in the combination (IF+HIIT) group suggests that HIIT when combined with IF can improve GLUT4 translocation and skeletal muscle glucose homeostasis. Lower expression of AS160 in IF only group in males suggests that IF hampers AS160 mediated insulin signalling and glucose uptake and it could also be the reason behind poor glucose tolerance in IF group reported in chapter 4.

6.4.4 Inflammation

Several studies have suggested that skeletal muscle inflammation occurs in obesity (Collins et al., 2016) and is evidenced by increased expression of pro-inflammatory cytokines like TNFα (Borst and Conover, 2005). In the present study, TNFα expression in ad libitum HF/S fed CON males was significantly ($p<0.01$) higher in comparison to all other intervention groups. Resistance training in human males has been reported to lower skeletal muscle TNFα gene and protein expression (Greiwe et al., 2001). Similarly, calorie restriction has also been reported to lower muscle TNFα expression (Phillips and Leeuwenburgh, 2005). These reports are in line with our findings that HIIT, IF and their combination is effective for reducing skeletal muscle inflammation in males. Although noticed earlier in other anthropometric and plasma parameters, females respond very different to males in context to TNFα expression with no significant difference between CON and/between any intervention group. Estrogen has been reported to affect TNFα expression (Rink, 1998), which could be the reason behind the difference in its expression in male and female mice observed in the present study.

6.4.5 Muscle atrophy

Muscle atrophy has been reported as one of the repercussions of obesity (Roy et al., 2016). Muscle ring finger 1 (MURF1) and muscle atrophy F-box (MAFbx) are important regulators of protein degradation and gene expression in muscle atrophy. Expression of MURF1 has been reported to increase in skeletal muscle of diet induced obese rodents (Sishi et al., 2011, Le et al., 2014). Expression of *MURF1* and *MAFbx* has been reported to upregulate after fasting (1-2 days) (Yamamoto et al., 2015) and calorie restriction (30 months) in mice (Hepple et al., 2008). However, voluntary exercise for 6-weeks has been reported to reduce the expression of both *MURF1* and *MAFbx* in mice

(Hojman et al., 2014). Contrary to this, males in HIIT group in the present study showed higher expression of both *MURF1* and *MAFbx* in comparison to CON group. However, IF group showed lower expression of both *MURF1* and *MAFbx* in comparison to HIIT only group in both males and females and CON group in females only. The discrepancy between results reported by this study and previous studies could be due to different modes of diet restriction and exercise training. Also, IF+HIIT showed lower expression of both *MURF1* and *MAFbx* in comparison to HIIT only group in males and both HIIT and CON group in females. This suggests that, despite HIIT inducing muscle atrophy, IF in this group prevented muscle atrophy by keeping down the expression of both *MURF1* and *MAFbx* despite simultaneous consumption of high calorie diet.

6.4.6 Muscle mass and fibre type

No significant difference in muscle mass was found between CON and/between intervention groups in both males and females. Although HIIT has been reported to enhance muscle mass of soleus and EDL muscle in chow fed mice after 16 weeks (Seldeen et al., 2018). No change in muscle mass could be due to difference in diet as the mice in the present study were on HF/S diet which might have hampered the effect of HIIT on muscle growth.

No significant difference in fibre type distribution was found between CON and/between intervention groups in both males and females. Although, moderate exercise training for 16 weeks has been reported to increase type I fibres (Dube et al., 2008, Pattanakuhar et al., 2016) in humans. Also, HIIT for 10 weeks (3 days per week) has been reported to reduce the proportion of type IIb fibres with no change in type I and type IIa fibres (Ross et al., 2016, Vogiatzis et al., 2005) in humans. However, in the present study, no change in fibre type distribution has been found either in type I, type IIa or IIb fibres

in EDL or soleus muscle. A plausible cause could be the volume of exercise (Pattanakuhar et al., 2016), which as mentioned previously was of lower volume in comparison to the studies showing change in fibre type distribution.

6.4.7 MyomiR expression

Skeletal muscle enriched miR-133a and 133b, regulate skeletal muscle development and differentiation (Koutsoulidou et al., 2011, Silva et al., 2017). MiR-133a and 133b has been reported to get upregulated in skeletal muscle of humans after acute endurance training (Russell et al., 2013). In the present study, HIIT female mice showed higher expression of miR-133a in comparison to IF+HIIT group. Higher expression of miR-133a has been linked to mitochondrial biogenesis in mice after six weeks of endurance training (Nie et al., 2016). This was also consistent with higher *PGC1α* expression found in female HIIT group.

Contrary to the above relationship, lower expression of miR-133a has been suggested to be beneficial as miR-133a can target members of the IGF-1/Akt pathway in vitro, including IGF-1, the IGF-1 receptor, as well as heat shock protein 70 (HSP70) (Fyfe et al., 2016, Elia et al., 2009). In females, IF+HIIT showed significantly lower expression of miR-133a in comparison to all other groups. Lower expression of miR-133a has been suggested to induce anabolic response by alleviating the repression of IGF-1/Akt signalling pathway and promoting muscle hypertrophy. Lower expression of these miRNAs in IF+HIIT in the present study suggests induction of muscle hypertrophy by combining IF with HIIT, although muscle weight was not significantly different from other groups.

MiRNA-696 has been reported as physical activity dependent miRNA and regulates the expression of PGC1α (Aoi et al., 2010). Expression of miR-696 in IF+HIIT

group was significantly higher compared to CON group in both males and females and compared to HIIT group in males, indicating that weight loss induced by IF+HIIT upregulates the expression of miR-696. Contrary to the expectation, no correlation was found between miR-696 and PGC1α. This could be attributed to translational mode of gene regulation rather than transcriptional gene regulation by miR-696. Translational regulation by miR-696 was also reported by another study which observed a correlation coefficient (R) of 0.64 between miR-696 expression and PGC1α protein expression (Aoi et al., 2010).

In addition to this, miR-696 has also been reported to contribute to insulin resistance by reducing mitochondrial function and antioxidant capacity in skeletal muscle cells (C2C12) (Queiroz et al., 2013). The study on C2C12 skeletal muscle cells reported elevated expression of miR-696 with simultaneous reduction in insulin sensitivity (Queiroz et al., 2013). Likewise, in the present study, miR-696 and insulin resistance (HOMA-IR) increased in IF group and IF+HIIT in females, further confirming the previous report on C2C12 cells and indicating that upregulation of miR-696 in IF mice could be involved in the insulin resistance noticed in these animals by our study and other studies as well (Cerqueira et al., 2011). However, at the same time, increased expression of miR-696 in IF+HIIT males did not conform to above relationship between miR-696 expression and insulin sensitivity, as no significant difference in insulin resistance was noticed between IF+HIIT group and other intervention groups (chapter 4, section 4.3.6).

In silico gene target identification using miRWalk2.0 online database showed that miR-133a targets *TNFα*, and miR-133b targets *MURF1*. However, no significant correlation was found between these miRNAs and their presumed target genes in the

present study. Just like miR-696, these miRNAs could have been regulated at translational level and need more specific studies to elaborate their function.

6.5. CONCLUSION

This study was aimed to investigate the effect of IF and/or HIIT on skeletal muscle gene expression related to oxidative metabolism, inflammation, muscle growth and atrophy, fibre type distribution and miRNAs related to metabolism and muscle growth. This study suggests that HIIT induce higher mitochondrial biogenesis, oxidative metabolism, fatty acid oxidation and ATP production. However, IF alone had a very different and opposite response which lowers mitochondrial biogenesis, oxidative metabolism, fatty acid oxidation, ATP production and insulin sensitivity suggested by the expression of marker genes related to them. The combination of IF and HIIT did not show any synergistic effect on the muscle, however various marker genes especially related to metabolism (*SIRT1*, *AMPK*, *CPT1*, *AS160*) showed the expression level higher than IF only group indicating that HIIT was able to rescue their expression level and mitigating the effect of IF on metabolism. However, this role is reversed in terms of atrophy genes as induced by HIIT was mitigated by IF in IF+HIIT in both males and females. Although the effect was very gender specific, both IF and HIIT alone or in combination showed decline in obesity induced inflammation. Even though no superior effect of combining IF with HIIT has been observed, combination of these two interventions can induce beneficial adaptations in skeletal muscle with either one of them compensating the unfavourable effects of other and keeping the balance. Expression of miR-696, 133a and 133b although did not show any significant correlation with their reported or *in silico* identified targets, their differential expression between different groups and their gender specificity suggests their role in fine tuning the adaptive changes induced by IF and HIIT.

CHAPTER 7

CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

The overall purpose of this thesis was to investigate the effect of two lifestyle interventions, IF and HIIT, alone or in combination, in high fat/sugar diet induced obese mice, on body composition, glucose homeostasis, plasma lipid profile (discussed in chapter 4), molecular changes underpinning adaptations in adipose tissue (discussed in chapter 5) and skeletal muscle (discussed in chapter 6). This chapter will summarise the main findings of this study in the first part of the chapter. Last two sections of the chapter will discuss limitations and will also provide future direction.

7.1. KEY FINDINGS

7.1.1 Combination of IF and HIIT prevents weight gain by less accumulation of fat mass and maintenance of lean mass

Twelve weeks of IF combined with HIIT prevented weight gain in both males and females while concurrently consuming a high fat/sugar diet in mice. These mice showed no significant gain in body weight over 12-weeks, despite concurrently consuming high caloric diet. Whereas, ad libitum CON group on the same diet showed significant increase in body weight over this period. In males, IF alone also showed very similar results, with significantly no weight gain over 12-weeks intervention period. Contrary to this, females in IF group showed significant gain in body weight. Although previous studies on intermittent fasting have shown reduced weight gain (Higashida et al., 2013, Gotthardt et al., 2016), the discrepancy was due to less intensive fasting regime with few fasting days used in this study compared to the previous studies. Also, HIIT alone led to gain in body weight in both males and females. This does not agree with literature as previous HIIT studies in similar animal models have reported less gain in body weight (Cho et al., 2015,

Lund et al., 2015). This was mainly due to more number of exercise training sessions performed by animals in the previous studies in comparison to the present study. Interestingly, despite low intensity of exercise and less number of fasting days, combination of these interventions led to reduced weight gain in both males and females. The change in body weight noticed in IF+HIIT group in both males and females is mainly due to low fat mass as lean mass in this group did not show any significant difference from *ad libitum* CON group. This suggests that combination of IF and HIIT can be beneficial for preventing weight gain mainly by reduced fat accumulation while maintaining lean mass. The combination of IF and HIIT also showed lower plasma TAG levels in females and lower LDL levels in both males and females.

7.1.2 Combination of IF with HIIT leads to less fat accumulation due enhanced fatty acid oxidation and efflux

Molecular investigation in adipose tissue showed that IF+HIIT group displayed lower expression of genes related to the formation of unilocular lipid droplets (CIDEC) in females, suggesting fragmentation of lipid droplets and enhanced lipolysis (Marcinkiewicz et al., 2006). This was accompanied by higher expression of marker genes for fatty acid oxidation (HADH) relative to IF only group in both males and females and higher expression of fatty acid trafficking marker (FABP4) in comparison to other intervention and CON groups in males. These results suggest that reduced fat mass accumulation in adipose tissue of IF+HIIT group could be due to higher oxidation and efflux of fatty acids (Verhoef et al., 2013). Lower expression of leptin and pro-inflammatory markers were also observed in the combined diet and exercise group supporting the findings in chapter 4.

7.1.3 Intermittent fasting and high intensity exercise had opposite effect on skeletal muscle molecular markers

In skeletal muscle, HIIT induced higher mitochondrial biogenesis, oxidative metabolism, fatty acid oxidation and ATP production. Contrary to this, IF alone lowered mitochondrial biogenesis, oxidative metabolism, fatty acid oxidation, ATP production and insulin sensitivity suggested by lower expression of marker genes related to them. Fasting group also showed lower expression of AMPK-SIRT1-PGC1α axis. However, IF group showed lower expression of MURF1 and MAFbx indicating lower muscle atrophy, whereas HIIT showed higher expression of these genes. Although IF and HIIT did not show any synergistic effect on various molecular markers, combination of IF and HIIT can promote favourable adaptations in skeletal muscle with either one of them compensating the unfavourable effects of other and keeping the balance.

7.1.4 Intermittent fasting perturbed the systemic glucose homeostasis

Surprisingly, after 12-weeks intervention period, IF group in both males and females and IF+HIIT group in females showed higher fasting glucose, plasma insulin, higher insulin resistance and poor glucose tolerance. This is very unexpected as most of the studies (Barnosky et al., 2014) have reported improvement in glucose homeostasis. This could be due to redox imbalances and insulin receptor nitration (Cerqueira et al., 2011) or could be due to frequent feeding/fasting cycle which has been suggested as a risk factor for age-associated obesity and insulin resistance (Park et al., 2014). Besides hampered systemic glucose homeostasis, skeletal muscle, one of the major contributing tissues towards systemic glucose disposal, also showed poor glucose handling indicated by lower expression of AS160 in IF group. Although more intensive investigations are required to back up this finding, SIRT1 expression in adipose tissue of IF group was found to be lower in both males and females. Expression of SIRT1 in adipose tissue has recently

been correlated to systemic glucose homeostasis, and lower expression of SIRT1 with poor glucose homeostasis in IF group in the present study, further confirms the previous report by Stefanowicz et al. (2018).

7.1.5 Sexually dimorphic response to IF and/or HIIT

Males and females responded very differently to IF and/or HIIT. Males showed higher changes in body composition in comparison to females. This was also in line with some previous studies where differences between sexes has also been reported in response to lifestyle interventions (Williams et al., 2015, Baillot et al., 2015, Gallagher et al., 2017). This has been attributed to differences in adipose tissue distribution and functionality and also hormonal differences between sexes with males exhibiting higher levels of circulating leptin and females exhibiting higher levels of circulating adiponectin (Valencak et al., 2017). Also at the molecular level, several genes and miRNAs showed different trend in two genders. Especially in case of miRNAs, in females, miR-24, 222, 143 in adipose tissue and in males, muscle specific miR-133a and 133b did not show any significant differences with IF/HIIT. Translating this to humans, care must be taken while recommending these interventions to males and females as both these interventions have to be optimised separately for two sexes.

7.2. LIMITATIONS

A number of limitations exist in this thesis. To determine the time effect of IF and/or HIIT, plasma and tissue samples before and after intervention period from the same animal could not be obtained due to animal ethics limitations. Therefore, plasma insulin, glucose, HOMA-IR, lipid panels, muscle fibre type distribution and expression of mRNA and miRNAs were only measured at the end of the 12-week intervention period.

Moreover, due to housing constrains of ethical approval, mice from different intervention groups were housed together, more specifically, mice undergoing fasting (IF and IF + HIIT) were housed together and mice undergoing no fasting (HIIT and CON) were housed together. Thus, food intake of individual mice could not be obtained and the effect of IF or exercise on diet intake per se could not be determined.

Also, though mice were acquired from the same inbred strains, it was noticed that there was variability within the same intervention groups in terms of gain in body weight, body composition, plasma glucose and lipid profiles, expression of genes and miRNAs etc, and thus we can only assume the mice had similar genotype.

7.3. FUTURE DIRECTIONS

There are several questions that arose from the findings of this thesis which will inform future research in this area. Firstly, despite several claims by most previous studies both male and female mice in IF group showed poor glucose homeostasis. This was also reported by very few previous animal studies (Cerqueira et al., 2011). It is still unknown, if it is something peculiar to animal models, diet or is age related disorder with IF. Long term intermittent fasting studies with proper time point controls are required to answer this anomaly.

Secondly, both diet and exercise interventions have been reported to influence structure and function of central nervous system (Pinilla, 2006). It would be interesting to know how IF and/or HIIT influence various functions especially in hypothalamic region of the brain, since this part of the brain is called “control centre” of energy homeostasis (O'Brien et al., 2017). This might also give a clue about insulin resistance and poor glucose handling found in IF mice in this study. Also, how combination of these two intervention affect cognitive functions is still unknown.

Thirdly, the caloric intake between fasting and non-fasting groups were found to be similar. This indicates that body compositional changes induced by IF may not just be induced by negative energy balance, as despite consuming same amount of diet, mice in fasting group weighed less. Future studies can be targeted to explore the reason behind this observation.

Moreover, only limited miRNAs can be investigated in the present study and their roles at the level of translational regulation were not determined due to limitations of tissue availability and resources. More robust investigations are required to determine the exact role of signature miRNAs in response to these lifestyle interventions. MiRNAs have been suggested as a potential therapeutic targets (Krützfeldt, 2016) and identification of candidate miRNAs, that can be used as therapeutic targets for treatment of obesity and associated comorbidities would be very novel.

Finally, the findings of the present study can be used as a foundation for clinical research on the combination of these interventions in obese humans. Since, the response to IF and HIIT was different in males and females, separate studies for males and females must be designed and optimised to maximize outcome.

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