Identification of novel DNA methylation signatures in the development of cardiovascular disease

Tracey Jooste

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Supervisor: Prof R. Johnson

Co-supervisors: Dr L. Mabasa, Dr E. Marais and Dr C. Pheiffer

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Declaration

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Abstract

Cardiovascular diseases (CVDs) remain the leading cause of death worldwide, claiming approximately 17.9 million lives annually. The last few decades have seen an exponential increase in the prevalence of major CVD risk factors, such as obesity, insulin resistance (IR), and type 2 diabetes mellitus (T2DM) in underdeveloped countries, including South Africa. This increase is concomitant to escalating CVD incidence and in part due to increased consumption of diets high in fat and sugar, and sedentary lifestyles. Several mechanisms have been implicated in the pathogenesis of diabetic-induced CVD and recently, increasing evidence suggests that dysregulation of the epigenome may play an important role in the development of T2DM and related cardiac complications. More specifically, aberrant DNA methylation has been extensively investigated and implicated in the development of chronic diseases such as obesity, T2DM and CVD. Nonetheless, it has been suggested that some active dietary compounds may reverse this epigenetic phenomenon which allows for the modification of the transcription of critical genes associated with the progression of CVD. Several plant-polyphenols have been reported to influence gene transcription by altering DNA methylation status. Thus, targeting DNA methylation could provide a promising approach for alternative prevention strategies.

This study explored the gene expression networks activated during diet-induced CVD and the ability of a green rooibos extract, Afriplex GRT™, to alter this consequence. In addition, the study aimed to evaluate aberrant DNA methylation associated with diet-induced CVD to further elucidate pathophysiology. To this end, cardiomyocytes exposed to high glucose and palmitate (HG + Pal) displayed a diminished antioxidant defence system, mitochondrial dysfunction and increased apoptosis, indicative of cardiac stress. Additionally, the combinatory treatment with HG + Pal induced transcriptional changes associated with inflammation, oxidative stress, altered lipid metabolism and increased contractile dysfunction, ultimately promoting the development of atherosclerosis and hypertrophic cardiomyopathy. Interestingly, post treatment with Afriplex GRT™ or Aspalathin had no significant effect on the metabolic and molecular derangements induced under HG + Pal stress. Similarly, RNA sequencing conducted on cardiac tissue of Wistar rats that received a high fat, high sugar (HFHS) diet, revealed the downregulation of differentially expressed genes (DEGs) involved in host antioxidant activity and inflammatory response, accompanied with an increase in hypertrophic gene expression possibly affecting cardiac muscle functionality. Supplementation with Afriplex GRT™ yielded no high confidence results for

the amelioration of the transcriptomic signatures resulting from HFHS diet feeding. To profile DNA methylation throughout disease progression, cardiac tissue of male Wistar rats maintained on a HFHS diet were subjected to whole genome bisulfite sequencing (WGBS). The latter revealed aberrant DNA methylation of genes linked to the phagosome, platelet activation, toll-like receptor signalling and diabetic cardiomyopathy. Furthermore, hypomethylation within the intergenic and gene body regions of several differentially methylated genes (DMGs) overlapped with DEGs identified in the RNA sequencing analysis. Collectively these results demonstrate the ability of the HFHS diet to act as a pathological stimulus capable of inducing altered gene expression and DNA methylation associated with a heightened proinflammatory and lipid metabolism response that increased the risk of CVD development.

Opsomming

Kardiovaskulêre siektes (KVSs) bly wêreldwyd die grootste oorsaak van sterftes en eis jaarliks ongeveer 17,9 miljoen lewens. Die afgelope paar dekades het 'n eksponensiële toename in die voorkoms van belangrike KVS-risikofaktore, soos vetsug, insulienweerstandigheid (IW) en tipe 2-diabetes mellitus (T2DM) in onderontwikkelde lande, insluitende Suid-Afrika, plaasgevind. Hierdie toename gaan gepaard met die toenemende voorkoms van KVS, en is deels die gevolg van verhoogde verbruik van diëte met baie vet en suiker, en onaktiewe lewenstyle. Verskeie meganismes is in die patogenese van diabetes-geïnduseerde KVS geïmpliseer en onlangs het toenemende bewyse daarop gedui dat wanregulering van die epigenoom 'n belangrike rol in die ontwikkeling van T2DM en verwante hartkomplikasies kan speel. Meer spesifiek is afwykende DNA-metilering breedvoerig ondersoek en in die ontwikkeling van kroniese siektes soos vetsug, T2DM en KVS geïmpliseer. Nietemin word dit voorgestel dat sommige aktiewe dieetverbindings hierdie epigenetiese verskynsel kan omkeer en die transkripsie van kritieke gene, wat verband hou met die progressie van KVS, kan modifiseer. Daar word berig dat verskeie plant-polifenole geentranskripsie beïnvloed deur die status van DNA-metilering te verander. Dus, deur te fokus op DNA-metilering kan dit 'n belowende benadering tot alternatiewe voorkomingstrategieë bied.

Hierdie studie het ondersoek ingestel na die gene uitdrukkings netwerke wat geaktiveer word tydens dieet-geïnduseerde KVS en die vermoë van 'n groen rooibos-ekstrak, Afriplex GRT™, om hierdie gevolg te verander. Daarbenewens het die studie ten doel gehad om afwykende DNA-metilering geassosieer met dieet-geïnduseerde KVS te evalueer om patofisiologie verder toe te lig. Vir hierdie doel het kardiomiosiete wat aan hoë glukose en palmitaat (HG + Pal) blootgestel was, 'n verminderde antioksidant-verdedigingstelsel, mitochondriale disfunksie en verhoogde apoptose getoon, wat dui op kardiale spanning. Verder het die kombinasiebehandeling met HG + Pal transkripsionele veranderinge veroorsaak wat verband hou met inflammasie, oksidatiewe spanning, veranderde lipiedmetabolisme en verhoogde kontraktiele disfunksie, wat uiteindelik die ontwikkeling van aterosklerose en hipertrofiese kardiomiopatie bevorder.

Dit was interessant dat post-behandeling met Afriplex GRT™ of Aspalatien geen noemenswaardige effek op die metaboliese en molekulêre afwykings wat onder HG + Pal spanning veroorsaak is, gehad het nie. Net so het RNA-volgordebepaling, wat uitgevoer was op hart weefsel van Wistar-rotte wat 'n dieet met 'n hoë vet, hoë suiker (HVHS) ontvang

het, die afregulering van differensieel uitgedrukte gene (DUG'e) wat betrokke is by die gasheer-antioksidantaktiwiteit en inflammatoriese reaksie, aan die lig gebring, met 'n gepaardgaande toename in hipertrofiese geenuitdrukking wat moontlik die hartspier funksionaliteit kon beïnvloed.

Aanvulling met Afriplex GRT™ het geen hoë vertrouensresultate opgelewer in die verbetering van die transkriptomiese aanwysings as gevolg van HVHS-dieetvoeding nie. Om die DNA-metilering profiel gedurende die hele siekteprogressie te neem is hartweefsel van manlike Wistar-rotte, wat op 'n HVHS dieet gehou was, onderwerp aan 'n volledige genoom-bisulfiet-volgordebepaling. Laasgenoemde het afwykende DNA-metilering van gene aan die lig gebring wat gekoppel is aan die fagosoom, bloedplaatjie-aktivering, tol-agtige reseptor seinpaaie en diabetiese kardiomiopatie. Verder het hipometilering in die intergeniese streke en geenliggaam van verskeie differensieel gemetileer gene (DMG’e) oorvleuel met DUG'e wat geïdentifiseer is in RNA-volgordebepaling. Gesamentlik toon hierdie resultate die vermoë van die HVHS-dieet om as 'n patologiese stimulus op te tree wat veranderende gene-uitdrukking en DNA-metilering kan veroorsaak, wat gepaard gaan met verhoogde inflammatoriese reaksies en lipiedmetabolisme wat uiteindelik die risiko vir KVS-ontwikkeling verhoog.

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Research Outputs

This study contributed to the following publications, conference proceedings and poster presentations.

Publications

- Glanzmann, B., **Jooste, T.**, Ghoor, S., Gordon, R., Mia, R., Mao, R., Li, H., Charls, P., Douman, C., Kotze, M., Peeters, A., Loots, G., Esser, M., Tiemessen, C. T., Wilkinson, R. J., Louw, J., Gray, G., Warren, R. M., Möller, M. & Kinnear, C. (2020) Human whole genome sequencing in South Africa. Scientific Reports. 11(1), 606.

  This paper contains work on optimisation and validation of the sequencing platform used in the current study. Ms Jooste shares first authorship and was involved in study conceptualisation, responsible for the optimisation and execution of sequencing experiments, and aided with drafting and editing of the manuscript.

- Johnson, R., Nxele, X., Cour, M., Sangweni, N., **Jooste, T.**, Hadebe, N., Samodien, E., Benjeddou, M., Mazino, M., Louw, J. & Lecour, S. (2020) Identification of potential biomarkers for predicting the early onset of diabetic cardiomyopathy in a mouse model. Scientific Reports. 10(1), 12352.

  This paper contains work on the identification and validation of potential biomarkers for diabetic cardiomyopathy, a disease state of particular interest to the current study. Ms Jooste performed the *in silico* biomarker prediction, mRNA expression analysis, and aided with drafting and editing of the manuscript.

Conference proceedings

- **Jooste, T.L**., Glanzmann, B., Marais, E., Mabasa, L., Johnson, R. & Kinnear, C. Whole genome bisulfite sequencing in South Africa.

  Ms. T.L Jooste presented the whole genome bisulfite sequencing analysis described in this study at the 10th Annual Biomedical Research and Innovation Platform (BRIP) Symposium. Cape Town, South Africa, 19-20 October 2020. Ms Jooste received a “Best Young Researcher” award for the work presented at this event.

Posters

- **Jooste, T.L**., Marais, E., Mabasa, L., Frederiksen, M. & Johnson, R. Profiling chromatin accessibility associated with high fat (HF) diet-induced CVD.

  The bioinformatic pipeline described in this study contributed to a poster presented by Ms T.L. Jooste at the 9th Annual Biomedical Research and Innovation Platform (BRIP) Symposium, Cape Town, South Africa, 21 August 2019.

- **Jooste, T.L**., Marais, E., Mabasa, L., & Johnson, R. Gene expression and DNA methylation changed associated with high fat, high sugar diet-induced CVD in Wistar rats.

  The transcriptomic sequencing analysis described in this study contributed to a poster presented by Ms T.L. Jooste at the ICGEB Workshop for Epigenetics of Infectious and Non-communicable Diseases, Cape Town, South Africa, 16-19 September 2019. Ms Jooste received an award for the second-best poster presentation at this event.

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List of abbreviations

%	Percentage
Afriplex GRT™	Aspalathin-rich rooibos extract
ALT	Alanine aminotransferase
Asp	Aspalathin
AST	Aspartate transaminase
ATP	Adenosine triphosphate
AUC	Area under the curve
BH	Benjamin-Hochberg
bp	Base pair
BP	Blood pressure
BSA	Bovine serum albumi
CAD	Coronary artery disease
cDNA	Complementary DNA
CpG	Dinucleotide: cytosine followed by guanine
CVD	Cardiovascular disease
DAVID	Database for annotation, visualisation, and integrated discovery
DCFH-DA	2’,7’-Dichlorofluorescin Diacetate
DEG	Differentially expressed gene
DMC	Differentially methylated cytosine
DMEM	Dulbecco’s modified eagle’s medium
DMG	Differentially methylated gene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco’s phosphate-buffered saline
dsRNA	Double-stranded RNA
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FBG	Fasting blood glucose

FBS	Fetal bovine serum
g	Gram
GB	Gigabase
GO	Gene ontology
H&E	Hematoxylin and eosin
HBBS	Hank's balanced salt solution
HDL	High-density lipoprotein
HF	Heart failure
HFHS	High fat, high sugar
HG	High glucose
HOMA-IR	Homeostatic model assessment of insulin resistance
IF	Immunofluorescence
IR	Insulin resistance
kb	Kilobase
KEGG	Kyoto encyclopaedia of genes and genomes
LDL	Low-density lipoprotein
M	Molar
MI	Myocardial infarction
min	Minute
mL	Millilitre
mM	Millimolar
mmol	Millimole
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NG	Normal glucose
NGS	Next generation sequencing
nM	Nanomolar
nt	Nucleotide
oC	Degrees celsius
OCR	Oxygen consumption rate
OD	Optical density
OGTT	Oral glucose tolerance test
Pal	Palmitate
PCR	Polymerase chain reaction

pmol	Picomole
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative PCR
SDS	Sodium dodecyl sulfate
sec	Second
STD	Standard
T2DM	Type 2 diabetes mellitus
TAE	Tris-acetic acid/ EDTA
Tris-HCL	2-amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloric acid
UTR	Untranslated region
WGBS	Whole genome bisulfite sequencing
ΔΨM	Mitochondrial membrane potential
µg	Microgram
µL	Microliter
µM	Micromolar

Chapter 1: Introduction

1.1 Research rationale

Cardiovascular diseases (CVDs) involving the heart and blood vessels, are the leading cause of mortality worldwide (Benjamin et al., 2019; NHS, 2018; WHO, 2021). According to the latest World Health Organization (WHO) report, an estimated 17.9 million people died from CVD in 2019, representing approximately 32% of all global deaths (WHO, 2021). Alarmingly, CVD is projected to claim 23.6 million lives by 2030 (Benjamin et al., 2017; Donnan et al., 2006; Kucharska-Newton et al., 2010; Lloyd-Jones et al., 2010). Over the past few years, the historical association of CVD with developed nations has shifted towards underdeveloped countries such as South Africa (WHO, 2021; Yusuf et al., 2014). This increase in CVD prevalence in low- and middle-income countries can in part be attributed to a rise in the incidence of known CVD risk factors such as obesity and type 2 diabetes mellitus (T2DM) (Cavan et al., 2016; Humphries et al., 2010; Kim et al., 2010; Yusuf et al., 2004). Urbanisation and sedentary lifestyles bring about a nutritional shift, characterised by the increased consumption of western diets rich in dietary fat, sugar and carbohydrates (Cavan et al., 2016; Humphries et al., 2010; Kim et al., 2010; Stelmach-Mardas et al., 2016; Walsh et al., 2017; Zong et al., 2016). This transition in diet results in metabolic distortions such as increased production of reactive oxygen species (ROS), development of insulin resistance (IR) and low-grade inflammation, ultimately increasing CVD risk (Kruger et al., 2005; Micklesfield et al., 2013; Mirmiran et al., 2017; Oikonomou et al., 2018).

Despite the implementation of global initiatives aimed at reducing CVD prevalence through lifestyle and nutritional interventions, South African women have amongst the highest rates of obesity worldwide (Joubert et al., 2007; Ng et al., 2014; Zhou et al., 2017). Presently, T2D is the leading cause of micro- and macro vascular complications, with diabetic cardiomyopathy (DCM) being one of the major causes of end stage heart failure throughout the world. Furthermore, there is no pharmacological treatment to avert the onset of CVD and drug therapies aimed at treating primary conditions such as T2DM tend to lose their efficacy to protect the diabetic heart over time. From a South African perspective, the increased mortality associated with the poor prognosis of diabetic heart failure does not only impose a heavy cost in terms of suffering and death, but also places a colossal financial burden on individuals (poor households), the health system as well as the national budget. Therefore, the importance of improving preventive and therapeutic measures against CVD

is constantly growing, driving the need for a more thorough understanding of disease pathophysiology.

Several mechanisms have been implicated in the pathogenesis of diabetic-induced cardiac dysfunction and recently, increasing evidence suggests that dysregulation of the epigenome may play an important role in disease development (Baccarelli et al., 2010; Meaney, 2014). More specifically, aberrant DNA methylation has been extensively investigated and implicated in the development of chronic diseases such as obesity, T2DM and CVD (Kim et al., 2010; Khalil, 2014, Meany, 2014; Ramos et al., 2016; Stenvinkel et al., 2007). Of particular interest to this study is the role of altered DNA methylation associated with hyperglycaemia and glucose intolerance resulting from increased dietary fat intake as reported in human and animal fetal programming models (Cannon et al., 2014; Moody et al., 2017; Seki et al., 2017; Soubry et al., 2015; Zang et al., 2019). Furthermore, the adverse effects brought on by increased sugar intake, specifically fructose, has also been suggested to be mediated by DNA methylation (Davegårdh et al., 2018; Yamanda et al., 2017). However, limited evidence exists evaluating the combinatory ability of high fat and high sugar to stimulate DNA methylation-induced transcriptional changes. Nonetheless, it has been suggested that some active dietary compounds may reverse this epigenetic phenomenon which allows for the modification of the transcription of critical genes associated with CVD (Crider et al., 2012; Jamaluddin et al., 2007; Nazki et al., 2014). In particular, several plant-polyphenols have been reported to influence gene transcription by altering DNA methylation status (Crescenti et al., 2013; Feil & Fraga, 2012; Pan et al., 2013; Pandey et al., 2009; Parks & Booyse, 2002; Schiano et al., 2015; Switzeny et al., 2012). Thus, targeting DNA methylation signatures associated with CVD could provide a promising approach for developing early prognostic or diagnostic markers to aid alternative prevention strategies.

The use of indigenous medicines for health and custom purposes is not a foreign concept for South Africans, highlighting the need to further tap into South Africa's rich biodiversity to develop safe and cost-effective natural products with the ability to effectively reduce the risk of cardiac dysfunction. Of interest to this study is the indigenous plant, rooibos (Aspalathus linearis), known for its antioxidant and anti-inflammatory properties. Previous research demonstrated the ability of rooibos and its polyphenolic constituents to exert protective effects against metabolic disorders such as insulin resistance (IR) and T2DM, both risk factors of CVD (Dludla et al., 2014; Johnson et al., 2017; Mazibuko et al., 2013; Son et al., 2013). However, to characterise plant-polyphenols in relation to the amelioration of

metabolic ailments and cardiac complications, research providing a better understanding of disease pathophysiology and how metabolic factors contribute to the clinical manifestation of CVD is necessary.

The evolution of next generation sequencing (NGS) has significantly impacted genomic research, aiding the comprehension disease aetiology and the identification of diagnostic, prognostic, and therapeutic markers. Concerning CVD, NGS technologies have been utilised to demonstrate the association of aberrant gene expression and genetic variants with underlying CVD pathophysiology (Jneid et al., 2012; Liao et al., 2019; Lundmark et al., 2015). Coupling RNA sequencing with whole genome bisulfite sequencing (WGBS) as applied in this study, allows for the evaluation of genome wide interactions between transcriptomic and epigenetic alterations. Exploration the correlation of these alterations with CVD not only adds towards understanding of the disease pathology, but also provides possible new therapeutic targets.

1.2 Aims and Objectives

This study aimed to explore the gene expression networks associated with cardiac dysfunction in a high fat, high sugar (HFHS) diet Wistar rat model, and the ability of a green rooibos extract, Afriplex GRT™, to alter these consequences. Additionally, this study aimed to evaluate aberrant DNA methylation during the development and progression of HFHS diet-induced CVD to further elucidate pathophysiology.

In order to achieve these aims, the following objectives were identified:

- To evaluate the effect of combinatory treatment with high glucose plus palmitate (HG + Pal) using an *in vitro*, rat derived H9c2 cardiomyocyte model and the ability of Afriplex GRT™ and Aspalathin to mitigate these effects.
- To utilise an *in vivo*, HFHS diet-induced IR Wistar rat model in order to characterise CVD development and progression.
- To profile transcriptional changes associated with HFHS diet-induced CVD and Afriplex GRT™ supplementation using RNA sequencing.
- To profile genome wide DNA methylation changes related to HFHS diet-induced CVD using whole genome bisulfite sequencing (WGBS).

1.3 Dissertation Structure

This dissertation is comprised of seven, individually introduced chapters. Supplementary information relating to the individual chapters is provided at the end of the thesis.

1.3.1 Chapter 1: Introduction

A general introduction to the study and its implication, including an overview of the aims and objectives, layout as well as research outputs.

1.3.2 Chapter 2: Literature Review

An overview of the literature pertaining to this study is provided including the underlying pathophysiology of CVD, metabolic risk factors, epigenetic modifications and the high throughput technologies used to investigate the discussed abnormalities.

1.3.3 Chapter 3: High glucose and palmitate-induced decrease in antioxidant capacity and mitochondrial bioenergetics in H9c2 cardiomyocyte: the role of Afriplex GRT™

This chapter describes the metabolic and molecular derangements in cardiomyocytes exposed to a combined insult of high glucose plus palmitate (HG + Pal). Additionally, the effect of Afriplex GRT™ or Aspalathin treatment on HG + Pal induced stress is discussed.

1.3.4 Chapter 4: A high fat, high sugar model to investigate diet-induced CVD in Wistar rats

The influence of a high fat, high sugar (HFHS) diet on physiological parameters of male and female Wistar rats is described. The use of this model as a diet-induced CVD risk model and the effect of Afriplex GRT™ on the described changes was also evaluated.

1.3.5 Chapter 5: Transcriptome profiling of diet-induced CVD in Wistar rats through high-throughput mRNA sequencing

This chapter describes the use of transcriptome sequencing to evaluate the cardiac gene expression profile associated with high fat, high sugar (HFHS) diet feeding and the ability of Afriplex GRT™ to modulate the observed derangements.

1.3.6 Chapter 6: Whole genome bisulfite sequencing to assess aberrant DNA methylation associated with diet-induced CVD in Wistar rats

The cardiac DNA methylation profiles of HFHS diet fed male Wistar rats, was surveyed using whole genome bisulfite sequencing (WGBS). The differentially methylated cytosines (DMCs), overlapping transcripts and pathway enrichment is described. A comparison of WGBS and transcriptome sequencing data is also provided.

1.3.7 Chapter 7: Conclusions, limitations, and prospects

A concluding summary describing the main study findings, along with the strengths and limitations of this study. Proposals for future research is also provided.

1.5 References

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Chapter 2: Literature Review

2.1 Cardiovascular disease

Cardiovascular disease (CVD) is a blanket term used to refer to a group of disorders which either results from narrowed or blocked blood vessels or involves defects of the heart muscle (Benjamin et al., 2019; Lloyd-Jones et al., 2010; WHO, 2021). These disorders include, but are not limited to cerebrovascular disease (e.g., stroke); coronary artery disease (CAD); myocardial infarction (MI); hypertensive heart disease (e.g., aneurysm); peripheral arterial disease; venous thrombosis; atrial fibrillation; rheumatic heart disease; inflammatory heart disease (e.g., pericardial disease); cardiomyopathy; congenital heart disease; cardiac arrhythmias; and heart failure (HF) (Benjamin et al., 2019; Wattanapitayakul & Bauer, 2001; WHO, 2021). Amongst the afore-mentioned, CAD and stroke are the two most prevalent types, accounting for approximately 42% and 38% of CVD cases respectively (Benjamin et al., 2019; NHS, 2018; WHO, 2021). CVDs irrespective of the origin are the leading causes of premature mortality, responsible for more deaths than cancer worldwide (Benjamin et al., 2019; WHO, 2021). Over the past few decades there has been an increase in CVDs in developing nations with over three quarters of all CVD deaths occurring in low- and middle-income countries such as South Africa (WHO, 2021; Yusuf et al., 2014).

2.2 Risk factors

Since the Framingham study, the concept of factors increasing the risk of developing CVD has played an integral role in prevention and treatment strategies (Mahmood et al., 2014). The various risk factors for CVD can broadly be classified into those that can be managed, referred to as modifiable, and those which cannot be managed, known as non-modifiable risk factors. Some of the non-modifiable risk factors include gender, race, genetic predisposition, and aging (IDF, 2019; Pencina et al., 2019; WHO, 2021). In contrast, hypertension, smoking, obesity, unhealthy diets, metabolic disorder, diabetes, and physical inactivity form part of the modifiable risk factors (Díaz-Vegas et al., 2018; Mandini et al., 2018; WHO, 2021). While possessing multiple risk factors does increase the possibility of developing CVD, it does not however imply that CVD development is imminent (BHF, 2019).

2.2.1 Non-modifiable risk factors

The rise in population aging observed globally, accompanies increased prevalence in non-communicable diseases including CVD. The risk of developing CVD has been proven to increase proportionally with age, with the lifetime risk of developing CAD and stroke doubling after the age of 40 and 50, respectively (ADA, 2018; Brown et al., 2020; Sanchis-Gomar et al., 2016). Likewise, men tend have greater risk of developing CVD than a premenopausal woman, possibly linked to the protective role of female cyclic hormones. Ongoing research further supports the concept of certain CVD risk factors being more prevalent or having a more prominent effect in certain genders (ADA, 2018; Maas & Appelman, 2010; Möller-Leimkühler, 2007; Regitz-Zagrosek et al., 2016). Additionally, the influences of ethnicity and family history cannot be underestimated as risk factors for diseases with a known genetic component such as CVD. To this end, higher risk of CVD mortality has been observed in individuals with a family history of premature CVD, as well as those of African, Asian, or Caribbean ancestry (Allport et al., 2016; Carnethon et al., 2017; Chaturvedi, 2003; Rodriguez et al., 2014; Volgman et al., 2018). Other non-modifiable determinants of CVD include socioeconomic status with specific emphasis on stress and limited access to proper nutrition.

2.2.2 Modifiable risk factors

Modifiable risk factors, often referred to as behavioural risk factors, are characterised by their ability to be reduced or controlled through the effective implementation of lifestyle modifications. As is the case with numerous other diseases, the use of tobacco products has been shown increase the risk of CVD development and mortality (BHF, 2021; Mons et al., 2015; Pan et al., 2015). This increased risk, to a lesser extent, is also often conveyed to individuals regularly exposed to second-hand smoke (BHF, 2021; Critchley & Capewell, 2003; Japuntich et al., 2015). Another prominent modifiable risk factor is physical inactivity, often promoted by sedentary lifestyles. According to the WHO, there has been an increase in sedentary behaviours with approximately 31% of the population participating in insufficient physical activity (Park et al., 2020; WHO, 2010; WHO, 2020). Furthermore, with clinical recommendations aimed at increasing physical activity levels rather than addressing forces driving sedentary behaviour, these modifiable risk factors continue to affect global mortality (ADA, 2018; Brown et al., 2020; Yusuf et al., 2004). The consequences of modifiable risk factors may present in the form of measurable, intermediate risk factors such as overweight

or obesity, hypertension and elevated blood glucose or lipid levels (Ades & Savage, 2017; Bastien et al., 2014; Wilson et al., 2002). These intermediate risk factors often act as independent modifiable risk factors of cardiovascular events, while simultaneously increasing the risk of developing other CVD risk factors (Brown et al., 2020; Poirier et al., 2009; Romero-Corral et al., 2006). While numerous studies have revealed that the onset of CVD in individuals can be prevented through lifestyle interventions, adherence to such recommendations remains suboptimal (Gleissner et al., 2007; WHO, 2021). In a developing country such as South Africa, the vulnerabilities related to modifiable CVD risk factors are further heightened by an overburdened health care system (StatisSA, 2020). For the purpose of the current study, the focus will be on diet-induced metabolic syndrome as a risk factor for type 2 diabetes mellitus (T2DM) and related cardiovascular complications.

2.2.3 Metabolic syndrome as a risk factor

The last few decades have seen an increase in the consumption of unhealthy diets and the development of metabolic syndrome in underdeveloped countries, including South Africa (Brunner et al., 2007; Ng et al., 2013; Retelny et al., 2008). This increase is concomitant to escalating CVD incidence and in part due to increased urbanisation (Maruthur et al., 2009; Retelny et al., 2008; Stenvinkel et al., 2007; Vorster, 2002). Both clinical and basic research has shown that diets rich in fat and sugar lead to elevated blood glucose levels, obesity, hypertension, and hyperlipidaemia (Amani et al., 2010; Arnett et al., 2019; Brown et al., 2020; Chobanian et al., 2003; Jang & Serra, 2014; Narain et al., 2016). These conditions often cluster together as metabolic syndrome, known to predispose individuals to the clinical manifestation of T2DM and CVD (Kim et al.,2010; Zong et al., 2016). Per the American Heart Association, diabetic adults are two to four times more likely to die from cardiovascular complications, making it the primary cause of diabetic-related deaths. IR, which is characterised by the compromised ability of cells to respond to insulin, is often considered as the driving force behind T2DM and is accompanied by an increase in cytokines, free fatty acids (FFAs), and oxidative stress (Bian et al, 2008; Inoguchi et al., 2000; Rutter et al., 2005). The latter leads to the activation of damaging pathways involved in impaired endothelial function, inflammation, and apoptosis (Festa et al., 2003; Frankel et al., 2008; Laakso, 2010). Chronic elevated blood glucose levels as a result of T2DM further augments these irregularities, ultimately leading to diabetes-induced cardiac dysfunction (Bruno et al., 2005; de Jager et al., 2006; Soinio et al., 2006; Voelter-Maholknecht, 2016). Additionally, IR has also been shown to be directly linked to atherosclerosis and CAD through the increase

in thrombotic events and the suppression of fibrinolysis (high PAI-1 concentrations), even in non-diabetic subjects (Grant, 2007; Laakso, 1996; Laakso, 2010; Natali et al., 2006; Turner et al., 1998).

2.3 CVD pathophysiology

2.3.1 Shift in substrate preference

The antagonistic relationship between the use of FFA or glucose as the preferred metabolic substrate in the heart is depicted by the Randle cycle (Bae et al., 2021; Hue & Taegtmeyer, 2009; Kolwicz et al., 2013; Marcelino et al., 2013). Under normal physiological conditions, FFAs are the preferred substrate for ATP production in the adult heart, accounting for approximately 60-70% of the energy generation (Chanda et al., 2016; Lopaschuk, et al., 2010; Ragavan et al., 2017; Stanley et al., 2005). The remaining energy is acquired from the oxidation of glucose, lactate and to a lesser extent, amino acids, and ketone bodies (Abdurrachim et al., 2015; Chanda et al., 2016; Lei et al., 2004; Ormazabal et al., 2018; Stanley et al., 2005). The cardiac network alternates between these various substrates depending on numerous factors including substrate availability, energy demand, physiological and pathological conditions (Abdurrachim et al., 2015; Akki et al., 2008; Bastiaansen et al., 2016; Goodwin & Taegtmeyer, 2000).

Under cardiac stress such as exercise, hypertrophy and diabetic-induced cardiac dysfunction, glucose becomes the preferred substrate to accommodate the increased energy demand (How et al., 2006; Mandavia et al., 2013; Nagoshi et al., 2011; Ormazabal et al., 2018; Shao & Tian, 2015). This adaptive metabolic shift relies heavily on efficient transport of glucose into cardiac cells, which is mediated by two major glucose transporter (GLUT) proteins namely, GLUT1 and GLUT4 (Figure 2.1). While GLUT1 is predominantly responsible for the uptake of basal glucose, GLUT4 is the more prominent mechanism of glucose uptake in the heart (Augustin, 2010; Shao & Tian, 2015; Tsirka et al., 2001). Research has shown that insulin stimulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane when there is an increased demand for glucose metabolism (Goodwin & Taegtmeyer, 2000; Malfitano et al., 2015; Ormazabal et al., 2018; Stanley et al., 2005;). Similarly, increased glucose uptake has been associated with GLUT4 overexpression in the heart under the above-mentioned stress conditions, simulating a fetal-like metabolic profile (Chen et al., 2007; Watson et al., 2004; Zisman et al., 2000). These

observations are further supported by the reappearance of a fetal-like gene expression profiles, which play a vital role in pathological cardiac remodelling (Doenst et al., 2010; Kolwicz & Tian, 2011; Razeghi et al., 2001).

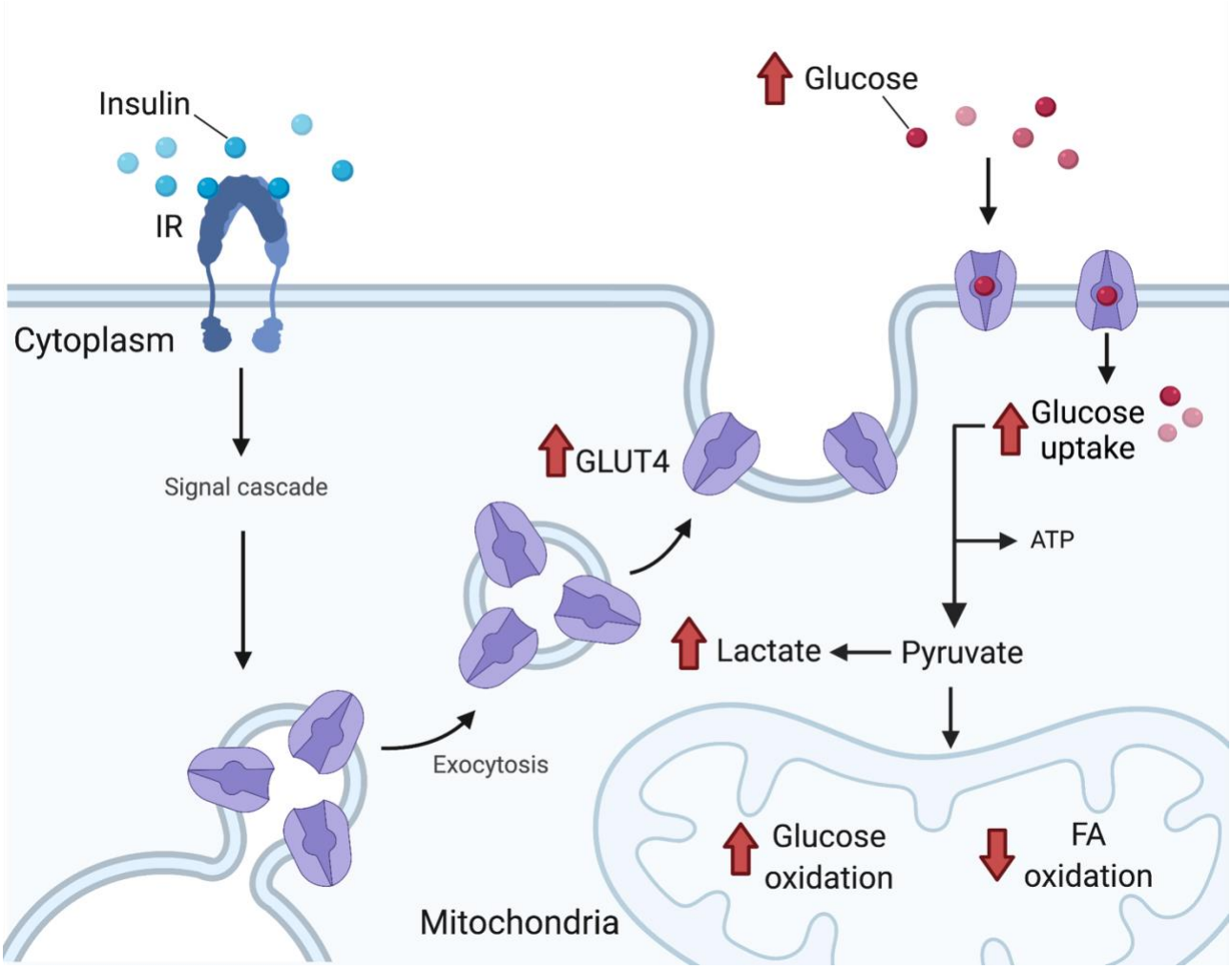

Figure 2.1. Schematic diagram illustrating the metabolic shift in cardiac energy metabolism in favour of glucose utilisation. Under cardiac stress such as **hypertrophy** and **heart failure** (HF) glucose becomes the preferred substrate for energy generation. Increased glucose uptake is mediated by the main glucose transporter in the heart, GLUT4. GLUT4 is translocated from intracellular compartments to the cell membrane through insulin stimulation. Altered glucose metabolism inhibits fatty acid (FA) oxidation to accommodate for the increased energy demand during cardiac hypertrophy. Red arrow labelling illustrates the effect on the respective components or genes where an up-arrow indicates increase, and a down-arrow represents decrease. **IR**, insulin receptor; **GLUT4**, glucose transporter 4; **ATP**, adenosine triphosphate; **FA**, fatty acid. Adapted from "Insulin Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

During IR however, the heart is nested in an environment characterised by elevated FFAs and glucose levels resulting in the inhibition of glucose uptake and the generation of energy almost exclusively (≥ 90% ATP) through FFA uptake and oxidation (Figure 2.2) (Aroor et al., 2012; How et al., 2006; Mandavia et al., 2013; Marfella et al., 2009; Wright et al., 2009). Of interest to this study is the demonstration of decreased glycolysis concomitant with increased FFA oxidation specifically in diabetic animal models utilising a high fat diet (Belke et al., 2000; Nisr & Affourtit, 2010; Sikder et al., 2018; van den Brom et al., 2009; van den Brom et al., 2010). Increased FFA uptake into cardiac cells is facilitated through the upregulation of the cluster of differentiation 36 (CD36) transporter protein and leads to activation of genes encoding proteins responsible for intracellular FFA transport and oxidation (Ajith & Jayakumar, 2016; Kolwicz et al., 2013; Lopaschuk et al., 2010; Malfitano et al., 2015; Ormazabal et al., 2018; Su & Abumrad, 2009). Following entry into cardiac cells, fatty acids are often transformed into triacylglycerols (TAGs), also known as triglycerides (DeGuire & Weiler, 2013; Kaçar, 2019; Naviglio et al., 2017; Van Der Vusse et al., 2000).

As the disease phenotype continues to progress or additional stresses are introduced, the heart's ability to utilise FFAs as a metabolic substrate decline, consequently leading to intracellular accumulation of lipids such as ceramide and diacylglycerol (DAG) (D'Souza et al., 2016; Oakes et al., 2006; Park et al., 2008). The latter has been shown to induce protein kinase C (PKC) activation, mediating altered insulin signalling, inflammation, and apoptosis, ultimately contributing to contractile dysfunction and cardiomyopathy (Connelly et al., 2009; Geraldes & King, 2010; Goldberg et al., 2012; Kota et al., 2011; Liu et al., 2016; Salabei et al., 2016; Unger & Orci, 2002). Conversely, the continued inhibition of glycolysis can lead to the induction of glucotoxicity through the excess accumulation of advanced glycation end-products (AGEs) and the activation of detrimental pathways including the polyol- and hexosamine pathway within the heart (Daroux et al., 2010; Folli et al., 2011; Kresge et al., 2005; Ormazabal et al., 2018).

Collectively, investigations regarding the use of FFAs as a metabolic substrate compared to glucose suggest that the ability of the heart to alternate between multiple substrates depending on the energy demands is essential for normal function. Therefore, therapeutic interventions should aim to modify cardiac substrate utilisation to increase metabolic efficiency while simultaneously preventing glucolipotoxicity.

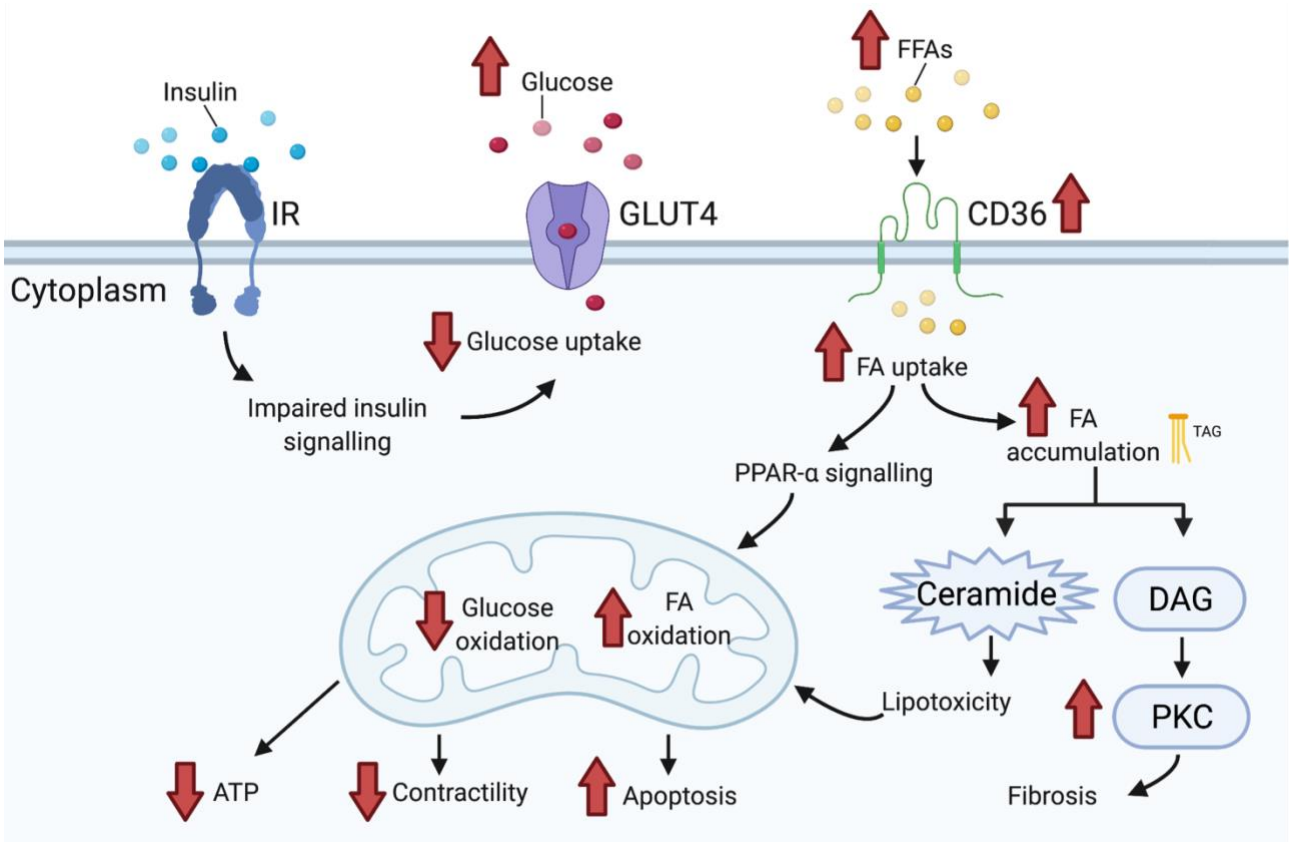

Figure 2.2. Overview of metabolic derangements during **insulin resistance** (IR) facilitated by an abnormal increase in FFA oxidation. Increased FFA uptake through the CD36 transporter activates PPAR-mediated signalling, leading to the upregulation of genes involved in FA oxidation. Concurrently, the accumulation of intracellular lipids such as DAG and ceramide is associated with lipotoxicity. Impaired insulin signalling further decreases the uptake and oxidation of glucose. This shift in substrate preference plays a critical role in activating pathways involved in diabetes-induced cardiac dysfunction including reduced ATP production and cardiac muscle contraction, as well as increased fibrosis and apoptosis. Red arrow labelling illustrates the effect on the respective components or genes where an up-arrow indicates increase, and a down-arrow represents decrease. **FFAs**, free fatty acids; **IR**, insulin receptor; **GLUT4**, glucose transporter 4; **CD36**, cluster of differentiation 36; **PPAR-α**, peroxisome proliferator activated receptor alpha; **FA**, fatty acid; **TAG**, triacylglycerol; **DAG**, diacylglycerol; **PKC**, protein kinase C. Adapted from “Insulin Pathway”, by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

2.3.2 Oxidative stress

Indeed, reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are naturally generated during anaerobic energy production, with basal levels being an essential requirement for biological functions (Finkel, 2011; Goszcz et al., 2015; Senoner & Dichtl, 2019). The increased production of ROS has been extensively implicated in the pathogenesis of numerous diseases including CVD (Guldiken et al, 2009; Lapolla et al., 2007; Senoner & Dichtl, 2019; van Campenhout et al., 2006). Mitochondria, along with the oxidative enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), are considered major driving forces of intracellular ROS production (Bedard & Krause, 2007; Holmström et al., 2014; Zhou et al., 2016). Following a cardiac insult such as chronic hyperglycaemia, the shift in substrate metabolism increases ability of mitochondria to produce energy which also increases ROS production. These elevated ROS levels results in a state of oxidative stress capable of triggering detrimental effects on cellular function (Zhou et al., 2018). In addition to oxidative stress leading to the depolarisation of the mitochondrial membrane, it is also a major stimulator of pro-inflammatory responses which further augments mitochondrial dysfunction and induces apoptosis within the heart (Kim et al., 2005; Montaigne et al., 2013; Sabri et al., 2003; Sack, 2009; Zorov et al., 2014).

Indeed, oxidative stress has been associated with endothelial dysfunction, atherosclerosis, diabetic cardiomyopathy, cardiac hypertrophy, hypertension, and heart failure (Boudina & Abel, 2010; Dludla et al., 2014; Ingwall, 2009; Joseph et al., 2014; Kattoor et al., 2017; Kuroda et al., 2010; Mapanga & Essop; 2016; Senoner & Dichtl, 2019; Tahhan et al., 2017; Tarquini et al., 2011). Nonetheless, the body is equipped with an endogenous antioxidant defence network, comprised of both enzymatic and non-enzymatic compounds with the potential to circumvent free radical damage (Balaban et al., 2005; Senoner & Dichtl, 2019). These protective measures, however, have been known to become deficient following chronic exposure to pathological stimuli such as hyperglycaemia (Bhatt et al., 2013; Marnewick et al., 2011; Padmalayam, 2012). This imbalance in favour of pro-oxidants significantly contributes to continued stress induction through the activation of proinflammatory cytokines and chemokines, and myocardial apoptosis (Kim et al., 2005; Sabri et al., 2003; Wang et al., 2007).

2.3.3 Inflammation

As an integral part of the immune response, inflammation has been associated with the pathophysiology of numerous diseases including obesity, metabolic disorder, T2DM and CVD (Buckley et al., 2013; Hansson, 2005; Medzhitov, 2008; Sastre et al., 2011; Willerson & Ridker, 2004). More specifically, vascular inflammation has been extensively investigated for its underlying role in the development of atherosclerosis and cardiac remodelling (Montecucco et al., 2017; Packard & Libby, 2008; Sharma et al., 2016). The activation of inflammatory responses is often facilitated by the inflammasome multi-protein platform which consists of three proteins namely NOD-like receptor (NLR), apoptosis-associated speck-like protein (ASC) and cysteine-aspartic protease (Caspase) (Broz & Dixit, 2016; Kanneganti, 2015; Mariathasan et al., 2004; Martinon et al., 2002). Of interest to the current study is the widely evaluated NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome as increase in its function has been suggested to play a role in diabetes, atherosclerosis, hypertrophy, ischemia, and cardiomyopathy (Duewell et al., 2010; Kawaguchi et al., 2011; Liu et al., 2014; Mezzaroma et al., 2011; Sandanger et al., 2013; Shaw et al., 2010; Toldo et al., 2016; Vandanmagsar et al., 2011). In the absence of a microbial pathogen, as is the case with CVD, several signals resulting from cardiac injury and apoptotic or necrotic cells can activate the NLRP3 inflammasome (Beg, 2002; Chen et al., 2018; Opie et al., 2006). While the presumed role of the NLRP3 inflammasome in cardiac cells has been poorly defined, the complete signalling cascade has been shown to be active in cardiac fibroblasts (Kawaguchi et al., 2011; Sandanger et al., 2013).

The binding of injury associated ligands to the toll-like receptor 4 (TLR4) mediates pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway, inducing the transcription of NLRP3 (Lawrence, 2009; Lorenzo et al., 2011; Miguel-Carrasco et al., 2010). Furthermore, stimuli such as increased intracellular calcium and ROS facilitates NLRP3 inflammasome assembly and the activation of caspase 1 (Casp1) (Franchi et al., 2009; He et al., 2016; Lee et al., 2015; Ridker et al., 2008). The latter results in increased production of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 18 (IL-18) which augments inflammation, leading to fibrosis, cardiac remodelling and ultimately cell death (Goyal et al., 2008; He et al., 2015; Lee et al., 2015; Nguyen et al., 2017; Shi et al., 2015; Yu & Finlay, 2008). Furthermore, while hyperglycaemia is known to promote NLRP3 inflammasome assembly through ROS, glucose itself has also been shown to be an activator (Gordon et al., 2011; Mariappan et al., 2010; Shi et al., 2015; Zu et al., 2015). Similarly, a high fat diet

has been associated with increased cardiac NLRP3, ASC, Casp1 and IL-1β expression in diabetic rats (Chen et al., 2018; Luo et al., 2014). Along with excessive ROS production, increased expression of the NLRP3 inflammasome stimulates inflammation-induced cell death, in the form of pyroptosis (Qiu et al., 2019; Yang et al., 2018; Yang et al., 2019). The latter is known to play a critical role in the development of T2DM and related cardiovascular complications. Emerging studies have suggested the potential of targeting pyroptosis and inflammasome signalling pathways as a novel therapeutic avenue worth exploring (Chen et al., 2020; Luo et al., 2017; Mamun et al., 2021).

Considering the overwhelming evidence supporting the role of inflammation in CVD, serum inflammation markers such as C-reactive protein (CRP) and TNF-α have been investigated for CVD diagnosis and prognosis (Fichtlscherer et al., 2004; Hulthe et al., 2006; Morrow et al., 2006; Ridker et al., 2000; Ridker, 2003; Tsmikas et al., 2006). Additional pro-inflammatory cytokines and chemokines previously shown to be involved in the diabetes-induced cardiac inflammatory response include interleukin 6 (IL6), chemokine (C-C motif) ligand 3 (CCL3) and monocyte chemotactic protein 1 (MCP1) (Goyal et al., 2008; Melter et al., 2001; Shanmugam et al., 2003).

2.3.4 Apoptosis

The regulated activation of signalling cascades leading to programmed cell death in the form of apoptosis, is an essential component within biological systems (Renehan et al., 2001; Tower, 2015). However, aberrant levels of apoptosis often result in the development diseases and has been linked to ischemia, MI, cardiomyopathy, cardiac remodelling, and HF (Abbate et al., 2003; Baldi et al., 2002; Cotter, 2009; Cummins & Badley, 2010; Kang & Izumo, 2000; Kim & Kang, 2010; Silvestri et al., 2002; Teringova & Tousek, 2017). To this end, the chronic elevated glucose levels accompanying IR and T2DM has been shown to mediate myocardial apoptosis (Cai et al., 2002; Kang et al., 2000; Rajamani & Essop, 2010). The initiation of apoptosis signalling cascades occurs either through the mitochondrial- or death receptor pathway, which both have been shown to be involved in diabetic cardiomyopathy and HF (Figure 2.3) (Giacco & Brownlee, 2010; Kim & Kang, 2010; Li et al., 2007).

The extrinsic death receptor pathway is triggered through the binding of apoptotic ligands such as proinflammatory cytokines TNF-α and cluster of differentiation 95 ligand (CD95L)

to their corresponding death receptors located on the cell membrane (Asgeri et al., 2015; Fertin et al., 2012; Lee et al., 2003; Monden et al., 2007). This interaction leads to the formation of a signalling domain and the activation of caspase 8 (CASP8) (Ashkenazi & Dixit, 1998; Kim & Kang, 2010). Downstream effector caspases, CASP3 and CASP7, are subsequently cleaved thereby inducing the terminal apoptotic pathway (Elmore, 2007; Teringova & Tousek, 2017). Conversely, the intrinsic mitochondrial pathway is stimulated by ROS accumulation, resulting in decreased mitochondrial membrane potential and the release of apoptotic proteins (Kim & Kang, 2010; Rajamani & Essop, 2010). This insult induces the release of cytochrome c (CYCS), which together with caspase 9 (CASP9) and apoptotic protein activating factor 1 (APAF1) forms the apoptosome (Acehan et al., 2002; Bao & Shi, 2007; Johnson et al., 2016; Reubold et al., 2011; Webster, 2012; Yuan et al., 2010). The release of CYCS from the mitochondria is known to be regulated by pro- and anti-apoptotic proteins such as B-cell lymphoma 2 (BCL2) and BCL2 associated X (BAX) (Brocheriou et al., 2000; Chen et al., 2001; Hochhauser et al., 2007; Youle & Strasser, 2008). Interestingly, increased intracellular CYCS, even in the absence of mitochondrial membrane depolarisation, has been associated with apoptosis during ischemic and hyperglycaemic conditions (Borutaite & Brown, 2003; Yu et al., 2008). The apoptosome activation complex subsequently promotes the cleavage of downstream caspases including CASP3 (Kim & Kang, 2010; Teringova & Tousek, 2017). It therefore becomes evident that the extrinsic and intrinsic pathways intersect at a terminal effector, CASP3 or CASP7, to implement apoptotic mediated changes within in the cell. Consequently, increased caspase activity in animal models have been associated with mitochondrial dysfunction, cardiac remodelling, hypertrophy, and ischemia (Cai et al., 2002; Condorelli et al., 2001; Joseph et al., 2014; Liu, 2014; Schwarz et al., 2006). The overlap in apoptotic pathway stimuli and those involved in oxidative stress and inflammation, suggest a synergetic role of these mechanisms in the pathophysiology of diabetes related cardiac complications. Therefore, the prevention of cardiomyocyte loss through the inhibition of apoptosis becomes crucial in the management of diet-induced CVD.

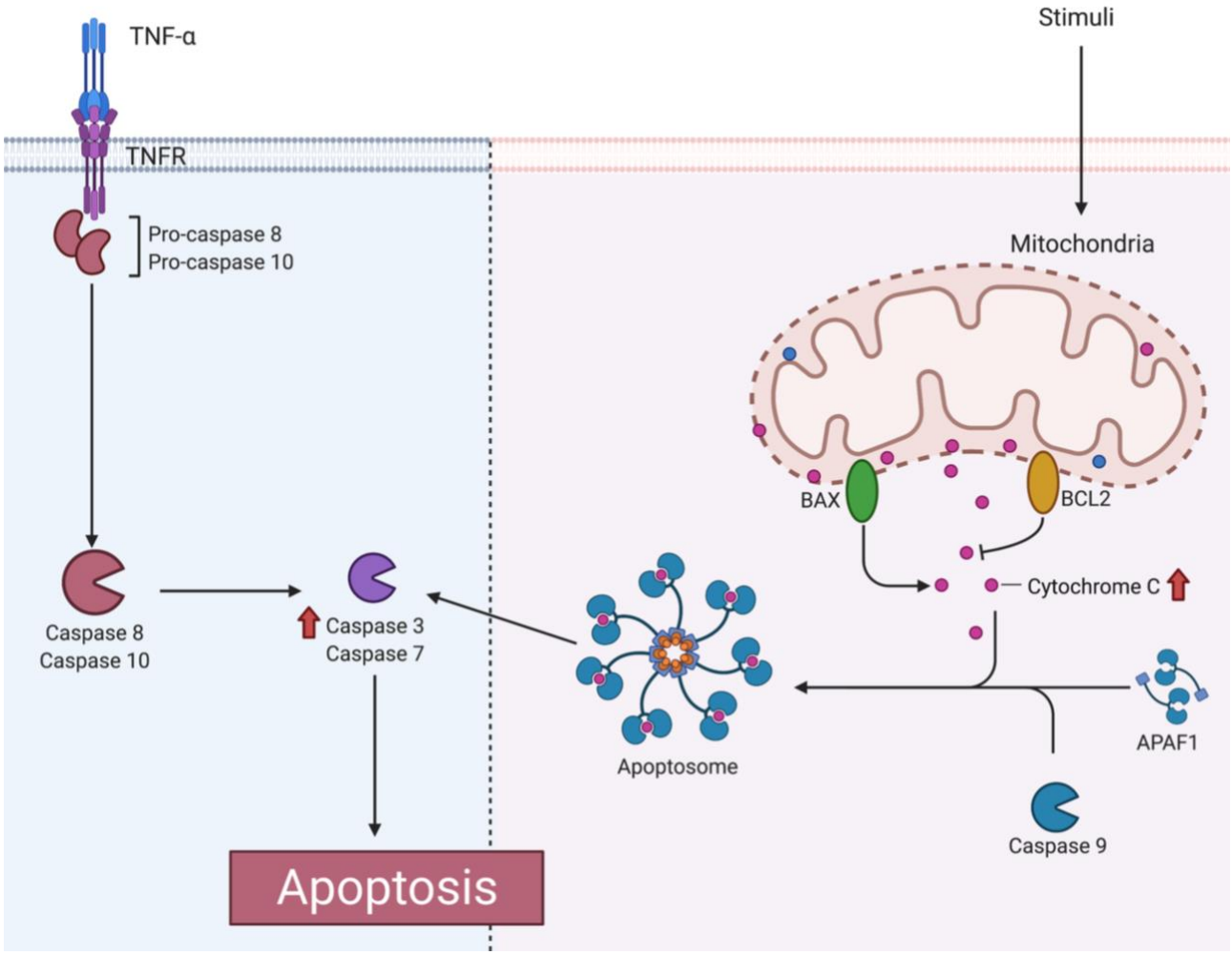

Figure 2.3. The role of apoptosis in diet-induced cardiovascular complications. Initiation of the apoptotic signalling occurs either through death receptor- or mitochondrial pathway. While the extrinsic death receptor pathway is activated through binding of apoptotic ligands (TNF- α or FAS), the intrinsic mitochondrial pathway is initiated through stimuli such as ROS accumulation. **TNF- α**, tumor necrosis factor alpha; **TNFR**, tumor necrosis factor receptor; **BAX**, BCL2 associated X; **BCL2**, B-cell lymphoma 2; **APAF1**, apoptotic protein activating factor 1. Adapted from “Apoptosis Extrinsic and Intrinsic Pathways”, by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

2.4 Epigenetic modifications and CVD

The emergence of epigenetics has provided a new approach for the comprehension of biology and disease (Feinberg, 2010; Jaenisch & Bird, 2003; Khalil, 2014; Portela & Esteller, 2010; Stenvinkel et al., 2007). Epidemiological studies have revealed a definite interaction between environmental factors and genetic components in CVD, suggesting that epigenetics may play a vital role in disease development (Baccarelli et al., 2010; Meaney, 2014; Ordovás & Smith, 2010). Epigenetics refer to the ability of alterations in the cell environment to trigger in changes in chromosomes without altering the DNA sequence (Egger et al., 2004; Rodenhiser & Mann, 2006). These alterations affect the way genes are regulated, are stably inherited, and often lead to the development and progression of disease (Baccarelli et al. 2010; Beekman et al., 2010; Fraga et al., 2005; Ji et al., 2010; Kelsey & Feil Soler, 2013; Miao et al., 2008; Soler-Botija et al., 2019; Ushijima et al., 2003). Some of the major epigenetic modifications include histone modifications, DNA methylation and microRNA (miRNA) related alterations (Khalil, 2014; Soler-Botija et al., 2019; Wilson, 2008). These mechanisms have all been directly associated with the development of CVDs such as atherosclerosis, CHD, hypertrophy, diabetes induced cardiac complications and HF (Baccarelli et al., 2011; El-Osta, 2008; Friso et al., 2012; Latronico & Condorelli, 2009; Mao et al., 2013; Movassagh et al., 2010; Pirola et al., 2011; Stenvinkel et al., 2007; Zhang et al., 2011). Furthermore, several studies have linked alterations in the epigenome with CVD risk factors such as diet, smoking, age, IR, T2DM and hypertension (Buro-Auriemma et al., 2013; Fuke et al., 2004; Khalil, 2014; Ling et al., 2008; Ordovás & Smith, 2010; Rivière et al., 2011). Thus, further investigations into the influence of epigenetic modification on CVD development may therefore aid in understanding disease pathophysiology. Amongst the various epigenetic features investigated, DNA methylation remains the most widely studied and the focus of this study (Michels, 2012; Zhong et al., 2016).

2.4.1 DNA methylation

DNA methylation refers to the addition of a methyl group to the 5th carbon position of the cytosine ring, in humans, this primarily occurs in the context of CG dinucleotides referred to as CpGs (Figure 2.4) (Bird, 2002; Wilson, 2008). CpGs are unevenly distributed throughout the genome and often cluster together forming CpG islands (Khalil, 2014). DNA methylation process is catalysed by DNA methyltransferases (DNMTs) and plays an imperative role in maintaining genomic stability as well as regulating gene expression (Jaenisch & Bird, 2003;

Kirchner et al., 2013; Kurdyukov & Bullock, 2016). Indeed, DNA methylation also alters protein binding sites on DNA, often leading to transcriptional activation or silencing of genes, which in turn may affect phenotype and lead to the development of disease (Moore, Le & Fan, 2013; Nikitina et al., 2007; Suzuki & Bird, 2008). While DNA methylation within the promoter and enhancer regions have been shown to correlate inversely with gene expression, current research has also demonstrated the association of inter- and intragenic DNA methylation with gene expression (Costantino et al., 2018; Fernández-Sanlés et al., 2018; Jeschke et al., 2015; Moore et al., 2013; Muka et al., 2016; Shenker & Flanagan, 2012). Numerous studies have linked altered DNA methylation to biological processes underlying CVD such as arrhythmias, inflammation, hypertension, and endothelial dysfunction (Baccarelli et al., 2010; Friso et al., 2008; Ikram et al., 2009; Joehaneset al., 2016; Turunen et al., 2009; Zeilinger et al., 2013). Nonetheless, it has been increasingly reported that this process can be reversed to restore normal function through lifestyle modifications including diet (Crider et al., 2012; Jamaluddin et al., 2007; Nazki et al., 2014). This reversible nature allows for the use of DNA methylation signatures as possible novel targets in the development of alternative therapeutic strategies to prevent and treat CVD.

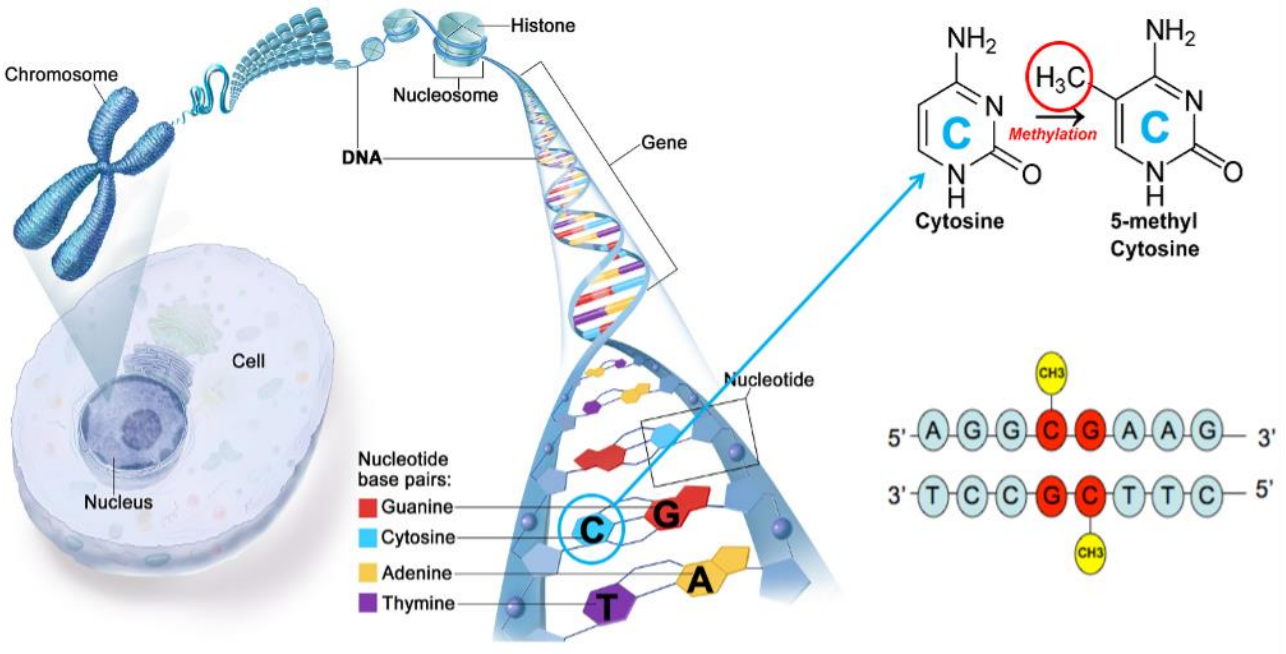

Figure 2.4. Schematic diagram illustrating the concept of DNA methylation. A methyl group is added to the 5th carbon position of the cytosine ring. In humans this primarily occurs in the context of CG dinucleotides.

2.4.2 Measuring DNA methylation

In addition to its gene regulatory role, DNA methylation also plays a significant role in cellular differentiation, and inactivation of the X chromosome (Altun et al., 2010; Condon et al., 2018; Beard et al., 1995; Sun et al., 2015). Given its role in cellular processes and disease, the accurate identification and quantification of changes in DNA methylation within biological ailments has gained considerable attention. One of the first methods used to study DNA methylation entailed the use of methylation sensitive, restriction enzymes such as MspI and HpaII in restriction fragment length polymorphism (RFLP) analysis (Dupont et al., 2009; Jin et al., 2010; Lopez et al., 1997; Vos et al., 1995). While this technique is particularly beneficial in organisms lacking a reference genome, it is limited in the depth of information provided. Furthermore, several commercial kits currently enable the quantification of overall DNA methylation levels using an enzyme-linked immunosorbent assay (ELISA) or microarrays (Benoukraf et al., 2013; Fouse et al., 2010). The lack of information regarding the context specific methylation (CpG- or non-CpG) and genomic location of methylated sites demonstrated by the previously mentioned detection methods has shifted the focus of methylation detection towards more sequence-based techniques.

The advancements in next generation sequencing (NGS) technologies and the availability of complete reference genomes have jointly aided the revolution of DNA methylation profiling on a genome wide scale (Laird, 2010). These techniques can broadly be classified into two categories namely enrichment-based and bisulfite sequencing based methods (Harris et al., 2010; Shafi et al., 2018; Sun et al., 2015; Taiwo et al., 2012). Enrichment-based methods, also referred to as region-based methods, utilise antibodies that bind to methylated DNA prior to sequencing and include techniques such as methyl-DNA immunoprecipitation (MeDIP) or methyl binding domain sequencing (MBD-seq) (Bock et al., 2010; Otto et al., 2012; Ruike et al., 2010; Serre et al., 2010; Weber et al., 2005;). These techniques provide a cost-effective alternative to assess differential methylation levels but are often prejudiced towards highly methylated regions (Lister & Ecker, 2009; Otto et al., 2012; Shafi et al., 2018; Sun et al., 2015). On the other hand, bisulfite sequencing methods entails the sequencing of DNA following chemical modification (i.e., bisulfite conversion), allowing for the evaluation of methylation on a single nucleotide resolution. The bisulfite treatment involves the conversion of unmethylated cytosines to thymine whereas methylated cytosines remain unchanged. The resulting bisulfite converted DNA is then used to evaluate the methylation status of cytosines throughout the genome through whole genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS)

or Infinium methylation microarrays (e.g., Illumina's Methylation450K) (Bock et al., 2010; Cokus et al., 2008; Dedeurwaerder et al., 2011; Harris et al., 2010; Meissner et al., 2005; Sandoval et al., 2011; Sun et al., 2015). While there has been an increase in the number of studies utilising Illumina's microarrays and RRBS due to its reduced cost, the evolution of NGS over the past few years have increased the economic feasibility of WGBS studies. The increased resolution and depth of genome coverage afforded by WGBS has led to it being considered the gold standard approach to evaluate DNA methylation and therefore the method of choice in the present study (Bock et al., 2010; Otto et al., 2012; Shafi et al., 2018; Xi & Li, 2009).

2.5 Therapeutic interventions

2.5.1 Role of anti-glycaemic agents in protecting the heart

While numerous studies have revealed that the onset of heart failure in diabetic individuals can be prevented through lifestyle interventions, adherence to such recommendations remains suboptimal (Gleissner et al., 2007; WHO, 2021). There has been an exponential increase in research evaluating the use of anti-diabetic therapies, with the primary agenda of maintaining normal blood glucose levels, for their ability to prevent or treat related cardiac complications. These secondary therapies are particularly relevant to the current study, as first line cardio-protective therapies are often limited in their ability to account for the extent of environmental influences such as nutritional shifts. The treatment landscape for CVD in patients with T2DM has therefore changed tremendously over the past decade, to now include well-known biguanides such metformin, as well as novel anti-diabetic medications such as glucagon-like peptide-1 (GLP-1) receptor agonists and sodium-glucose cotransporter 2 (SGLT-2) inhibitors. As each class of medication varies in their degree of efficacy and underlying mechanism of action, they will be discussed separately to comprehend their potential cardio-protective effects.

- **Biguanides**

Metformin, the only drug in this class currently available on the market, is the first line treatment for type 2 diabetes (Inzucchi et al., 2015). This group of drugs primarily target the liver to improve sensitivity to insulin and restore glucose metabolism through the reduction of FA oxidation (Viollet et al., 2012). Research involving the cellular mechanism of metformin

suggests its glucose-lowering effects result from the inhibition of the mitochondrial enzyme, complex I (El-Mir et al., 2000; Miller & Birnbaum, 2010). Although the exact mechanism has not been fully elucidated, the interaction of metformin with complex I prohibits the influx of reduced nicotinamide adenine dinucleotide (NADH), leading to a reduction in the cellular energy charge and ultimately impairing mitochondrial oxidative phosphorylation (ATP production) (Foretz et al., 2005; Zhou et al, 2001;). The latter activates adenosine monophosphate-activated protein kinase (AMPK), which was shown to be the major mediator of the reduced gluconeogenic gene expression and glucose production associated with metformin therapy (He et al., 2009; Miller & Birnbaum, 2010; Shaw et al., 2005; Viollet et al., 2012). However, over the past few years AMPK-independent mechanisms have also been implicated (Foretz et al., 2010; Rena et al., 2017). The activation of the primary kinase upstream of AMPK, liver kinase B1 (LKB1), as well as a reduction in the expression of genes encoding gluconeogenesis proteins such as PPAR-γ coactivator 1α (PGC-1α) and phosphoenolpyruvate carboxykinase (PEPCK), are all examples of the abovementioned AMPK-independent pathways (Foretz et al., 2010). These acute changes in gene expression levels, however, did not directly cause a reduction in glucose output and therefore it's level of contribution to the pharmacological action of metformin requires further investigation (Miller & Birnbaum, 2010).

Numerous studies have indicated that the cardiovascular benefits associated with metformin therapy are significantly greater than those exhibited by other diabetic medications such as sulfonylureas (Bennett et al., 2011; American Diabetes Association, 2013; Rao et al., 2008; Rena & Lang, 2018; Roumie et al., 2012). The metformin-related cardioprotective benefits generally result from its metabolic action to diminish IR, ultimate reducing the risk of HF and mortality (Kinsara & Ismail, 2018; Wang et al., 2011). However, studies investigating its role in inflammation have revealed the involvement of the suppression of plasma cytokine levels and the nuclear NF-κB inflammatory signalling pathway (Cameron et al., 2016; Griffin et al., 2017). In addition to this, animal studies have demonstrated the ability of metformin to reduce TNF-α and fibroblast growth factor, thus reducing left ventricular volume and the degree of cardiac remodelling (Eriksson & Nystrom, 2014; Murtaza et al., 2019). Despite the multiple benefits of the drug in CVD, further research is required regarding the mechanisms through which this is exerted in the absence of T2DM.

- **Incretin based therapies (IBT)**

These therapeutics rely on the modulation of two major gut-derived proteins secreted often post-meal, namely, GLP-1 and glucose-dependent insulinotropic peptide (GIP) (Baggio & Drucker, 2007; Inzucchi & McGuire, 2008; Ussher & Drucker, 2014). Release of these incretins occur in a glucose-dependent manner and stimulates insulin output from pancreatic beta cells, to regulate blood glucose concentrations (Advani et al., 2013; Campbell & Drucker, 2013; Lovshin & Drucker, 2009). The two classes of medicines belonging to this therapy method, GLP-1 receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors, exert their anti-diabetic effects by ameliorating the partially deficient incretin system observed in patients with T2DM (Knop et al., 2007; Lovshin & Drucker, 2009). The first drug class, GLP-1 analogues, mimics the function of the natural occurring hormone by binding to GLP-1 receptors found on pancreatic beta cells (Advani et al., 2013; Garg et al., 2019). Incretins are expeditiously degraded by DPP-4 enzymes, expressed in many tissues including the kidney, liver, lung, and the small intestine (Ahren, 2007). As such, the second class of IBT, DPP-4 inhibitors, act as oral blockades of these enzymes, preventing their breaking down and ultimately increasing endogenous concentrations of GLP-1 and other incretins (Garg et al., 2019; Inzucchi & McGuire, 2008; Lovshin & Drucker, 2009). The use of these incretin-based agents to lower blood glucose levels has been extremely successful and they either do not affect body weight (DPP-4 inhibitors) or promote weight loss (GLP-1 receptor agonists) (Garg et al., 2019; Inzucchi & McGuire, 2008; Lovshin & Drucker, 2009; Murtaza et al., 2018).

Therapy with incretin-based drugs can indirectly affect the cardiovascular (CV) system through the metabolic mechanisms resulting from increased insulin levels, as well as decreased glucagon and FFA levels (Garg et al., 2019; Murtaza et al., 2018; Ussher & Drucker, 2014). By mediating these traditional risk factors for CVD, these agents improve myocardial metabolism and alleviate contractile dysfunction (Hernandez et al., 2018; Holman et al 2017; Marso et al., 2016a; Marso et al., 2016b; Pfeffer et al., 2015). Through acting on inflammatory pathways and GLP-1 receptors that are expressed on vascular smooth muscle and myocardial cells, IBT also have the potential to exert direct functional effects on cardiac myocytes and coronary vasculature (Garg et al., 2019; Inzucchi & McGuire, 2008; Ussher & Drucker, 2014). IBT not only promotes coronary blood flow and increases glucose uptake, but also reduces inflammatory myocardial damage (Inzucchi & McGuire, 2008; Ussher & Drucker, 2014). Pre-clinical and animal studies have revealed that systemic treatment with GLP-1 analogues and DDP-4 inhibitors exert beneficial effects on

left ventricular function (McMurray et al., 2013; Murtaza et al., 2018). Remarkably, the two classes of drugs do not demonstrate the same levels of CV effects in large outcome studies, with GLP-1 agonists showing increased efficacy regarding myocardial infarction (MI), stroke and CVD death (Gerstein et al., 2018; Hernandez et al., 2018; Holman et al 2017; Marso et al., 2016a; Marso et al., 2016b). Whereas DPP-4 inhibitors on the other hand, demonstrate the potential to affect additional peptides, including stromal cell derived factor-1 (SDF-1) which can have adverse CV effects such as cardiac inflammation, fibrosis, adverse remodelling, and increased risk for HF (Meneilly et al., 2003; Schwartz et al., 2010; Xiao et al., 2012). The multiple differences in cardiovascular mechanisms of action between incretin-based drugs emphasise the importance of individual clinical trials to examine the safety and efficacy of each agent and the underlying mechanism involved (Andrew et al., 2019; Bain et al., 2019).

- **Gliflozins**

SGLT-2 inhibitors collectively referred to as gliflozins, represent a relatively new class of drugs that exert anti-hyperglycaemic effects by augmenting the occurrence of glucosuria, as well as natriuresis in an insulin-independent manner (Anderson & Marrs, 2012; Saleem, 2017; Shubrook et al., 2015). As the name suggests, this class of medication can bind to low affinity SGLT-2 receptors, predominantly situated in the proximal convoluted tubules of the kidneys (Chao, 2014; Kalra, 2014; Lytvyn et al., 2017). These transporter proteins are responsible for approximately 90% of the kidney's glucose reabsorption thus through its inhibition, gliflozins prevent the reuptake of glucose, promoting the excretion of glucose in urine (glucosuria) and subsequently leading to a decrease in blood glucose levels (Anderson & Marrs, 2012; Li et al., 2011; Murtaza et al., 2018). Additionally, SGLT-2 receptors also facilitate sodium reabsorption at the proximal convoluted tubule, which has been shown to be increased under diabetic conditions (Lytvyn et al., 2017). Treatment with SGLT-2 inhibitors thus increases the concentration of sodium in the tubule and aids its elimination from the body in urine (Garg et al., 2019). This natriuretic effect is further supported by a decrease in total body sodium content observed in patients receiving SGLT-2 inhibitor treatment (Garg et al., 2019; Schneider et al., 2017).

Gliflozins, although moderate compared to GLP-1 receptor agonists, indirectly reduces CVD risk factors such as obesity, hypertension, and hyperlipidaemia in T2DM patients (Buse et al., 2013; Kalra, 2014; Katout et al., 2014; Lytvyn et al., 2017; Neal et al., 2017; Pozo et al., 2019; Schork et al., 2019; Wiviott et al., 2019; Zinman et al., 2015). On a molecular level,

activation of the renin–angiotensin–aldosterone system (RAAS) leads to an increase in the expression of SGLT-2 transcripts (Bautista et al., 2004; Lim et al., 2018). Inhibition of SGLT-2 could hypothetically have inhibitory effects on the regulation of intravascular system and blood pressure (BP) for which the RAAS is responsible (Garg et al., 2019). Numerous studies have reported on the ability of SGLT-2 inhibitors to decrease both systolic and diastolic BP in T2DM patients with or without hypertension (Frías et al., 2016; Kario et al., 2018; Neal et al., 2017; Wiviott et al., 2019; Zinman et al., 2015). This phenomenon is amplified in the instance of combined therapy with GLP-1 receptor agonists to exceed the ability of each medication when administered as a monotherapy (Frías et al., 2016; Kario et al., 2018; Tikkanen et al., 2015). Although the exact mechanisms through which SGLT-2 inhibitors exert direct cardioprotective effects have not been fully elucidated, current available clinical and animal study data demonstrates the ability of this therapy to reduce the risk of nonfatal MI and stroke, hospitalisation due to HF, as well as CVD mortality (Cheng et al., 2019; Neal et al., 2017; Wiviott et al., 2019; Zinman et al., 2015). Further studies regarding unravelling the molecular mechanisms and pathways behind the cardiovascular benefits of SGLT2 inhibitors will not only contribute to a better understanding of disease pathophysiology but will also promote repurposing of gliflozins for the treatment of HF in diabetic patients as a potential alternative to metformin.

Nonetheless, despite availability of various primary and secondary cardioprotective drug classes, a significant gap remains in the treatment of CVD. Additionally, current available drugs have been reported to have numerous undesirable consequences, therefore alternative therapies such as nutraceutical have been identified as promising targets.

2.5.2 Polyphenols as an alternative cardioprotective therapy

Several clinical and epidemiological studies support the concept that a diet rich in polyphenols is associated with reduced metabolic syndrome and CVD risk (Pan et al., 2013; Pandey & Rizvi, 2009; Parks & Booyse, 2002; Schiano et al., 2015; Switzeny et al., 2012;). These polyphenols are present in dietary components such as fruit, vegetables, herbal tea, red wine and cocoa, and their beneficial effects have been attributed to their antioxidant and anti-inflammatory capacities (Crescenti et al., 2013; Feil & Fraga, 2012). Correspondingly, Mediterranean diets comprising of plant-based fruits and vegetables have been investigated for its ability to prevent chronic diseases (Castro-Barquero et al., 2018; Pernice et al., 2006; Romagnolo & Selmin, 2017). Amongst the dietary antioxidants explored, plant-derived

polyphenols have gained much interest for their potential health benefits against the development of metabolic complications including diabetes and CVD, possibly through the modulation of aberrant DNA methylation (Chen et al., 2013; Feil & Fraga, 2012; Marnewick et al., 2011; Muller et al., 2012; Schiano et al., 2015). Indeed, polyphenols have been recognised as potent DNMT inhibitors with the ability to modulate the expression of a wide range of targets including long interspersed nucleotide repetitive elements-1 (LINE1), insulin-like growth factor 2 (IGF2), sirtuin 1 (SIRT1) and TNF-α known to be associated with CVD (Baccarelli et al., 2010; Chung, et al., 2010; Hoyo et al., 2011; Zhang et al., 2011). Similarly, the plant-derived polyphenol, resveratrol can improve inflammation and CAD through the upregulation of SIRT1 (Chung et al., 2010; Xu et al., 2014). Additionally, a recent study also reported on the ability of a cocoa extract to inhibit the expression levels of genes encoding DNMTs *in vitro* (Crescenti et al., 2013).

2.5.3 Rooibos as a nutraceutical

Of particular interest to this study is the potential therapeutic role of rooibos (*Aspalathus linearis*), a fynbos plant indigenous to South Africa used to manufacturer an internationally popular herbal tea. Rooibos and its polyphenolic compounds have been shown to exert a protective effect on metabolic disorders through its high antioxidant and anti-inflammatory properties (Dludla et al., 2014; Joubert & de Beer, 2011; Mazibuko et al., 2013; Son et al., 2013). Some of the major polyphenolic compounds of therapeutic value in rooibos include C-linked dihydrochalcones (Aspalathin and Nothofagin), flavones (e.g., Orientin and Apigenin), as well as flavanols (Quercetin and Rutin) (Figure 2.5) (Joubert et al., 2012; Shimamura et al., 2006). Amongst these, Aspalathin and Quercetin have been implicated as some of the major contributors to the therapeutic potential of rooibos (Joubert et al., 2009). Following processing, rooibos can be consumed in either its fermented or unfermented form, which differ both in colour and phenolic content (Del Rio et al., 2013; Joubert & Schulz, 2006; von Gadow et al., 1997). To date, fermented rooibos, characterised by its red colour, has been well studied and demonstrated to increase insulin signalling and glucose metabolism while decreasing inflammation in pre-clinical as well as human studies (Joubert & de Beer, 2011; Kotzé-Hörstmann & Sadie-Van Gijsen, 2020; Marnewick et al., 2011; Sanderson et al., 2014; Standley et al., 2001; Villano et al., 2010). However, due to the known decrease in antioxidant content resulting from the fermentation process, research has shifted towards evaluating unfermented, green rooibos for its potentially greater therapeutic effect (Joubert & de Beer, 2011; Marnewick et al., 2000; Standley et al., 2001;

Schulz et al., 2003). In this regard, green rooibos extracts have been demonstrated to improve ROS, hyperglycaemia and hyperlipidaemia *in vitro* utilising skeletal muscle, cardiac and fat cell lines (Johnson et al., 2016; Mazibuko et al., 2013; Mazibuko et al., 2015; Muller et al., 2012). Similarly, numerous studies have also evaluated the role of isolated phenolic compounds such as Aspalathin to ameliorate metabolic disorders such as IR and T2DM (Dludla et al., 2014; Mazibuko et al., 2013; Son et al., 2013). Taken together, current scientific evidence suggests that rooibos and its bioactive compounds exerts its potential cardioprotective effect through modulating major CVD risk factors which will further be discussed individually.

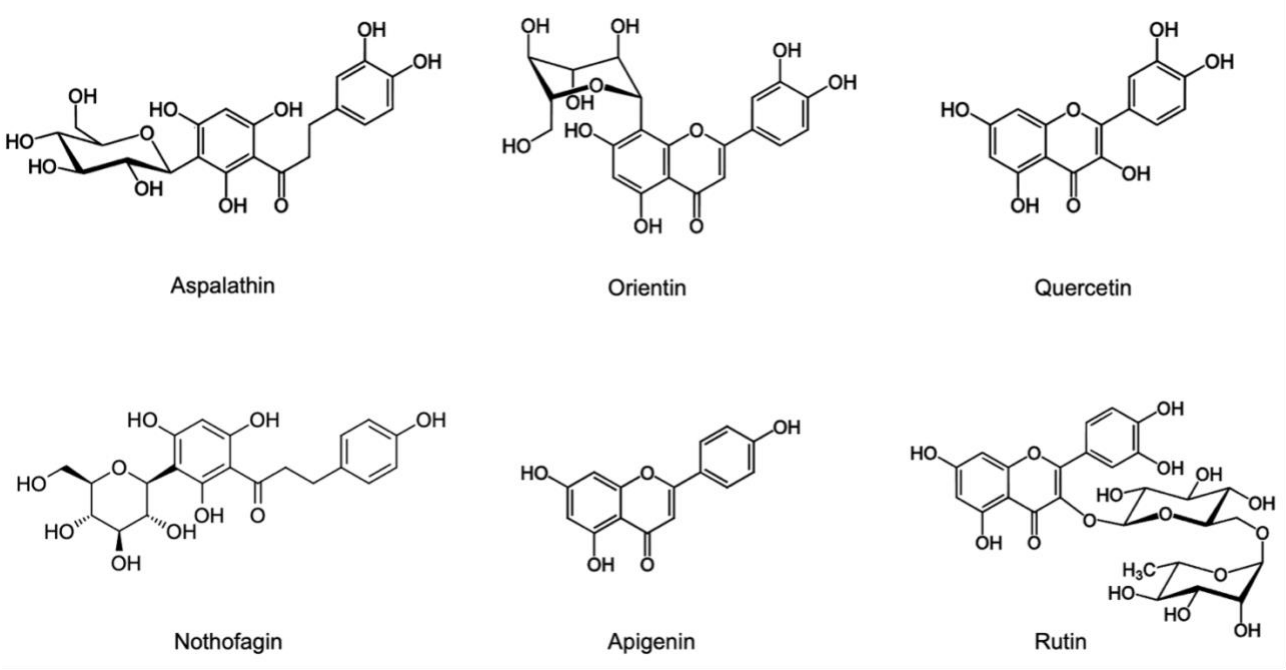

Figure 2.5. The chemical structure of some of the major dihydrochalcones, flavones and flavanols present in Aspalathus linearis (rooibos).

2.5.3.1 Effect on hyperglycaemia and hyperlipidaemia

The evaluation of blood glucose and lipid levels are considered standard practice in CVD risk assessment. Several previously described studies have revealed a significant association between increased glucose and FFA levels, and diet-induced CVD. The *in vitro* effect of fermented and unfermented rooibos on glucose homeostasis has been contradictory, specifically with respect to protein kinase B (PKB) activation which is required for insulin dependent GLUT4 translocation (Garofalo et al., 2003). While Son et al. (2013)

found no significant differences in PKB activation of normal myocytes, a study performed by Mazibuko and colleagues (2013) demonstrated the ability of rooibos to ameliorate decreased PKB levels induced by palmitate exposure in skeletal muscle cells, thereby facilitating glucose uptake through GLUT4 translocations. Similarly *in vivo* studies have also been conflicting where rats receiving fermented and unfermented rooibos extracts displayed no differences in cardiac PKB levels, while mice and humans consuming fermented rooibos displayed enhanced glucose uptake resulting in decreased blood glucose levels (Kamakura, 2015; Marnewick et al., 2011; Pantsi et al., 2011). Furthermore, Aspalathin was able to enhance glucose uptake and insulin signalling *in vitro* as well as in diabetic mouse and rat models (Kawano et al., 2009; Mikami et al., 2015; Muller et al., 2012; Smit et al., 2018; Son et al., 2013). Interestingly, research comparing the effect of both rooibos extracts and Aspalathin, has found the improvement of hyperglycaemia to be more prominent when treating with the extract, suggesting that a possible synergistic interaction of the polyphenolic compounds in rooibos could be responsible to efficiently execute its glucose lowering abilities (Kawano et al., 2006; Mikami et al., 2015; Miller et al., 2018). Additionally, treatment with both rooibos and Aspalathin has been shown to improve lipid profiles through lowering LDL-cholesterol and triglyceride levels in humans, as well as diabetic mice and rats, ultimately reducing CVD risk (Marnewick et al., 2011; Najafian et al., 2016; Son et al., 2013).

2.5.3.2 Effect on oxidative stress and inflammation

As previously discussed, oxidative stress and inflammation in response to stress play a vital role in the underlying pathophysiology of diet-induced metabolic syndrome and its related cardiovascular complications. Briefly, the excessive production of ROS is often accompanied by a reduction in naturally occurring antioxidant levels, resulting in increased cellular damage in the form of oxidative stress. *In vitro* experiments conducted using both fermented- and unfermented rooibos extracts demonstrated their ability to improve antioxidant capacity and reduce intracellular ROS under hyperglycaemic and ischemic conditions (Chen et al., 2013; Dudla et al., 2014; Prasad et al., 2002; Son et al., 2013). These findings were confirmed *in vivo* where the consumption of rooibos tea or extracts resulted in increased antioxidant levels (SOD and GSH) and decreased lipid peroxidation (Akinrinmade et al., 2017; Baba et al., 2009; Marnewick et al., 2011; Orlando et al., 2019; Pantsi et al., 2011). Similarly, the polyphenol Aspalathin was also shown to reduce oxidative stress through the downregulation of NRF2 and subsequently inhibit apoptosis in high

glucose exposed cardiomyocytes as well as diabetic mice (Dludla et al., 2017; Johnson et al., 2016; Simpson et al., 2013). Moreover, the consumption of rooibos tea has also been associated with reduced expression of proinflammatory cytokines TNF-α and IL-6 in an *in vitro* and animal model (Smith & Swart, 2016). Considering the close interaction between oxidative stress mediated mechanism and the inflammatory response in CVD, these findings further support the concept of the antioxidant and anti-inflammatory properties of rooibos contributing to its potential cardioprotective effect.

2.5.3.3 Cardioprotective effect

In addition to exhibiting protective effects against cardiac dysfunction through modulating underlying mechanisms of metabolic risk factors such as obesity, IR and T2DM, rooibos has also been shown to improve cardiac function. Treatment with rooibos extracts have been shown to prevent ischemic damage and inhibit apoptosis by decreasing the activity of apoptotic proteins such as poly ADP ribose polymerase (PARP) and CASP3 (Dludla et al., 2014; Pantsi et al., 2011). Similarly, a recent study performed by Johnson et al. (2017), reported on the ability of Aspalathin to attenuate doxorubicin-induced cardiotoxicity through increased autophagy, while simultaneously increasing the expression of the anti-apoptotic BCL2 protein, suggesting its involvement in regulation of cell death processes in cardiomyocytes (Johnson et al., 2017).

Although several studies have provided evidence that polyphenols mediate their ameliorative effects against disease through epigenetic mechanisms, particularly by reversing aberrant DNA methylation, the transcriptional and epigenetic mechanism(s) by which rooibos mediates its protective effects against diseases remains to be fully elucidated.

2.4 Conclusions

The detrimental impact of non-communicable diseases such as CVD on the health care sector thus drives the need for a more comprehensive understanding of disease pathophysiology and in the role of environmental factors such as diets comprising of excess fat and sugar in disease development. While several underlying mechanisms have been implicated in CVD pathogenesis including oxidative stress, inflammation and apoptosis, there is still a need for investigating the gene regulatory networks and epigenetic modifications associated with these causal factors. Recent improvements in NGS

technologies provides approaches with the required sensitivity to facilitate detailed research regarding these associations. Additionally, the potential cardioprotective properties of plant polyphenols described in this review, further strengthens the evaluation of the Afriplex GRT™ extract in the current study as a nutraceutical to be used as an adjuvant to existing medications in the treatment of diabetic-induced cardiac dysfunction.

2.6 References

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Chapter 3: High glucose and palmitate-induced decrease in antioxidant capacity and mitochondrial bioenergetics in H9c2 cardiomyoblasts: the role of Afriplex GRT™

3.1 Introduction

Cardiovascular disease (CVD), collectively referring to a group of disorders that affect the heart tissue or involve the obstruction of blood vessels, is the leading cause of premature mortality worldwide (Benjamin et al., 2019; Lloyd-Jones et al., 2010; WHO, 2021). An estimated 17.7 million people died from CVDs in 2015, representing 31% of all global deaths and it has been projected to claim 23.6 million lives by 2030 (Benjamin et al., 2019; Donnan et al., 2006; Lloyd-Jones et al., 2010; WHO, 2021). Over the past few decades, CVD incidence in developing nations has escalated tremendously in low- and middle-income countries such as South Africa (Stenvinkel et al., 2007; Vorster, 2002). This increase was concomitant with an increase in the prevalence of major CVD risk factors, including obesity, insulin resistance (IR), and type 2 diabetes (T2D). Increased urbanisation and sedentary lifestyles bring about a nutritional shift, characterised by increased dietary fat and energy-dense diet (Cavan et al., 2016; Humphries et al., 2010; Kim et al., 2010; Stelmach-Mardas et al., 2016; Zong et al., 2016). The latter has previously been linked to both glucose and lipid overload which is associated with decreased cardiac efficiency and subsequent hyperlipidaemic-induced cardiac dysfunction (Kruger et al., 2005; Micklesfield et al., 2013).

A large body of evidence suggests that chronic hyperglycaemia, linked to augmented reactive oxygen species (ROS) levels and oxidative stress, plays a significant role in the development and progression of deteriorating cardiac function (Davargaon et al., 2019; Giacco & Brownlee, 2010; Kayama et al., 2015; Tiwari et al., 2013). The chronic glucose exposure is accompanied by an increase in the release of proinflammatory markers, diminished cytosolic adenosine triphosphate (ATP) production, depolarisation of the mitochondrial membrane and ultimately myocardial apoptosis (Johnson et al., 2016; Johnson et al., 2017; Thomas et al., 2014; Yu et al., 2016). Similarly, the excessive intake of diets high in saturated fatty acid content, such as palmitic acid, has been implicated in the underlying pathology of CVDs (Cetrullo et al., 2020; Haffar et al., 2016; Nobuhara et al., 2013). Both in vitro and in vivo studies have demonstrated the ability of increased palmitic acid concentrations to result in the accumulation of lipotoxic intermediates, ultimately leading to IR, hypertrophy, and cardiac cell death (Chang et al., 2015; Kuwabara et al., 2015;

van de Weijer et al., 2011). Furthermore, free fatty acids (FFA) become the preferred energy substrate in the failing heart, which further exacerbates the decline in heart function (Giacco & Brownlee, 2010; Schulze et al., 2016). Understanding the mechanisms by which saturated fatty acids such as palmitate induces cardiac dysfunction, may lead to the identification of prognostic markers and/or putative therapeutic targets that can prevent premature heart failure. Furthermore, whilst both high levels of saturated fatty acids (such as palmitate) and chronic glucose exposure, have been individually associated with the impairment of cardiovascular functions, very few studies have evaluated their combinatory effect on markers of oxidative stress, inflammation and hypertrophic cardiomyopathy using an *in vitro* H92 cell culture model (Yang et al., 2019).

In the last decade, there has been significant interest in the health benefits of dietary plant polyphenols and their potential to ameliorate diet-induced glucolipotoxicity by increasing the body's endogenous antioxidant defence network (Bahadoran et al., 2013; Darvesh et al., 2010; Feil & Fraga, 2012; Pandey & Rizvi, 2009; Schiano et al., 2015). Of particular interest to the current study is the polyphenolic compounds found in the popular herbal tea, rooibos (Aspalathus linearis), which have previously been demonstrated to reduce oxidative stress and exhibit glucose-lowering, anti-inflammatory as well as cardioprotective properties (Baba et al., 2009; Chen et al., 2013; Dludla et al., 2014; Muller et al., 2012; Son et al., 2013). Therefore, this study proposed to 1) explore whether the combination of high glucose and palmitate induces increased oxidative stress, mitochondrial dysfunction, and cardiomyoblast apoptosis; 2) determine if Afriplex GRT™ and Aspalathin can ameliorate these events; and 3) provide a better understanding of the cardiac gene regulatory network activated. Taken together, the results provided by this study could aid the identification of signatures that could potentially serve as biomarkers for drug targets against diabetes-induced cardiac dysfunction.

3.2 Material and Methods

3.2.1 Cell culture and stress induction

Embryonic-derived rat cardiomyoblasts, H9c2 cells (American Type Culture Collection, CRL-1446), were cultured and maintained under standard conditions (37°C in humidified air and 5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM) (Lonza BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (Biochrom Ltd., Cambridge,

UK). The growth medium (DMEM + 10% FBS) contained 25 mM glucose which is the basal glucose level present in all the conditions listed. Cells were allowed to grow to a confluency of 70 – 80%, with the growth medium replenished every 2 to 3 days. Depending on the assay performed, cells were seeded into the appropriate plates at the required seeding densities specified in the sections below. Stock solutions of palmitic acid (50 mM) were prepared by dissolving the appropriate amount of palmitic acid in absolute ethanol. Prior to treatment experiments, working solutions of palmitic acid (0.5 mM) were prepared in high (33 mM) glucose treatment media, containing 2% fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich Corp., St. Louis, MO, USA) and heated to 37°C for an hour to facilitate the conjugation of palmitic acid with BSA. Confluent H9c2 rat cardiomyoblasts were then exposed to media containing either i) normal (5.5 mM) glucose which served as the control group or ii) high (33 mM) glucose and 0.5 mM palmitic acid specified as the stress-induced group, for 24 hours (Sigma-Aldrich Corp., St. Louis, MO, USA) (Jadaun et al., 2018). All treatments were performed in triplicate (n = 3 technical replicates), and each complete experiment was repeated three times (n = 3 biological replicates). Cell growth and integrity were monitored with microscopy during the growth phase and after the addition of the previously described treatment media. The treatment dosages used for the control and the stress-induced group were based on results obtained from previous studies (Davargaon et al., 2019; Dludla et al., 2017; Johnson et al., 2016; Johnson et al., 2017).

3.2.2 Post-treatment with Afriplex GRT™ and Aspalathin

The effects of an Aspalathin-rich green rooibos extract, Afriplex GRT™ (Afriplex® Pharmaceuticals, Paarl, South Africa) and its major phenolic compound, Aspalathin (Asp) were evaluated on glucolipotoxic conditions in cardiac cells. To this end, stock solutions of Afriplex GRT™ (10 mg/mL) and Asp (22.10 mM) were prepared in dimethyl sulfoxide (DMSO) and diluted to working concentrations using the high glucose plus palmitate (HG + Pal) treatment media (Muller et al., 2012). The toxic effect of DMSO at 0.001% was tested and ruled out in all the experiments performed. Cells pre-treated with HG + Pal for 24 hours were then treated with either Afriplex GRT™ (10 µg/mL) or Asp (1 µM) for an additional six hours. Treatment doses were obtained from previously performed in-house time and dose optimisation experiments (Hu et al., 2016; Johnson et al., 2016; Maeda et al., 2015).

3.2.3 Measurement of cellular metabolic activity

Adenosine triphosphate (ATP) content was used as a determinant of cellular metabolic activity and measured using the ViaLight™ plus ATP kit (Lonza, Basel, Switzerland), according to the manufacturer's instructions. Briefly, H9c2 cells were cultured in 96-well, white-walled luminometer plates at a density of 0.8 x 10^5 cells/mL. Upon completing stress induction and treatment reactions, the cells were lysed with 50 µL of the supplied cell lysis reagent and incubated at room temperature (RT) for approximately 10 min. Subsequently, 100 µL of the ATP monitoring agent, AMR plus, was then added to the lysate and the luminescence was quantified after approximately 2 min using a BioTek® FLx800 plate reader and Gen 5® software (BioTek Instruments Inc., Winooski, VT, USA).

3.2.4 Reactive oxygen species (ROS) detection

It is well established that the excessive production and accumulation of ROS molecules are responsible for oxidative stress-mediated cellular damage (Uy et al., 2011; Tiwari et al., 2013; Davargaon et al., 2019). The ability to detect and estimate ROS levels thus provides a valuable tool to assess the underlying mechanisms involved in the pathogenesis of disorders such as CVD (Giacco & Brownlee, 2010; Kayama et al., 2015). As per the guidelines of a previously established protocol, the intracellular production of ROS was detected in H9c2 cardiomyoblasts seeded in 24-well plates (1 x 10^5 cells/mL) using the Oxiselect™ Intracellular ROS Assay Kit (Cell Biolabs Inc., San Diego, CA, USA). This kit involves the exposure of cells to 20 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dye solution for 30 min, in the absence of light and under standard tissue culture conditions. Following incubation and aspiration of the fluorescent dye, the reaction was terminated by the addition of 150 µL Hank's balanced salt solution (HBBS). Cells were then dissociated from the plate surface using trypsinisation and collected using 400 µL DMEM medium (without FBS). To remove trypsin residue, the collected cell suspension was subjected to centrifugation at 390 rpm for 5 minutes and the cell pellet retained. After resuspension of the cell pellet in 150 µL Dulbecco's phosphate-buffered saline (DPBS) (Lonza, Basel, Switzerland), the DCFH-DA fluorescence was determined using a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) and measured on FL1 (FITC) using a minimum of 10 000 events per measurement.

3.2.5 Assessment of mitochondrial membrane potential (ΔΨM)

Over the past years, evaluating the ΔΨM with the labelling of fluorescent dyes has become a widely used technique as an indication of mitochondrial health. For the purpose of this study, the green, fluorescent dye, JC-1 (5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazoylcarbocyanine iodide) was used to assess mitochondrial depolarisation in H9c2 cardiomyoblasts following culturing and treatment in black, 96-well plates at a density of 0.8 x 10^5 cells/mL, as previously described (Shabalala et al. 2019). Briefly, upon completion of stress induction and treatment, the cells were washed with 100 µL DBPS before staining with 100 µL of 8 µM JC-1 solution reconstituted in glucose-free DMEM without phenol red (Sigma-Aldrich, Saint Louis, MO, USA). Following incubation for 45 min under standard culturing conditions, in the absence of light, the cells were washed with 100 µL DPBS once again and the fluorescence was measured and quantified using a BioTek® FLx800 plate reader and Gen 5® software, respectively. Additionally, fluorescence was visualised and imaged using a Nikon inverted fluorescence microscope (Nikon Inc., Tokyo, Japan).

3.2.6 Mitochondrial bioenergetics assessment

The heart is one of the most metabolically active tissues and therefore relies significantly on the role of mitochondria to ensure efficient function (Dott et al., 2014). The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of intact H9c2 cells were determined in real-time as a direct measurement of mitochondrial dysfunction, using a Seahorse XF96 extracellular flux analyser (Seahorse Bioscience, North Billerica, MA, USA). The use of this assay not only provides an understanding of the underlying cause of mitochondrial dysfunction but also the subsequent role it plays in metabolic pathways associated with CVD phenotypes (Brand & Nichollis, 2011). Undifferentiated H9c2 cells were cultured and seeded in 24-well XF96 cell culture microplates (Seahorse Bioscience, North Billerica, MA, USA) at a density of 1 x 10^5 cells/well using the above-mentioned growth medium. Cells were maintained under standard tissue culture conditions for 48 hours, followed by stress induction and treatment as described above. Key parameters of mitochondrial function in treated cells were assessed using the gold standard, Seahorse XF Mito Stress kit (Seahorse Bioscience, North Billerica, MA, USA), according to the manufacturer's guidelines (Figure 3.1) (Mazibuko-Mbeje et al., 2019). Briefly, cells were incubated in an unbuffered Seahorse XF DMEM base assay medium, containing 1 mM sodium pyruvate, 2 mM glutamine, and 25 mM glucose for 1 hour at 37^{o}C (without CO_2)

prior to conducting the extracellular flux assay. Mitochondrial OCR was measured over a period of 86 min. During this time, pharmacological modulators of oxidative phosphorylation including 1 mM oligomycin, 0.75 mM carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and a combination of 0.5 mM rotenone with 0.5 mM antimycin A were sequentially added to each well at specified time points (Figure 3.1). The compound injections, acting on the various components of the electron transport chain (Figure 3.2), were prepared at a concentration 10X higher than the required final concentration. Following completion of the assay, the bioenergetic profile is represented by OCR data expressed as pmol/min/ and ECAR data as mpH/min.

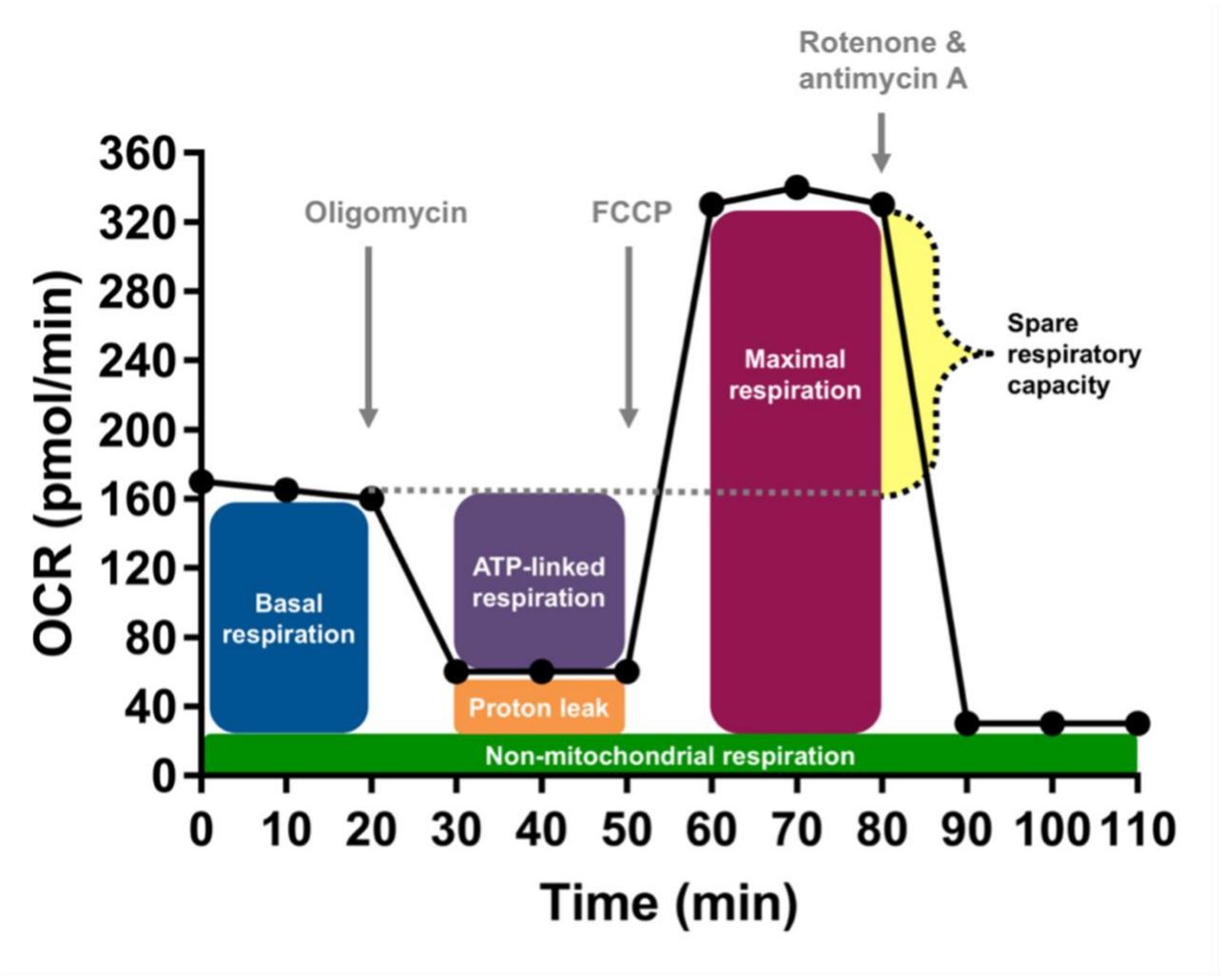

Figure 3.1. A representative of a Seahorse XT Mito Stress profile illustrating key parameters used to assess mitochondrial function. The oxygen consumption rate (OCR) is measured before and after the addition of several pharmacological modulators of mitochondrial respiration. Baseline respiration is used as a measure of the cell's energy demand under baseline conditions. Following this, oligomycin is added from which derive ATP-linked respiration and proton leak respiration can be derived. The addition of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), allows the electron transport chain to function at its maximal

capacity, while the addition of rotenone and antimycin A facilitates the measurement of non-mitochondrial respiration driven by alternative processes outside of the mitochondria. Finally, the spare respiratory capacity is deduced from basal respiration and maximal respiration, which indicates the cell's ability to respond to energy demands and can be a useful indicator of cell health (Seahorse XF Mito Stress Test Kit User Guide, 103016-400).

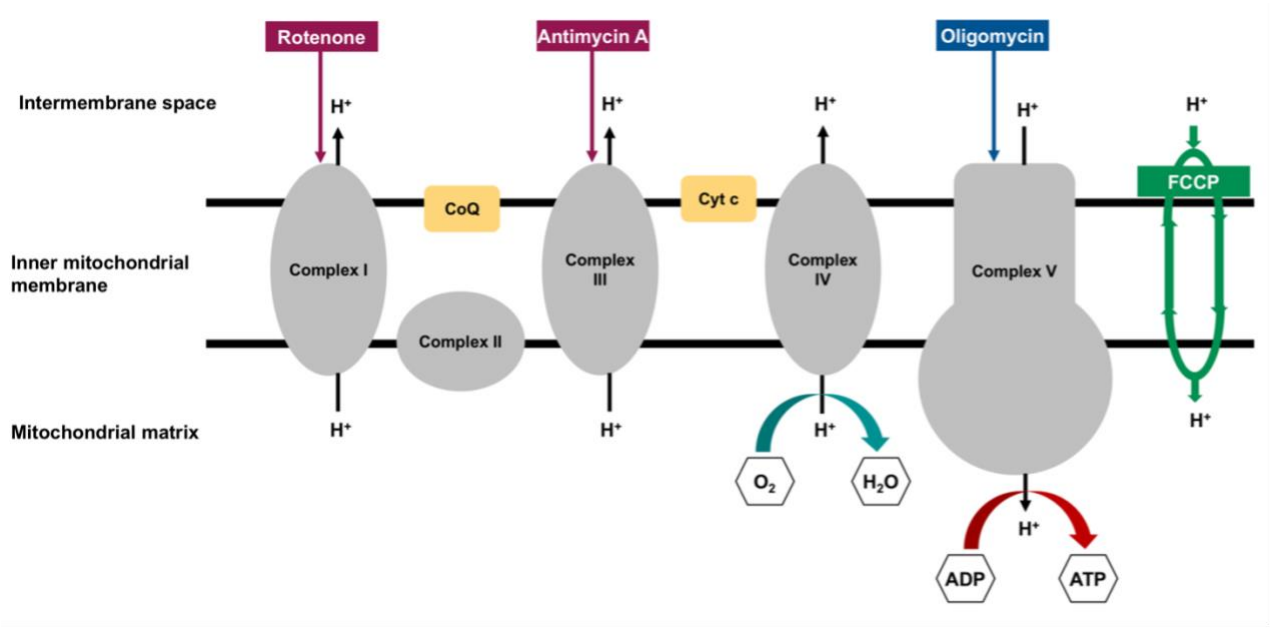

Figure 3.2. Illustration of inhibitory action resulting from pharmacological modulators on electron transport chain (ECT) used during the Seahorse XT Mito Stress assay. Following basal measurements, oligomycin is injected which inhibits ATP synthase (complex V). Secondly, the mitochondrial uncoupler, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is injected which disrupts the mitochondrial membrane and subsequently the proton gradient. The third injection is a combination of rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) which leads to a complete cessation of mitochondrial respiration. The addition of these compounds impacts the flow of electrons through the ECT and either decrease or increase mitochondrial respiration, altering the overall OCR profile.

The resulting OCR data was used to calculate assay parameters indicative of mitochondrial function namely: non-mitochondrial OCR, basal OCR, ATP production, proton leak, maximum respiration, and spare respiratory capacity according to guidelines stipulated in the Wave software version 2.61 (Agilent Technologies, Inc., Santa Clara, CA, USA). These parameters were then subsequently utilised to derive respiratory flux ratios including $State_{apparent}$, respiratory control ratio (RCR), phosphorylating respiration as well as, coupling efficiency as described by Dott et al., 2014. As the conventional respiratory state observed in isolated mitochondria (state 3 or state 4) is unlikely translated directly to intact cells, an intermediate state has been proposed which allows for the calculation of the apparent

respiratory state of the H9c2 cells using Equation 3.1 (Dranka et al., 2010; Brand & Nicholls, 2011; Dott et al., 2014). Similarly, the RCR, representing the relationship between respiration and oxidative phosphorylation was calculated using Equation 3.2 (Brand & Nicholls, 2011; Dott et al., 2014). While the proportion of respiration used to produce ATP under standard conditions was estimated as the phosphorylating respiration (Equation 3.3); the ratio of oxygen utilised for ATP synthesis compared to that driving the proton link was indicated by the coupling efficiency (equation 3.4) (Brand & Nicholls, 2011; Domenis et al., 2012; Dott et al., 2014). Taken together the determined assay parameters and flux ratios provide a comprehensive overview of the alteration of mitochondrial function following stress induction and/or treatment with the polyphenolic compounds of interest.

Equation 3.1

$$State_{apparent} = 4 - \left[\frac{Basal\ OCR - Proton\ leak}{Maximum\ respiration - Proton\ leak}\right]$$

Equation 3.2

$$Respiratory\ control\ ratio\ (RCR) = \frac{Maximum\ respiration}{Proton\ leak}$$

Equation 3.3

$$Phosphorylating\ respiration = \frac{ATP\ production}{Maximum\ respiration}$$

Equation 3.4

$$Coupling\ efficiency = \frac{ATP\ production}{Basal\ OCR}$$

3.2.7 Cellular apoptosis

A widely used combinatory staining process involving Annexin V-FITC (Invitrogen, Carlsbad, CA, USA) and Propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) was employed to

evaluate viable, apoptotic, and necrotic cell profiles. Briefly, the combined cell suspension of H9c2 cells treated in 24-well plates (1 x 10^5 cells/mL), containing both detached cells present in the supernatant and cells attached the surface area of the plate, was collected. Following centrifugation at 390 rpm for 5 minutes, the cell pellet was retained and resuspended in 150 µL staining buffer (DPBS supplemented with 10% FBS) prior to the addition of 1.5 µL Annexin V and 1 µL PI (2 µg/mL) staining solution. The staining reaction was allowed to occur for 10 min, in the absence of light, after which the cells were assessed on a BD Accuri™ C6 flow cytometer using the accompanying BD Accuri C6 Annexin V-FITC/PI template. Quantification of distinct cell populations (i.e live, apoptotic, or necrotic) was conducted with the BD Accuri™ C6 software (BD Biosciences, Franklin Lakes, New Jersey, USA) using the FITC signal detector FL1 (Ex 488 nm, Em 530 nm) for Annexin V positive cells and FL3 detector (Ex 488 nm, Em 670/LP) for PI-positive cells.

3.2.8 Gene expression analysis

3.2.8.1 RNA isolation and purification

Confluent H9c2 cells were seeded in 6-well plates at a density of 2 x 10^5 cells/mL. After completing the above-described stress induction and treatment exposure time, experiments were terminated by washing H9c2 cells with 1 mL DPBS. Total RNA was extracted from 3 wells per condition (n = 4 experiments) using Trizol lysis reagent (Invitrogen, Carlsbad, CA, USA) and treated with the Ambion Turbo DNase kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions to remove genomic DNA. In brief, H9c2 cells were dissociated with 1 mL lysis reagent, and homogenised using a TissueLyser (Qiagen, Hilden, Germany) to further promote cellular disruption. The resulting lysates were then subjected to centrifugation at 13 000 rpm for 10 min to remove cell debris. Phase separation of the previously retained supernatant was performed through the addition of 200 µL chloroform (Sigma-Aldrich Corp., St. Louis, MO, USA), followed by vigorous shaking (~15 seconds) and subsequent incubation at RT for 3 min. Following centrifugation, the clear, upper aqueous layer was attained and combined with 500 µL isopropanol (Sigma-Aldrich Corp., St. Louis, MO, USA) and incubated overnight at -20°C to enable the precipitation of RNA. The RNA was then pelleted with centrifugation at 13 000 rpm for 30 min and subjected to a series of wash steps using 70% ethanol, prior to air drying and resuspension in 60 µL nuclease-free water. All the steps involved in RNA extraction, including centrifugation, were conducted at 4°C unless otherwise stated. The yield and purity of isolated RNA was

quantified using fluorometric (Qubit™ RNA HS Assay kit) and spectrophotometric (Nanodrop) techniques, respectively. Furthermore, RNA integrity was assessed using the LabChip® GX Touch (PerkinElmer, Hopkinton, USA) and recommended RNA Assay, according to the guidelines provided by the manufacturer.

3.2.8.2 Real-time quantitative PCR (RT-qPCR) to confirm oxidative stress

A total of 1 µg total RNA was reversed transcribed to synthesise copy DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA) as described by the manufacturers. Thereafter, real-time quantitative PCR (RT-qPCR) analysis was performed using 5 µL TaqMan Universal PCR master mix, 0.5 µL predesigned TaqMan gene expression assays (*GPX2*, *NOX4*, *NRF2* and *SOD2*), 1 µL cDNA and RNase-free water to a final volume of 10 µL per reaction. All PCR reactions were carried out on a 7500 Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA) using the following cycling conditions: 50°C for 1 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The combination of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin-β (*ACT-β*) was used as endogenous controls and employed in the normalisation of expression levels of the genes of interest using the relative curve method.

3.2.8.3 RT^2 Profiler PCR Array analysis

The extracted RNA (400 ng) was reversed transcribed to generate cDNA with the RT^2 First Strand kit according to the manufacturer's protocol to subsequently employ in the RT^2 Profiler PCR Array system (Qiagen, Hilden, Germany). Accordingly, rat specific Atherosclerosis (PARN-038Z) and Skeletal Muscle Myogenesis and Myopathy (PARN-099Z) RT^2 Profiler PCR Arrays that evaluates the expression of 84 genes (including housekeeping genes and internal controls) were used. Contrary to pathway-specific arrays, disease-specific arrays were selected to analyse the differential expression of genes involved in multiple pathways such as apoptosis, remodelling, as well as stress and immune response, ultimately providing a more extensive overview of gene transcription. A master mix containing 20 µL cDNA; 650 µL RT^2 SYBR Green qPCR Master Mix and 639 µL nuclease-free water (NF-H_2O) was prepared for each sample (n = 4 per condition) and aliquoted into the respective wells of the array plate (384-well, 4 x 96, format E and G). The

QuantStudio™ Flex 7 Real-Time PCR instrument (ThermoFisher Scientific, Inc., Waltham, MA, USA) was used for mRNA quantification using the following cycling conditions: 50°C for 1 min; 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec; 60°C for 1 min and a 4°C infinite hold cycle. The best combination of endogenous controls was selected from the housekeeping gene panel included using geometric means. The geometric mean was selected as opposed to the arithmetic mean to account for the confounding effect. Relative expression levels were normalised to these controls using the delta-delta ($2^{-(\Delta\Delta Ct)}$) method. Analysis of PCR array data was done using a Microsoft Excel sheet with macros and the Gene Globe Data Analysis Centre (Qiagen, Hilden, Germany) made available by the manufacturer. Differentially expressed genes (DEG's) identified from the PCR array were selected based on statistical significance with a p-value less than or equal to 0.05 considered to be significant. The candidate list was further extended to additionally include genes displaying a fold regulation difference higher than or equal to 2, irrespective of the p-value and employed in further downstream analyses.

3.2.8.4 Gene ontology (GO) and pathway enrichment analysis

The candidate lists obtained from the two respective arrays were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg/pathway.html) mapper analysis to cluster DEG's on a biological function level (Kanehisa et al., 2017). Furthermore, the relationship between gene functions and biochemical processes was integrated using the Database for Annotation, Visualisation, and Integrated Discovery (DAVID, david.abcc.ncifcrf.gov) version 6.8 (Huang et al., 2009).

3.2.9 Statistical analysis

Data sets resulting from *in vitro* and gene expression experiments were analysed using a combination of Microsoft Excel and GraphPad Prism version 7.0 software (GraphPad Software, Inc., La Jolla, CA). The distribution of the groups was evaluated for normality and differences in variance, before performing any statistical comparisons. Differences between the means of groups were assessed for statistical significance using parametric tests (ANOVA and student t-tests) followed by a Bonferroni post hoc test, or non-parametric (Kruskal-Wallis) tests followed by a Dunn's post hoc test where applicable. In all instances, data is represented as the mean ± standard error of the mean (SEM), and a statistical difference of $p \leq 0.05$ was considered to be significant.

3.3 Results and Discussion

3.3.1 Cell viability

The availability of ATP has been known to drastically decrease upon cellular damage and has additionally, amongst other intracellular substances, been associated with the mode of resulting cell death (Atlante et al., 2005; Garcia and Massieu, 2003; Ulukaya et al., 2008). In this study, the ViaLight™ plus ATP kit was used to quantify light emission occurring from the chemical conversion of luciferin to oxyluciferin, catalysed by the luciferase enzyme. As ATP is present in all metabolically active cells, a linear relationship between the emitted luminescent signal and ATP concentration or effectively cell number can be observed (Crouch et al., 1993; Mueller et al., 2004). Cardiomyoblasts exposed to HG + Pal media for 24 hours displayed a significant decrease in cytoplasmic ATP levels compared to the NG control (49.29 ± 2.65 vs 100.80 ± 2.93; p = 0.0047) (Figure 3.3). Chronic exposure to glucose and saturated free fatty acids such as palmitic acid has previously been associated with detrimental effects on cell function through glucotoxicity and lipid toxicity (Dyntar et al., 2001; Guan et al., 2019; Mangali et al., 2019; Sears & Perry, 2015). Similarly, *in vitro* studies involving the use of H9c2 cells demonstrated that exposure to either palmitate or high glucose decreased intracellular ATP production, resulting in alterations in energy demands of H9c2 cells by decreasing major ATP consuming processes (Dludla, 2016; Okatan et al. 2019). Thus, the decreased cell viability in cells treated with HG + Pal stress induction media observed in this study is anticipated and confirmed previous findings of either Pal or HG exposure (Davargaon et al., 2019; Johnson et al., 2016; Nobuhara et al., 2013). Although the underlying mechanisms involved are not yet fully understood, recent evidence implicating the involvement of protein kinase R (PKR) mediated activation of c-Jun N-terminal kinase (JNK), nuclear factor kappa B (NF-kB), and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) pathways provide a possible mechanistic approach (Mangali et al., 2019). While treatment with Asp was able to slightly increase the metabolic activity of HG + Pal exposed cells (57.30 ± 2.92 vs 49.29 ± 2.65), the opposite was observed with Afriplex GRT™ treatment (39.16 ± 1.77 vs 49.29 ± 2.65) (Figure 3.3). Therefore, neither treatment with Asp nor Afriplex GRT™ was able to significantly improve the cytotoxic effects brought on by the combinatory insult of HG + Pal on cardiomyoblasts. The latter was incongruent with previous findings of Mazibuko et al (2013) demonstrating that Asp and a

green rooibos extract was capable of increasing ATP production in skeletal muscle cells under hyperglycaemic conditions.

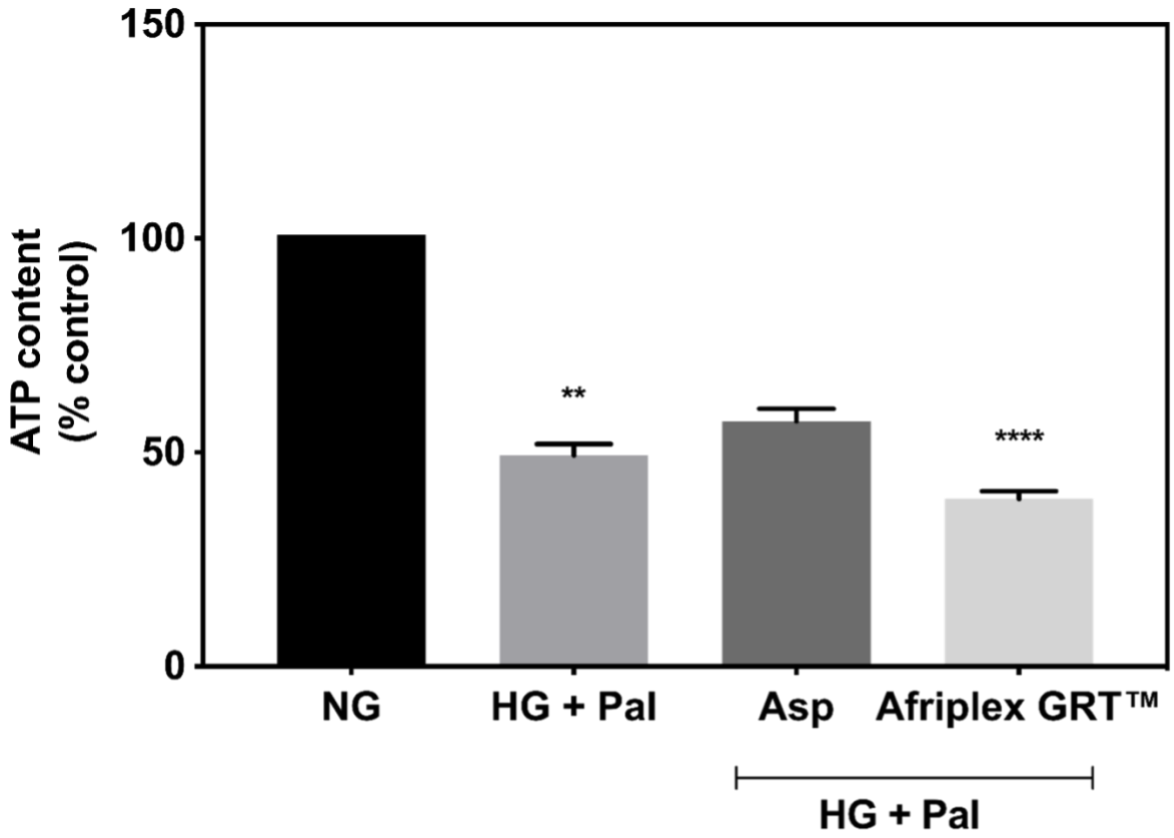

Figure 3.3. The effect of hyperglycaemic and hyperlipidaemic conditions on metabolic activity. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) plus palmitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments, with each experiment having 3 technical repeats (n = 9). Significance is depicted as ** $p < 0.005$ and **** $p < 0.0001$ versus NG control.

3.3.2 Oxidative stress

The ability of oxidative stress resulting from either high glucose- or palmitate-induced ROS accretion to damage DNA, and its involvement in numerous diseases such as diabetes, CVD and cancer has been well established (Görlach et al., 2015; Marnett, 2002; Togo et al., 2018; Wiseman & Halliwell, 1996). In this study, a cell-based assay using the DCFCH-DA fluorescent probe was used to measure hydroxyl, peroxyl, or other reactive oxygen species activity within the cytosol. In theory, DCFH-DA is rapidly oxidised by ROS to highly fluorescent DCF and the intensity of the resulting fluorescence is interpreted to be directly

proportional to levels of ROS within the cell. In this study, HG + Pal stimulation resulted in a substantial shift in the fluorescent peak (Figure 3.4) and a significant decrease in the intracellular ROS levels in comparison to the NG control (108159 ± 5723 vs 361425 ± 3251) (Figure 3.5). Subsequent treatment with Asp (99107 ± 4763 vs 108159 ± 5723), as well as Afriplex GRT™ (96614 ± 3648 vs 108159 ± 5723), had no significant effect on the mean fluorescence and accumulation of ROS when compared with non-treated cells exposed to HG + Pal (Figure 3.4 and 3.5).

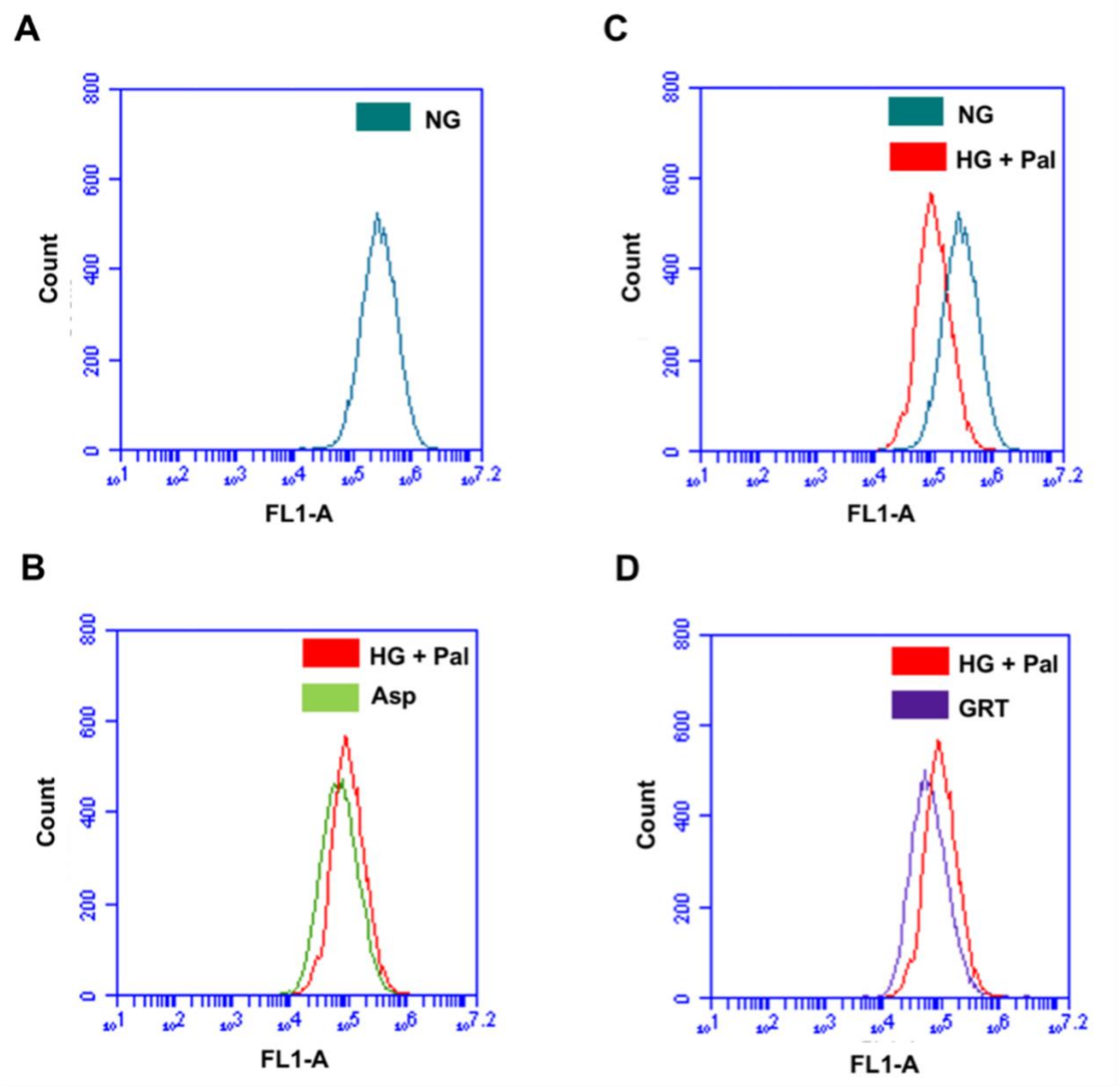

Figure 3.4. Flow cytometric depiction of fluorescent peak shifts obtained from DCFA-DA assay. In all instances, at least 10,000 events were used for each measurement.

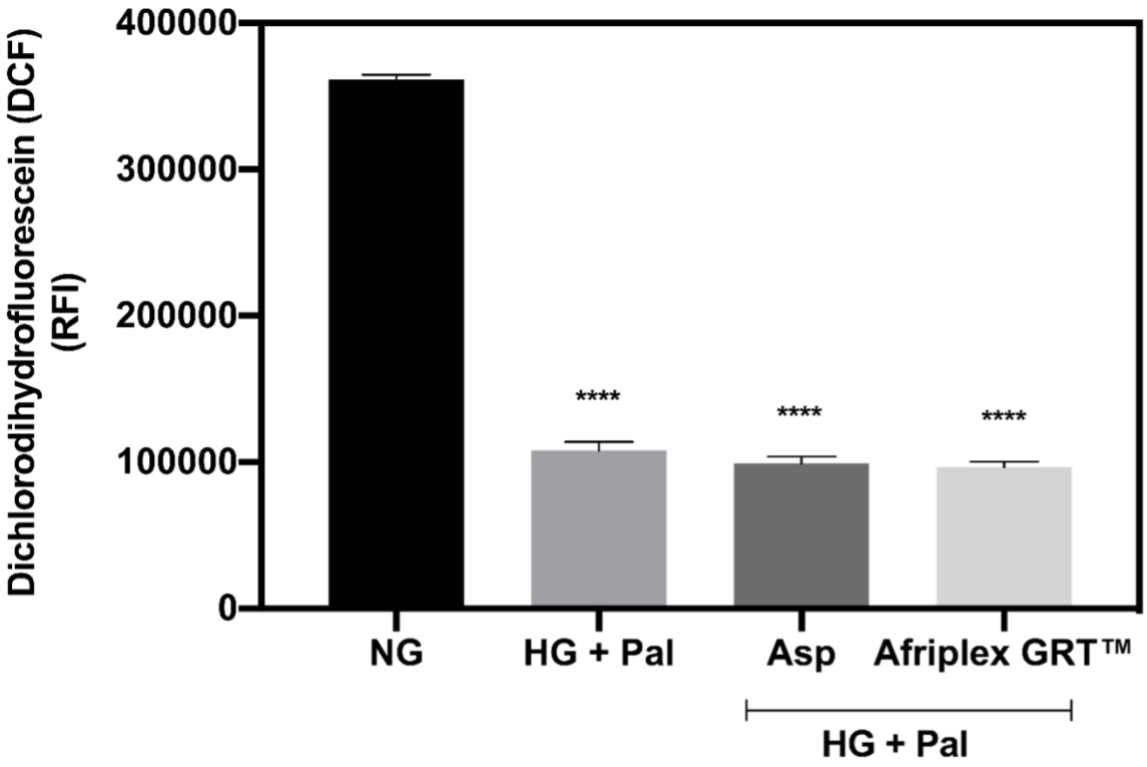

Figure 3.5. The effect of hyperglycaemic and hyperlipidaemic conditions on ROS production. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) plus palmitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments with each experiment having 3 technical repeats (n = 9). Significance is depicted as **** $p < 0.0001$ versus NG control.

Data obtained from pre-clinical and human studies demonstrate that the excess intake of carbohydrates and saturated fat, associated with most western diets, often lead to an elevation of ROS and oxidative stress, and the occurrence of insulin resistance (Basciano et al., 2005; Daly et al., 1997; Tan & Norhaizan, 2019; Vial et al., 2011). Furthermore, several studies, including previously conducted in-house studies by the current research group, have shown that exposure of H9c2 cells to either Pal or HG for 24 hours increases oxidative stress (Davargoan et al., 2019; Okatan et al., 2019; Wei et al., 2013). The results obtained from the current DCFH-DA assay therefore contradicts the bulk of scientific knowledge available with regards to cardiomyoblasts undergoing oxidative stress after chronic Pal or HG exposure. One possible explanation for this observation could be attributed to the large number of cells undergoing apoptosis following HG + Pal stress induction. With most of the cells in the suspension undergoing late-stage apoptosis (Figure 3.10), the incorporation of the DCFH-DA probe is severely diminished, ultimately resulting in a decrease in fluorescence. Given that the suitability of DCFH-DA to accurately measure intracellular ROS

and other oxidative species has been debated, the enquiry of performing this assay over a period, at set intervals, also arises (Kalyanaraman et al., 2012). A plausible alternative resolution could be a lack of protein kinase C (PKC) and NADPH oxidase (NOX) activation, previously demonstrated to potentially be required for palmitate-induced increased ROS production (Kuroda et al., 2010). A previous study conducted by Joseph et al (2016) indicated the inhibition of palmitate-induced ROS production in a genetic cardiomyocyte mice model through the obstruction of PKC or NOX expression. Additionally, Joseph et al (2016) reported that cardiomyocytes have several ROS sources, including NAPDH and nitric oxide synthase (NOS).

Considering this, total RNA extracted from untreated as well as treated cardiomyoblasts were used to evaluate the mRNA expression of pro-oxidant and antioxidant genes, to provide a more comprehensive view regarding the influence of HG + Pal treatment on the oxidative state. Although not significant, exposure of H9c2 cells to HG + Pal decreased the expression of glutathione peroxidase 2 (*GPX2*) ($p = 0.435$) and nuclear factor erythroid 2 related factor 2 (*NRF2*) ($p = 0.260$) by 1.5-fold relative to the NG control (Figure 3.5). Moreover, the mRNA expression of superoxide dismutase 2 (*SOD2*) and NADPH oxidase 4 (*NOX4*) was significantly decreased by 2.1-fold ($p = 0.0002$) and 1.8-fold ($p = 0.0047$), respectively following HG + Pal treatment when compared to the NG control. Palmitate is a known activator of PKC, which leads to an increase NOX4. Although the current study did not investigate PKC expression, the decreased mRNA expression of *NOX4* (Figure 3.6), could suggest that treatment with HG + Pal may have been insufficient to promote an augmented ROS production, however, this requires further investigation (Joseph et al., 2016; Thallas-Bonke et al., 2014). While post-treatment with Asp and Afriplex GRT™ was not able to ameliorate the previously described reduction in expression of the evaluated genes when compared to the HG + Pal treated cells, treatment with Asp significantly lowered the expression of *NRF2* ($p = 0.043$) when compared to the NG control (Figure 3.6). Furthermore, post-treatment with Asp and Afriplex GRT™ exacerbated the significant decrease of both *NOX4* and *SOD2* when compared to the NG control (Figure 3.6). Conversely, previous findings from Mazibuko et al (2013) using C2C12 muscle cells and Dludla et al (2017) using H9c2 cells, reported on the ability of a green rooibos extract (GRE) and Asp to ameliorate either HG- or Pal-induced oxidative stress. However, these studies involved the individual use of either HG or Pal as a stress, with none evaluating the combined effect of HG + Pal as assessed in the current study (Dludla et al., 2017; Mazibuko et al., 2013).

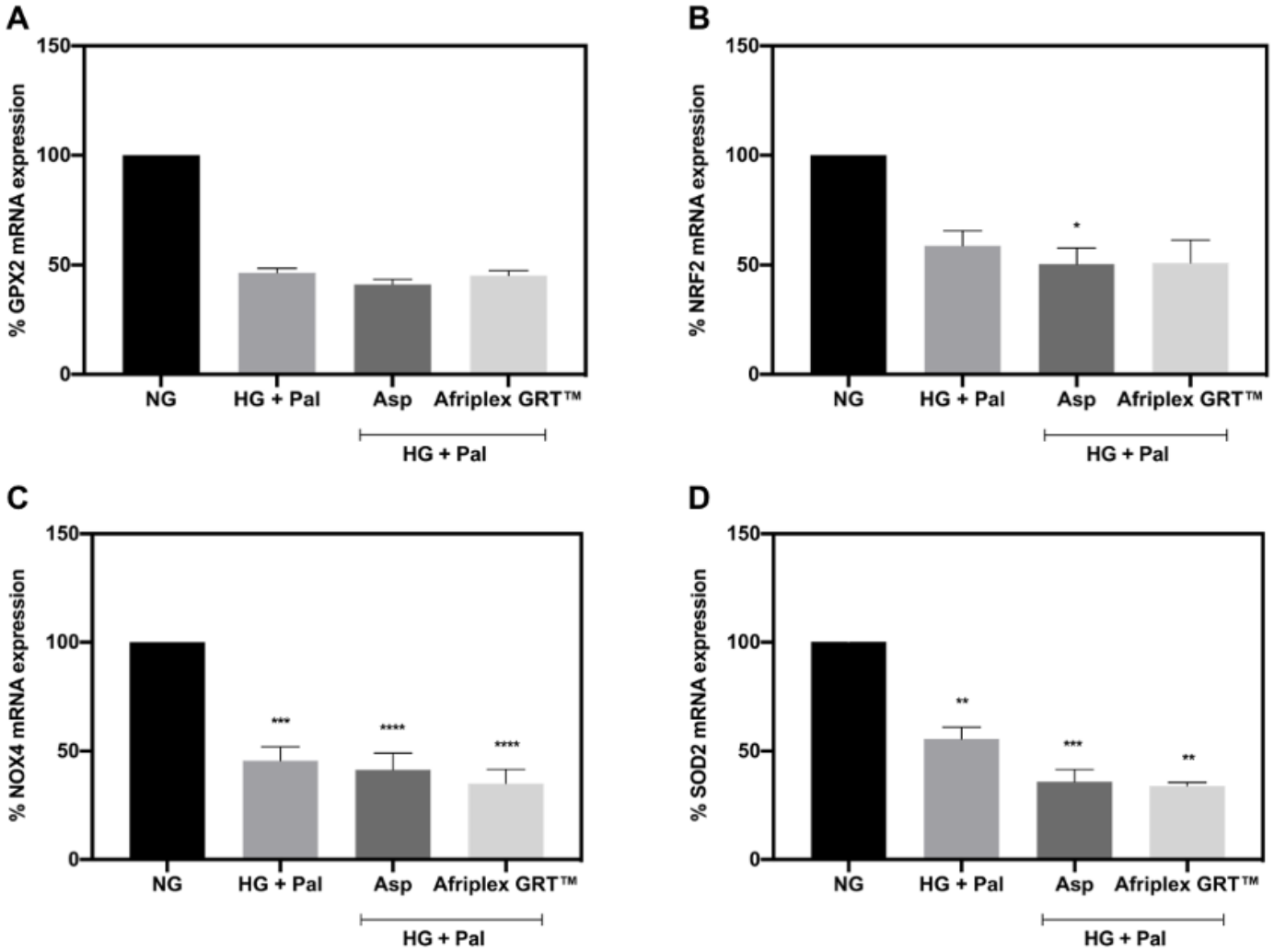

Figure 3.6. The effect of hyperglycaemic and hyperlipidaemic conditions on pro-oxidant and antioxidant mRNA expression. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) plus palmitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments, with each experiment having 3 technical repeats (n = 9). Significance is depicted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus NG control.

The master regulator, *NRF2*, is responsible for regulating the expression of numerous genes with antioxidant activity involved in an inflammatory response and free radical production. Previous studies have demonstrated both *GPX2* and *SOD2* expression are regulated through NRF2-mediated pathways (Banning et al., 2005; Dong et al., 2008; Krehl et al., 2012; Ma, 2013; Sun et al., 2015; Tonelli et al., 2018). Furthermore, *NRF2* exhibits an indirect effect on *NOX4* expression, which is regulated more prominently by the level of ROS as opposed to binding of *NRF2* to the *NOX4* promoter region (Bernard et al., 2017; Brewer et al., 2011; Kovac et al., 2015; Pendyala et al., 2011). The reduction of *NRF2* expression observed in H9c2 cells treated with HG + Pal, therefore, resulted in impaired *GPX2* and *SOD2* expression, ultimately leading to increased levels of hydroperoxides and a possible decline in antioxidant defence (Liu et al., 2017; Margis et al., 2008; Naiki et al., 2014; Toppo et al., 2009). As previously discussed, the unexpected decrease of the pro-oxidant gene, *NOX4*, following HG + Pal treatment could either suggest the possible occurrence of a counter-regulatory mechanism or be related to the low levels of viable cells present due to the severity of the stress conditions (Bernard et al., 2017). While *NOX4* is known to play an important role in cardiovascular pathophysiology, with its overexpression being directly linked to the exacerbation of mitochondrial oxidative stress, the present study was unable to demonstrate this. Therefore, further investigation is warranted to ascertaining the exact functional significance of this phenomenon (Kuroda et al., 2010).

3.3.3 Mitochondrial bioenergetics

Similarly, to previous studies, the effect of HG + Pal treatment on the bioenergetic profile of H9c2 cells was evaluated with the Seahorse XF96 extracellular flux analyser. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the cardiomyoblasts were used as a measurement of oxidative phosphorylation and glycolysis respectively. While cells treated with NG media were able to derive ATP from both oxidative phosphorylation and glycolysis, exposure of H9c2 to HG + Pal resulted in an increase in basal ECAR and a decreased oxidative phosphorylation (Figure 3.6). Under standard conditions, oxidative phosphorylation is the preferred method of ATP production in the heart. However, chronic exposure to elevated glucose and free fatty acids brings about a shift in substrate preference favouring glycolysis (Ait-Aissa et al., 2019; Rosca et al., 2008; Torsten et al., 2013). Treatment with neither Asp nor Afriplex GRT™ was able to alter the bioenergetic profile, with cells treated with these compounds displaying similar OCR and ECAR profiles to the HG + Pal treated cells (Figure 3.7).

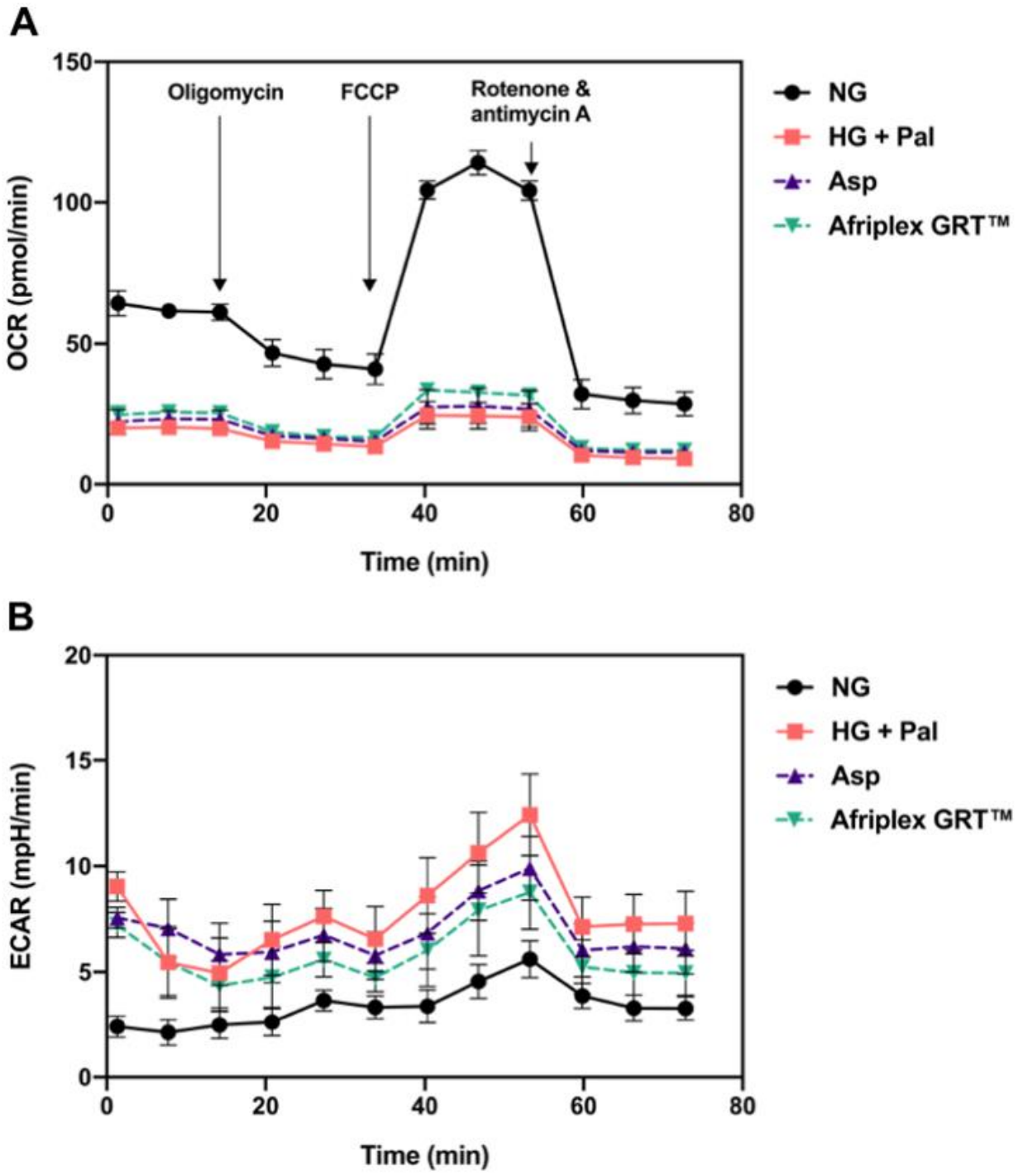

Figure 3.7. The effect of hyperglycaemic and hyperlipidaemic conditions on the bioenergetic profile as illustrated by raw **A)** oxygen consumption rate (OCR) and **B)** extracellular acidification rate (ECAR) measurements. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) plus palmitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments with each experiment having 3 technical repeats (n = 9).

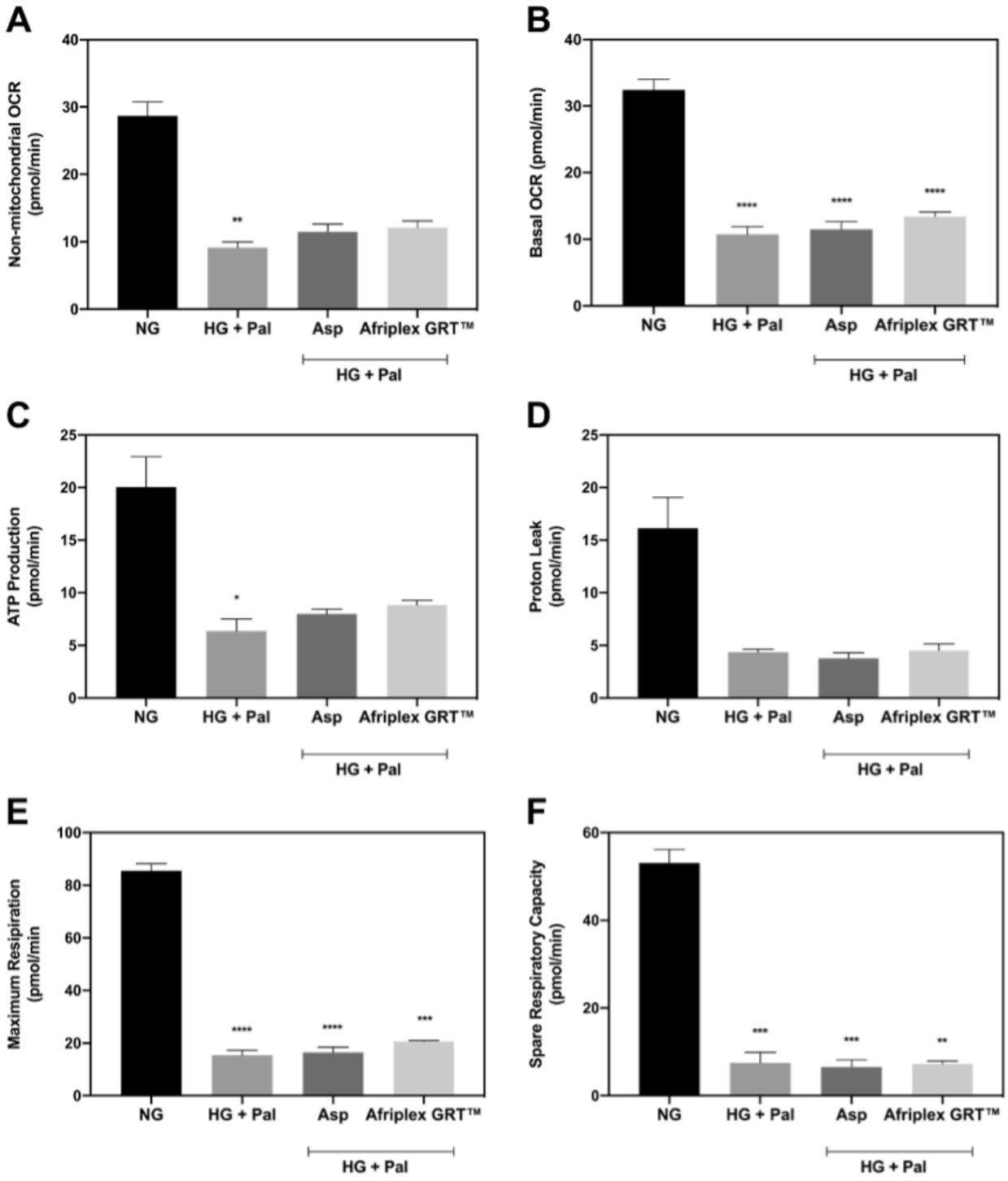

Figure 3.8. The effect of hyperglycaemic and hyperlipidaemic conditions on mitochondrial function assay parameters. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) plus palmitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments with each experiment having 3 technical repeats (n = 9). Significance is depicted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus NG control.

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For efficient energy production in the form of ATP, optimally maintained myocardial mitochondrial bioenergetics is required. To this end, the mitochondrial function of H9c2 cardiomyoblasts was measured through the sequential addition of inhibitors of the respiratory chain. In the current study, HG + Pal treatment was able to significantly decrease basal oxygen consumption ($p < 0.0001$); maximal respiration ($p < 0.0001$); ATP production ($p = 0.047$); as well as non-mitochondrial OCR ($p = 0.002$) compared to the NG control (Figure 3.8). Additionally, there was a tendency towards a decrease in the proton leak following HG + Pal treatment. Treatment with Asp as well as Afriplex GRT™ further suppressed all assay parameters of mitochondrial function to a similar degree than HG + Pal treatment, relative to the NG control (Figure 3.8). The resulting assay parameters were used to calculate internally normalised flux ratios to facilitate more accurate comparisons between the respective treatment groups.

Table 3.1. Respiratory flux ratios derived from mitochondrial parameters of cultured H9c2 cardiomyoblasts.

	NG	HG + Pal	Asp	Afriplex GRT™
$State_{apparent}$	3.73 ± 0.03	3.40 ± 0.04	**3.34 ± 0.05****	3.44 ± 0.03
Respiratory control ratio (RCR)	5.86 ± 1.22	3.55 ± 0.48	4.52 ± 0.35	5.02 ± 0.75
Phosphorylating respiration	0.24 ± 0.04	**0.41 ± 0.03***	**0.50 ± 0.03*****	**0.43 ± 0.02****
Coupling efficiency	0.63 ± 0.12	0.57 ± 0.04	0.69 ± 0.02	0.66 ± 0.03

Values are expressed as mean ± SEM. Each group consists of n = 6 independent replicates. Bold entries represent instances where a significant difference was observed. $State_{apparent}$: HG + Pal + Asp vs NG, ** $p < 0.005$. Phosphorylating respiration: HG + Pal vs NG, * $p < 0.05$; HG + Pal + Asp vs NG, *** $p < 0.001$ and HG + Pal + Afriplex GRT™ vs NG, ** $p < 0.005$.

Exposure of H9c2 cardiomyoblasts to HG + Pal decreased the $State_{apparent}$, respiratory control ratio (RCR) and coupling efficiency while increasing phosphorylation respiration. The $State_{apparent}$ value provides an estimation of the relative mitochondrial workload under basal conditions, and it can therefore be concluded that cells treated with HG + Pal displayed decreased mitochondrial workload (3.40 ± 0.04), which was significantly worsened by Asp treatment (3.34 ± 0.05, $p < 0.005$ compared to the NG control (Table 3.1). Likewise, the phosphorylating respiration following HG + Pal treatment (0.41 ± 0.03, $p < 0.05$) was significantly higher relative to the NG control (0.24 ± 0.04), demonstrating that a greater proportion of the maximal respiratory capacity (maximal OCR) was used for ATP production under basal conditions. This phenomenon persisted following treatment with Asp (0.50 ±

0.03, $p < 0.001$) and Afriplex GRT™ (0.43 ± 0.02, $p < 0.005$) when compared to the cells treated with normal glucose media (Table 3.1). Following the hypothesis that increased free fatty acid (FFA) uptake and oxidation associated with hypoglycaemic conditions, hinder the capabilities of the electron transport chain, several studies have targeted the improvement of mitochondrial function to alleviate cardiovascular complications (Bugger & Abel, 2010; Wu et al, 2018). In agreement, the present study thus demonstrated that the combination of elevated glucose and palmitate severely impaired mitochondrial bioenergetics, resulting in mitochondrial dysfunction. Treatment with neither Asp nor Afriplex GRT™ showed any promising results in attenuating these detrimental effects.

3.3.4 Membrane potential (ΔΨM)

The JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by green fluorescence emission at (~529 nm) for the monomeric form of the probe, which shifts to red (~590 nm) with a concentration-dependent formation of red fluorescent J-aggregates. In healthy cells, with normal mitochondrial membrane potential, JC-1 forms complexes known as J-aggregates producing an orange/red fluorescence. In cells with mitochondrial dysfunction, JC-1 remains in the monomeric form and exhibits a green fluorescence. Consequently, mitochondrial depolarisation is indicated by a decrease in the red/green fluorescence intensity ratio. In this study exposure of H9c2 cardiomyoblasts to HG + Pal resulted in significant depolarisation of the membrane, indicated by an in an increase in the accumulation of JC-1 monomers and a subsequent decrease in the ΔΨM when compared to the NG control (4.27 ± 0.11 vs 6.09 ± 0.27; $p < 0.0001$). Post-treatment with Asp did not affect the extent to which HG plus Pal altered the integrity of the mitochondrial membrane. Interestingly, post-treatment with Afriplex GRT™ further exacerbated mitochondrial depolarisation when compared with non-treated cells exposed to HG + Pal (2.83 ± 0.20 vs 4.27 ± 0.11; $p = 0.0001$) (Figure 3.9). During apoptosis, a significant loss in the permeability of the mitochondrial membrane and electron gradient is observed (Sivandzade et al., 2019; Zorova et al., 2018) Thus suggesting that the depolarisation of the mitochondrial membrane observed in this study could promote cardiomyoblast apoptosis.

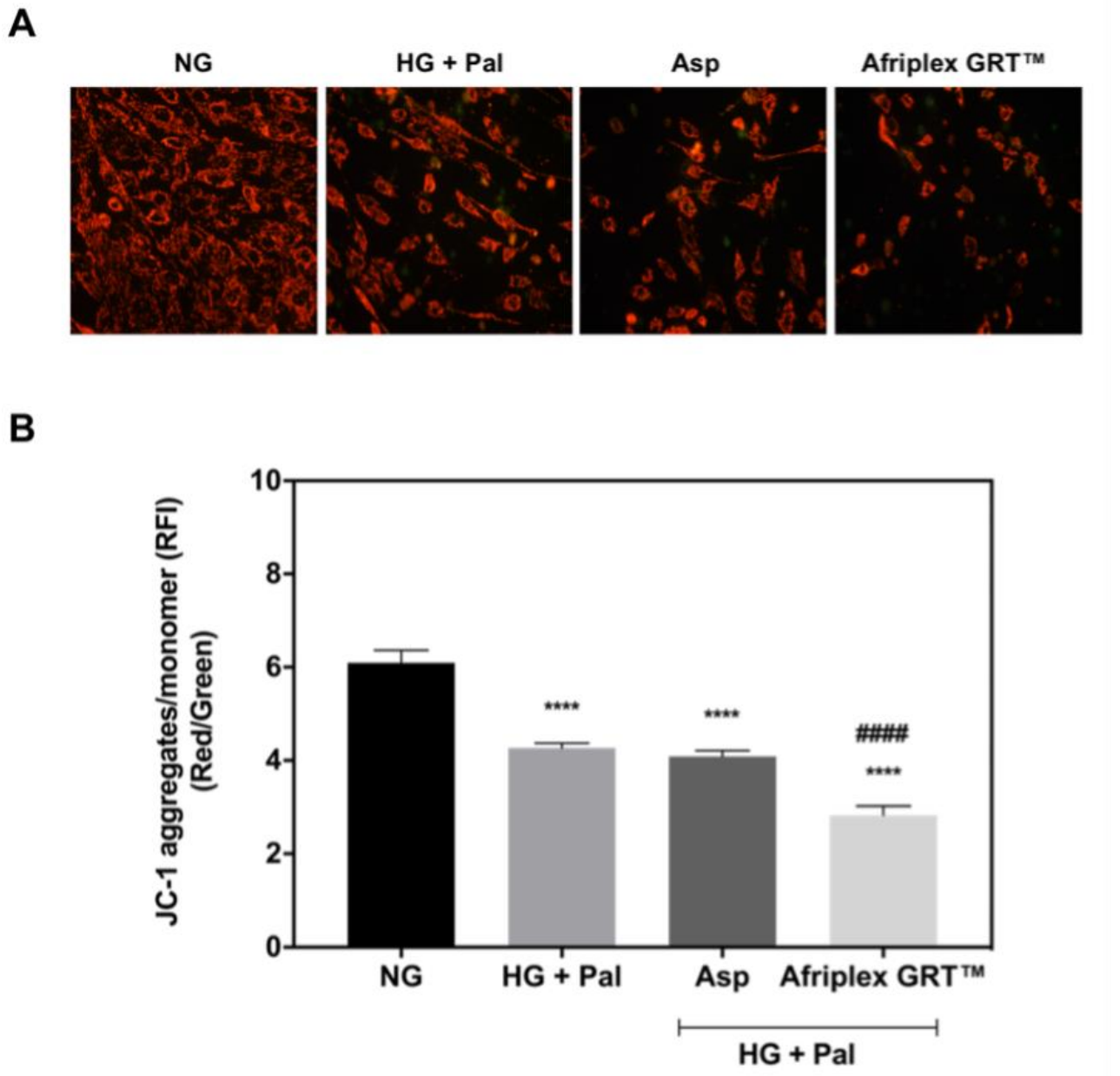

Figure 3.9. The effect of hyperglycaemic and hyperlipidaemic conditions on the mitochondrial potential. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) and palmitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments, with each experiment having 3 technical repeats (n = 9). Significance is depicted as **** $p < 0.0001$ versus NG control and #### $p < 0.0001$ versus HG + Pal group.

3.3.5 Cell death

In this study, the effect of hyperglycaemia plus hyperlipidaemia on the apoptotic state of cardiomyoblasts was evaluated with the commonly used combination of annexin V and PI fluorescent stains. In theory, annexin V is an apoptotic specific marker that binds to phosphatidylserine located on the outer membrane of apoptotic cells. The counterstain, PI, used for its ability to not bind to living cells, enters the cell, and intercalates to DNA only when the membrane has been disrupted. To this end, cells that are negative for both stains are considered “live”; annexin V-positive cells are specified as “early apoptotic”; PI-positive cells are denoted as “necrotic", and cells positive for both stains are symbolised as “late apoptotic” in the cell population being analysed (Figure 3.10 and 3.11). Flow cytometric analysis revealed a significant decrease in the percentage of live cardiomyoblasts after exposure to HG + Pal, compared to the NG control (41.66% ± 1.41 vs 78.31% ± 1.61; $p = 0.0009$). This finding is further supported by a significant increase in cells experiencing late cell death (annexin V and PI positive) (30.70 ± 1.10 vs 6.46 ± 0.70; $p < 0.0001$), as well as necrotic cell death (PI-positive only) (14.52 ± 1.63 vs 5.99 ± 0.29; $p = 0.0012$) following HG + Pal treatment. The increase in PI-positive cells is consistent with the results observed from the JC-1 staining experiments and can in part be attributed to the decrease in membrane potential and increased membrane permeability observed above (Figure 3.9). Although a slight increase in annexin V only positive cells (undergoing early apoptosis) were detected in cardiomyoblasts exposed to HG + Pal, this increase was non-significant compared to the NG control (13.13% ± 0.85 vs 9.24% ± 0.89). In all instances, secondary treatment with either Afriplex GRT™ or Asp had no significant effect on alleviating the degree of apoptosis or necrosis resulting from HG and Pal treatment (Figure 3.11). As the effect of HG + Pal was more prominent and consistent with late-stage cell damage, it could be speculated that simultaneous treatment with Afriplex GRT™ or Asp during the initial HG + Pal exposure as opposed to post-treatment, may have provided more comprehensive protection of the cardiomyoblasts.

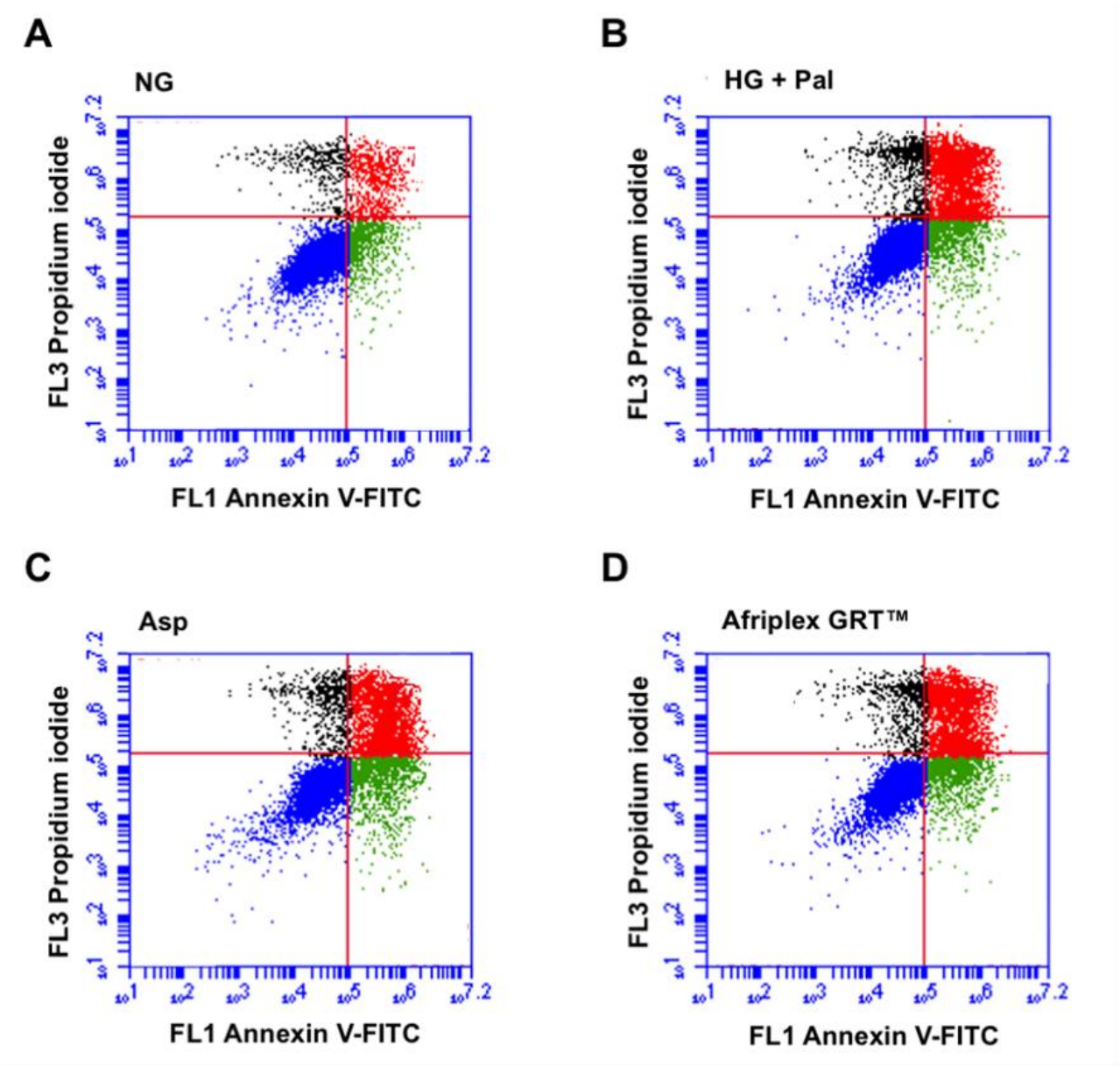

Figure 3.10. Flow cytometric depiction of the clustering of cells into four quadrants based on their apoptotic status. In all instances, “live” cells are depicted as blue; green cells are specified as “early apoptotic” (annexin V-positive); red cells are symbolised as “late apoptotic” (positive for both stains) while black cells represent “necrotic" cells.

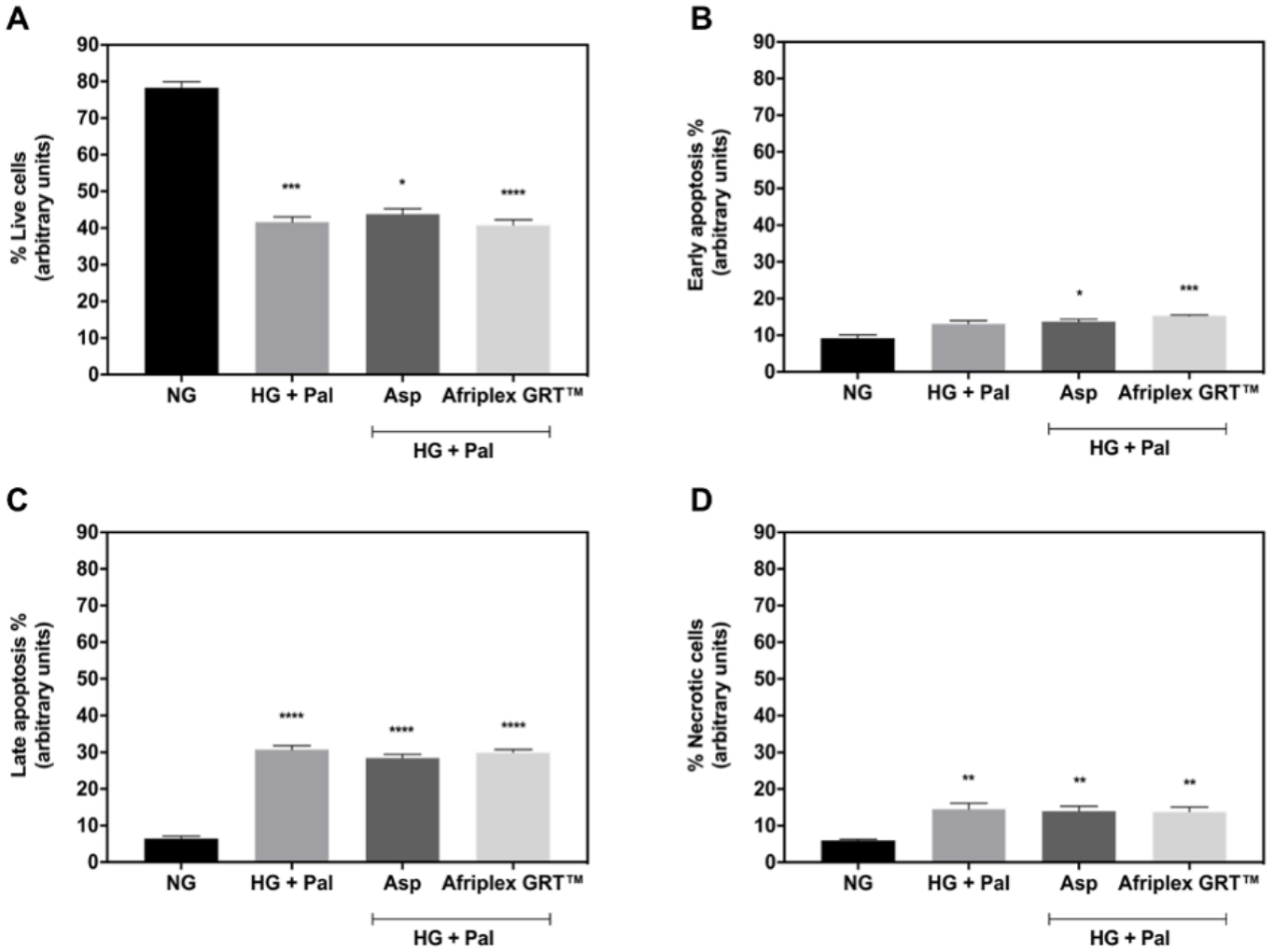

Figure 3.11. The effect of hyperglycaemic and hyperlipidaemic conditions on apoptotic profiles of the cell population. Cardiomyoblasts were cultured in normal glucose (NG) (5 mM) or high glucose (HG) (33 mM) and palimitate (Pal) (0.5 mM) for 24 hours. Thereafter, cells were treated with either Aspalathin (Asp) (1 µM) or Afriplex GRT™ (10 µg/mL) for 6 hours. Data are presented as the mean ± SEM of 3 separate biological experiments with each experiment having 3 technical repeats (n = 9). Significance is depicted as * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ and **** $p < 0.0001$ versus NG control.

3.3.6 Transcriptional profiling

In this study, the cardiac gene regulatory network that is activated under hyperglycaemic and hyperlipidaemic conditions on H9c2 cells was evaluated to better understand the pathophysiology that drives diabetes-induced cardiac dysfunction. To this end, gene expression profiling was employed using rat specific RT^2 Profiler PCR Arrays and differential gene expression analysis was performed using the Gene Globe Data Analysis Centre. Following normalisation to internal housekeeping genes and subsequent statistical analyses, 58 out of the 166 genes profiled were identified as differentially expressed ($p \leq 0.05$ or fold change ≥ 2) in HG + Pal treated group when compared to the NG control (Supplementary Table 1). Amongst these, 39 genes were upregulated (33%), and the remaining 19 genes (77%) were downregulated following HG + Pal exposure. Furthermore, evaluation of the 58 DEGs in cells treated with either Afriplex GRT™ or Asp revealed a similar trend in expression in terms of fold regulation, with neither being able to significantly alter mRNA expression when compared to HG + Pal treated cardiomyoblasts (Supplementary Table 1).

To provide biological significance, the DEGs were mapped onto key metabolic pathways involved in both disease and normal conditions using KEGG Mapper. Pathway enrichment analysis resulted in a total 213 pathway hits, identifying fluid shear and atherosclerosis (21 out of 58), and hypertrophic cardiomyopathy (18 out of 58) as the top two enriched pathways (Figure 3.12). In addition, the pathway enrichment of insulin resistance and immune-related terms such as TNF-, IL17- and NF-κB signalling, is of particular interest to the present study (Figure 3.12). Collectively, these results illustrate the association between the previously described changes in antioxidant capacity, mitochondria depolarization and apoptosis following HG + Pal exposure with underlying molecular mechanisms involved in the development and progression of CVD.

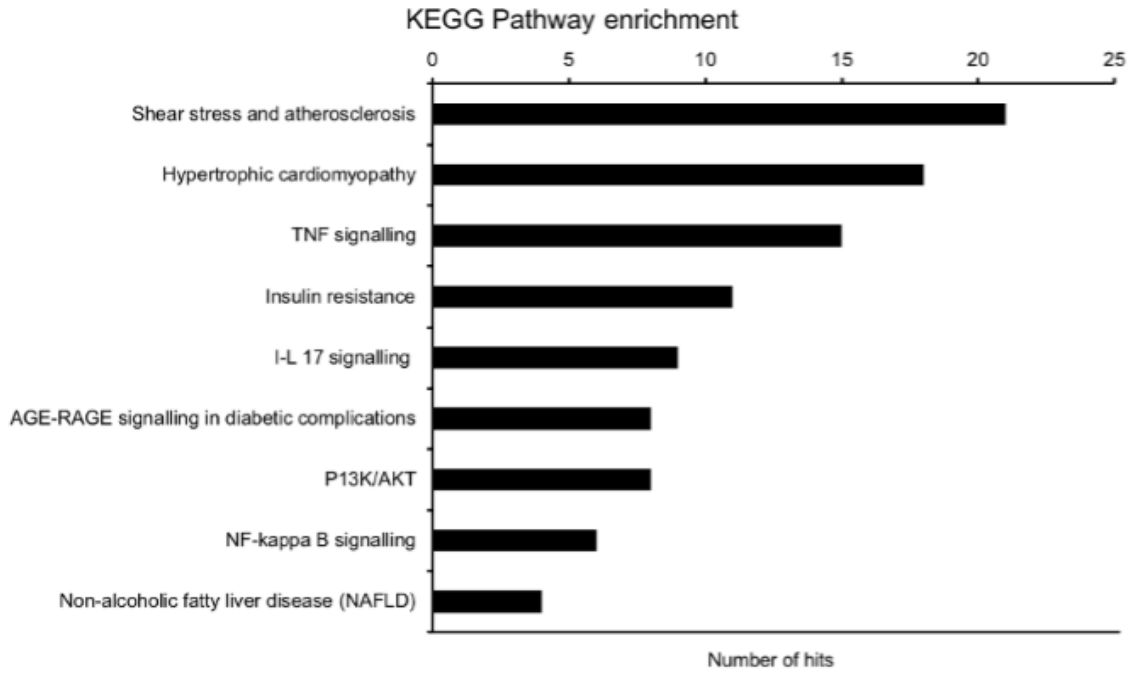

Figure 3.12. The top ten KEGG pathway enrichment terms associated with differentially expressed genes (DEGs).

3.3.6.1 Glucolipotoxicity alters shear stress and promotes atherosclerosis

Mechanistically, shear stress, which is representative of the frictional force of blood flow at the endothelial surface of the vessel wall, plays a central role in vascular function (Heo et al., 2014; Resnick et al., 2003; Souilhol et al., 2020; Wentzel et al., 2012; Zarins et al., 1983). While physiological high shear stress increases the expression of atherosclerotic-protective genes, disturbed or low oscillatory shear stress often localised to arterial bends and branches, promotes atherosclerosis in collaboration with several other risk factors (Cunnigham & Gotlieb, 2005; Garin et al., 2007; Heo et al., 2011; Koskinas et al., 2012; Reinhart-King et al., 2008; Won et al., 2007). However, the exact mechanisms through which various shear stress environments determine whether to regulate physiological or pathological responses remain to be fully elucidated. In this study, KEGG mapper analysis identified 21 of 58 (36%) DEGs to be involved in fluid shear stress and atherosclerosis, including tumor necrosis factor alpha (*TNF-α*), nitric oxide synthase 3 (*NOS3*) and vascular endothelial growth factor A (*VEGFA*) amongst others (Table 3.2). Furthermore, instances, where alterations in fluid shear stress are observed have been linked to alterations in the expression of genes and signalling proteins involved in antioxidant activity, inflammation, thrombosis, and endothelial dysfunction (Garin et al., 2007; Heo et al., 2011; Koskinas et al., 2012; Won et al., 2007).

Table 3.2. Condensed list of differentially expressed genes (DEGs) associated with shear stress and atherosclerosis pathway enrichment in treated H9c2 cardiomyoblasts.

Gene name	Symbol	Fold regulation		
		HG + Pal	Asp	Afriplex GRT™
Inflammation				
Interleukin 6	*IL6*	1.64	1.73	1.47
Chemokine (C-C motif) ligand 5	*CCL5*	-2.55	-2.20	-2.76
Tumor necrosis factor alpha	*TNF-α*	4.72	4.21	4.78
Colony stimulating factor 1	*CSF1*	2.83	2.56	2.10
Colony stimulating factor 2	*CSF2*	3.94	3.30	3.64
Early growth response 1	*EGR1*	3.10	3.52	3.60
Fibroblast growth factor 2	*FGF2*	2.51	2.23	2.67
Interferon gamma	*IFNG*	2.30	2.42	2.06
Interleukin 1 beta	*IL1B*	2.09	2.22	2.17
Vascular cell adhesion molecule 1	*VCAM1*	-3.24	-3.32	-3.01
Nitric oxide synthase 2	*NOS2*	4.87	5.01	4.93
Nitric oxide synthase 3	*NOS3*	2.53	2.00	2.10
Leukemia inhibitory factor	*LIF*	3.63	3.05	3.58
Titin	*TTN*	1.57	1.44	1.73
Troponin I type 2	*TNNI2*	1.58	1.26	1.84
Troponin T type 3	*TNNT3*	1.61	1.95	1.89
Nebulin	*NEB*	1.65	1.33	1.82
Platelet derived growth factor receptor beta	*PDGFRB*	-1.94	-1.44	-1.22
C-X-C motif chemokine ligand	*CXCL1*	2.29	2.53	2.62
Insulin resistance				
Serpin peptidase inhibitor, clade E, 1	*SERPINE1*	1.37	1.48	1.77
Vascular endothelial growth factor A	*VEGFA*	1.19	1.61	1.77
Protein kinase, AMP-activated, gamma 3	*PRKAG3*	7.06	7.53	7.18
Insulin-like growth factor binding protein 3	*IGFBP3*	2.91	2.87	2.45
Neuropeptide Y	*NPY*	6.42	6.61	6.06
Insulin-like growth factor binding protein 5	*IGFBP5*	1.84	1.36	1.94

This table represents a subsection of the complete differentially expressed gene (DEG) table provided as supplementary information. All DEGs associated with HG + Pal were either statistically significant ($p \leq 0.05$) or displayed a fold change ≤ 2 in either positive or negative direction. In all instances post-treatment with neither Afriplex GRT™ nor Asp was able to significantly impact gene expression derangements resulting from glucolipotoxicity.

- **Inflammation**

Numerous studies have provided evidence supporting the role of low shear stress in vascular inflammation and plaque formation through the modification of gene expression in favour of atherosclerosis (Brooks et al., 2004; Cheng et al., 2007; Cunnigham & Gotlieb, 2005; Libby et al., 2002; Orr et al., 2005; Seo et al., 2003). This study demonstrated that treatment with HG + Pal increased the expression of mRNA encoding the multifunctional cytokine TNF-α (4.72-fold change, $p = 0.002$), which in turn triggered the activation of proinflammatory cytokines interleukin 6 (IL6) and interleukin 1 beta (IL1B) by 1.64-fold ($p < 0.001$) and 2.09-fold ($p = 0.064$), respectively (Table 3.2). This study also demonstrated the significant upregulation of the chemokine ligand 1 (CXCL1) (2.29-fold change, $p < 0.00001$), which is known to be indirectly induced by IL6 or TNF-α via the activation of the NF-κB signalling pathway (Ma et al., 2018). The HG + Pal caused an increase in inflammatory cytokines, chemokines and growth factors subsequently promoted the expression of proliferative transcription factors VEGFA (1.91-fold change, $p < 0.015$), resulting in atherogenesis (Shaik-Dasthagirisaheb et al., 2013). Interestingly, the vascular adhesion molecule, VCAM-1 (-3.24-fold change, $p < 0.000097$), demonstrated contradictory decreased expression compared to related pro-inflammatory molecules. Overall, the results obtained illustrate the ability of HG + Pal to activate an inflammatory response and act in synergy with low oscillatory shear stress to alter transcriptional changes associated with the progression of atherosclerosis, however, post-treatment with neither Afriplex GRT™ or Asp was able to attenuate this effect (Table 3.2).

- **Nitric oxide (NO) and ROS**

The vascular wall redox state is modulated by shear stress, partially through the regulation of the expression of several oxidases. To this end, vascular endothelial cells displaying low oscillatory shear stress, demonstrate increased NAD(P)H activity and ROS production (Cunnigham & Gotlieb, 2005; Heo et al., 2014; McNally et al., 2003). The previously mentioned increased expression of NAD(P)H oxidase 4 (*NOX4*) specifically, was also associated with an increase in low density lipoprotein cholesterol (LDL-C) oxidation, promoting atherosclerosis (Hwang et al., 2003; Matsuura et al., 2008). Although this study observed a decrease in ROS production and *NOX4* expression following HG + Pal exposure, this was accompanied by a reduction in antioxidant gene expression, including *SOD2* and *GPX2* (Figure 3.6). Interestingly, a study conducted by McSweeney et al (2016) reported that the availability of ROS can have a direct effect on NOX4 expression and that

NRF2 can negatively regulate the expression of NOX4, decreasing ROS. However, this requires further investigation

Furthermore, this study provides evidence that HG + Pal increases the mRNA expression of both nitric oxide synthase 2 and 3 (*NOS2* and *NOS3*), by 4.87-fold ($p < 0.001$) and 2.53-fold ($p = 0.191$), respectively (Table 3.2). While increased NOS expression has been traditionally considered to be athero-protective, evidence exists supporting its significant contribution to ROS production should uncoupling of the electron transfer within the enzyme occur (Castier et al., 2005; Cunnigham & Gotlieb, 2005; Forstermann & Munzel, 2006; Heo et al., 2014; Verhaar et al., 2004). Accordingly, current study results allow for the speculation that HG + Pal exposure increased the bioavailability of NO (possible compensatory mechanism), forming reactive nitrogen species that could explain the suppressed ROS accumulation and subsequent NOX4 suppression. Nonetheless, because *NRF2* expression was also decreased following HG + Pal exposure, it was thus unable to diminish the suppression of antioxidant activity, that resulted in a loss of mitochondrial activity and subsequent myocardial apoptosis. Therefore, further studies are required to fully elucidate the signalling mechanisms by which decreased ROS and enhanced NO synthesis is coupled with the dysregulation of NRF2-mediated transcription of antioxidant genes, such as *SOD2* and *GPX2*.

3.3.6.2 Glucolipotoxicity promotes hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a myocardial disorder that is characterised by hypertrophy of myocytes of the left ventricle, myofibrillar disarray, and interstitial fibrosis, ultimately contributing towards the development of cardiac dysfunction (Kimura, 2016; Marian, 2010; Marian & Braunwald, 2017; Richard et al., 2003). To this end, numerous genetic mutations in genes that encode protein constituents of the sarcomere have also been associated with HCM (Kaski et al., 2009; Niimura et al., 2002). The asymptomatic nature of early onset HCM, along with the concomitant presence of other cardiovascular conditions such as hypertension, complicates the implementation of effective diagnostic and therapeutic interventions (Chimenti et al., 2004; Sachdev et al., 2002; Seo et al., 2016). In this study, pathway analysis revealed 18 out of the 58 (31%) identified DEGs to be associated with HCM, including desmin (*DES*), myosin heavy chain 2 (*MYH2*), ATP-binding cassette transporter A1 (*ABCA1*), protein kinase AMP-activated non-catalytic subunit gamma 3 (*PRKAG3*) and titin (*TTN*) amongst others (Table 3.2 and Table 3.3).

Table 3.3. Condensed list of differentially expressed genes (DEGs) associated with hypertrophic cardiomyopathy pathway enrichment in treated H9c2 cardiomyoblasts.

Gene name	Symbol	Fold regulation		
		HG + Pal	Asp	Afriplex GRT™
Lipid transport and metabolism				
ATP-binding cassette transporter A1	*ABCA1*	-3.44	-3.34	-3.75
Low-density lipoprotein receptor	*LDLR*	2.40	2.16	2.67
Nuclear receptor 1H3	*NR1H3*	-1.53	-1.22	-1.17
Prostaglandin-endoperoxide synthase 1	*PTGS1*	1.53	1.62	1.44
Perilipin 2	*PLIN2*	2.23	2.59	2.46
Retinoid X receptor alpha	*RXRA*	-6.01	-6.66	-6.48
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	*PPARGC1A*	2.20	2.15	2.87
Hypertrophy				
Actin alpha 1	*ACTA1*	-1.34	-1.66	-1.73
Activin A receptor type IIB	*ACVR2B*	1.51	1.22	1.19
Adrenergic beta 2	*ADRB2*	3.24	3.08	3.61
Agrin	*AGRN*	-1.54	-1.82	-1.39
Emerin	*EMD*	-1.49	-1.93	-1.84
Myosin heavy polypeptide 1	*MYH1*	1.62	1.78	1.99
Myosin heavy chain 2	*MYH2*	-5.39	-5.02	-5.85
Tripartite motif-containing 63	*TRIM63*	1.40	1.59	1.97
Desmin	*DES*	1.50	1.30	1.94
Elastin	*ELN*	-4.25	-4.56	-4.30
Catenin beta 1	*CTNNB1*	-1.27	-1.29	-1.47

This table represents a subsection of the complete differentially expressed gene (DEG) table provided as supplementary information. All DEGs associated with HG + Pal were either statistically significant ($p \leq 0.05$) or displayed a fold change ≤ 2 in either positive or negative direction. In all instances post-treatment with neither Afriplex GRT™ nor Asp was able to significantly impact gene expression derangements resulting from glucolipotoxicity.

- **Lipid transport and metabolism**

Scientific research suggests that increased cardiac lipid accretion often leads to fibrosis and ventricular stiffness, advancing HCM and cardiac dysfunction (Goldberg et al., 2012; Johnson et al., 2016; Sharma et al., 2004; Silver et al., 1999; Smit, 2016). Within the myocardium specifically, diabetes-induced lipid toxicity has been associated with left ventricular dysfunction, accompanied by increased mRNA expression of sterol regulatory element-binding protein (SREBF) and peroxisome proliferator-activated receptor gamma (PPARG) (Drosatos et al., 2013; Marfella et al., 2009). In the present study, the evaluation of gene expression following HG + Pal exposure revealed elevated mRNA levels of the transcriptional coactivator, PPARGC1A (2.04-fold change, $p = 0.211$), which interacts with PPARG to regulate energy metabolism (Table 3.3) (Dorn et al., 2015; Sanchis-Gomar et al., 2014; Valero, 2014).

Moreover, combined treatment with HG + Pal also resulted in the significant downregulation of mRNA encoding the membrane protein, ATP-binding cassette transporter A1 (ABCA1) (-3.44-fold change, $p < 0.001$). The latter is known to regulate cholesterol efflux and prohibit inflammation, by binding to apolipoproteins and activating several signalling pathways, ultimately exerting a cardioprotective effect (Liu & Tang, 2012; Tian et al., 2019). While this study confirms previous findings reporting on the ability of excess glucose to reduce both mRNA and protein ABCA1 levels, conflicting evidence exists for the effect of saturated fatty acids (SFAs) such as palmitate on ABCA1 expression, with the majority of studies lacking the use of HG + Pal as a combined stress (Albrecht et al., 2004; Cuffe et al., 2018; Hodson et al., 2001; Lewington et al., 2007; Mauerer et al., 2009; Mauldin et al., 2006; Passarelli et al., 2005; Uehara et al., 2007). Overall, the results obtained illustrate the ability of HG + Pal to alter lipid metabolism and cholesterol flux by increasing the expression of *PPARGC1A* and decreasing *ABCA1* expression, unable of being ameliorated by Afriplex GRT™ or Asp treatment (Table 3.3). The observed disordered lipid metabolism combined with the upregulation of low-density lipoprotein receptor (*LDLR*) (2.40-fold change, $p = 0.006$) potentially resulted in the development of atherogenic apolipoprotein and insulin resistance profiles, distinctive of HCM (Table 3.3) (Chan & Watts, 2006; DeFronza, 2010; Lee et al., 2006; Savage et al., 2007).

- **Cardiac muscle contraction and assembly**

During instances of chronic overload such as aging, exercise and metabolic insults the heart undergoes multiple changes in myocardial gene expression, influencing cardiogenesis and muscle contractability, ultimately promoting the development of HCM (Aoyama & Shibata, 2017; Barth et al., 2012; Lindskog et al., 2015; Nakao et al., 1997). In the current study, the expression of *DES* (1.50-fold change, $p = 0.022$) was increased to produce desmin, which is a protein required for muscle contraction within the sarcomere (Table 3.3) (Brodehl et al., 2018; McCormick et al., 2015). Dysregulation of DES results in an elevation of calcium sensitivity, possibly altering energy supply and demand withing in the heart under HG + Pal stress. In agreement, the present study previously demonstrated that the combination of HG + Pal, decreased cellular ATP content (Figure 3.3, **** $p < 0.0001$ versus NG control), severely impairing mitochondrial bioenergetics (Figure 3.7 and 3.8) and resulting in mitochondrial dysfunction unable of being attenuated with Afriplex GRT™ or Asp treatment. Furthermore, chronic exposure of H9c2 cells to HG + Pal significantly decreased the expression of myosin heavy chain 2 (*MYH2*) (-5.39-fold change, $p < 0.00001$), a crucial determinant of cardiac muscle contractile performance and fibre arrangement, irreversible by Afriplex GRT™ or Asp treatment (Table 3.3) (Allen et al., 2000; Allen et al., 2001; England & Loughna, 2013; Tajsharghi et al., 2010). Due to possible circadian regulation of myosin heavy chain isoforms, the significant association between decreased expression MYH2 and cardiac dysfunction warrants further investigation to exploit its role as a potential prediction or therapeutic biomarker (Aoyama & Shibata, 2017; Shavlakadze et al., 2013). The results of this study thus collectively illustrate the ability of HG + Pal to modify cardiac muscle gene expression signalling pathways, in favour of ventricular hypertrophy, altered muscle contraction and assembly, contributing towards the development of HCM and heart failure (Table 3.3).

3.4 Conclusions

The use of in vitro models to simulate disease phenotypes has been widely accepted throughout the scientific community as a preliminary strategy to aid in elucidating underlying mechanisms. One such model is the use of the clonal cell-line, H9c2, displaying the characteristics of primary cardiomyocytes, which renders it extremely valuable in the analyses of cardiotoxicity, myocardial injury, as well as hypertrophy (Cai et al., 2002; Hu et al., 2016; Kuznetsov et al., 2015; Watkins et al., 2011; Witek et al., 2016). A large body of

scientific evidence exists supporting the ability of excess glucose as well as high concentrations of the circulating saturated fatty acid, palmitate, to exert detrimental effects on cardiomyocytes (Davargaon et al., 2019; Johnson et al., 2017; Kayama et al., 2015; Thomas et al., 2014; Wang et al., 2017). However, limited information exists regarding the combinatory effect of high glucose plus palmitate (HG + Pal) on cardiovascular function and the transcriptional regulatory mechanism activated upon induction of a disease phenotype, using an *in vitro* H9c2 model.

Furthermore, this study is one of the first studies to investigate metabolic derangements induced under HG + Pal stress, and whether Afriplex GRT^{TM} and Asp have the potential to reverse the observed effects using undifferentiated H9c2 cells. The data obtained indicate that HG + Pal treatment was significantly associated with membrane depolarisation and impaired mitochondrial bioenergetics, ultimately resulting in mitochondrial dysfunction and a subsequent increase in the number of cells undergoing necrosis and apoptosis. Although conflicting results were obtained suggesting the inability of HG + Pal to increase ROS production, this study confirmed previous findings that either HG or Pal exposure modulates the antioxidant defence system, observed by a decrease in *GPX2*, *SOD2* and *NRF2* expression. The suppression of HG + Pal-induced ROS through increased NOS availability provides one possible explanation for the previously mentioned phenomenon; however, further studies are required in order to confirm this (McSweeney et al., 2015). Interestingly, this study also showed that post-treatment with Asp or Afriplex GRT^{TM}, had no significant beneficial effect on the parameters assessed and further exacerbated HG + Pal-induced mitochondrial dysfunction and apoptosis. While previous studies have demonstrated the ability of both Asp and rooibos extracts to ameliorate metabolic stress *in vitro*, these studies differ from the current study either in terms of the cell line-, the stress inducer-, or the specific rooibos extract used (Dludla et al., 2014; Dludla et al., 2017; Johnson et al., 2016; Kroukamp et al., 2018; Millar et al., 2020; Mazibuko et al., 2019; Mazibuko-Mbeje et al., 2019). Thus, allowing for the speculation that the combinatory insult of both high glucose and palmitate used in the current study, strongly contributed to the inability of Asp or Afriplex GRT™ to influence the metabolic perturbations evaluated.

Additionally, this study aimed to provide a better understanding of the cardiac gene regulatory network activated under high fat, high sugar (HG + Pal) conditions. Gene expression and functional enrichment analyses revealed that HG + Pal treatment was able to increase NOS and activate an inflammatory response which in combination with low oscillatory shear stress was able to induce transcriptional changes associated with the

development of atherosclerosis. Exposure to HG + Pal was also able to alter lipid metabolism and increase contractile dysfunction, thus promoting the development of hypertrophic cardiomyopathy (HCM). Along with shear stress, atherosclerosis and HCM; the transcriptional enrichment of insulin resistance, fibrosis and apoptosis was further observed following HG + Pal exposure. Treatment with Asp or Afriplex GRT^{TM} was once again unable to ameliorate the HG + Pal-induced transcriptional changes, possibly suggesting that the combined insult used in the current study may have resulted in a severe cardiac dysfunction phenotype unable of being remediated by the particular polyphenols. Future studies involving the treatment with Asp and Afriplex GRT^{TM} prior to stress induction, as well as combined therapy of the polyphenolic compounds with HG + Pal are advised to fully characterise the findings of the present study. To further characterise the role of an obesogenic diet in CVD risk development, the following chapters focus on the use of a Wistar rats maintained either on a high fat, high sugar (HFHS) or standard (STD) rodent diet, supplemented with or without Afriplex GRT^{TM}.

3.5 Supplementary data

Supplementary Table 1. Complete list of differentially expressed genes (DEGs) identified from RT^2 Profiler PCR Arrays.

3.6 References

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Chapter 4: A high fat, high sugar model to investigate diet-induced CVD in Wistar rats

4.1 Introduction

Cardiovascular disease (CVD), collectively referring to conditions that affect the heart tissue or involve the obstruction of blood vessels, is the leading cause of mortality worldwide. Annually, CVD claims approximately 17.9 million lives, accounting for almost a third of all global deaths (WHO, 2021). Over the past decade, there has been an exponential increase in the occurrence of CVD in low- and middle-income countries, transferring its historical association with affluent economies to underdeveloped countries (Yusuf et al., 2014; WHO, 2021). This increase is concomitant with a substantial increase in the prevalence of major CVD risk factors, including obesity, insulin resistance (IR), and type 2 diabetes mellitus (T2DM) (Cavan et al., 2016; Humphries et al., 2010; Yusuf et al., 2004). This can, in part be attributed to urbanisation and sedentary lifestyles accompanied by increased dietary fat intake and over nutrition (Cavan et al., 2016; Humphries et al., 2010; Kruger et al. 2005; Micklesfield et al. 2013).

Per the American Heart Association, diabetic adults are two to four times more likely to die from cardiovascular complications, making it the primary cause of diabetic-related deaths (Zhou et al., 2009). While the mechanisms whereby chronically elevated blood glucose levels, resulting from T2DM, elicits detrimental effects on the heart have not been fully elucidated, increased intracellular flux of free fatty acids (FFAs) have been widely implicated (Dyntar, 2003; Giacco & Brownlee, 2010; Kapur & De Palma, 2007; Opie, 2008; Schrieks et al., 2018). The excess availability of FFAs alters substrate preference in the heart, during which myocardial FFA uptake exceeds the rate of β-oxidation, which in addition to hyperglycaemia, further augments myocardial injury (Bugger & Abel, 2010; Schulze et al., 2016). The increased lipid storage within the diabetic heart correlates with the development of coronary artery disease (CAD) and diabetic cardiomyopathy, ultimately leading to diabetes-induced cardiac dysfunction (Borghetti et al., 2018). Currently, there are no prognostic markers or pharmacological drugs to prevent or treat diabetes-related cardiac complications. Furthermore, the asymptomatic nature of early onset CVD complicates the successful implementation of preventative and therapeutic strategies (Borghetti et al., 2018). Thus, as the burden of CVD continues to increase and the long-term prognosis remains poor, there is not only an imperative need to understand disease progression, but also to

investigate novel and more effective therapies to efficiently manage and ultimately alleviate the weight of this chronic disease.

Several clinical and epidemiological studies support the concept that a diet rich in polyphenols is associated with anti-diabetic and cardioprotective properties (Aryaeian et al., 2017; Chellan et al., 2004; Pan et al., 2013; Pandey & Rizvi, 2009; Parks & Booyse, 2002; Sun et al., 2020). These polyphenols are present in dietary components such as green tea, red wine and cocoa, and their beneficial effects have been attributed to their antioxidant capacity (Crescenti et al., 2013; Feil & Fraga, 2012). One such polyphenol, Aspalathin, which is unique to rooibos (a fynbos plant indigenous to South Africa), has been shown to exert protective effects against metabolic disorders such as insulin resistance (IR) and T2DM, both risk factors of CVD (Dludla et al., 2014; Mazibuko et al., 2013; Muller et al., 2013; Son et al., 2013). Additionally, the effectivity of Aspalathin in attenuating doxorubicin-induced cardiotoxicity through increased autophagy, while simultaneously increasing the expression of the anti-apoptotic BCL2 was also reported (Johnson et al., 2017).

This study aimed to establish a high fat, high sugar (HFHS) rodent model to allow for the investigation of metabolic factors contributing towards the clinical manifestation of diet-induced CVD. In addition, to ascertain whether an Aspalathin-rich green rooibos extract, Afriplex GRT™, could ameliorate the induced metabolic complications and infer cardioprotective effects. This animal model and resulting isolated samples will serve as the input for all subsequent chapters.

4.2 Materials and Methods

4.2.1 Study design

Three-week-old, male (n=40) and female (n=40) Wistar rats (*Rattus norvegicuss*) were caged in pairs according to gender and housed in a controlled animal facility at the Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (SAMRC). Rats of both genders were randomised into two main groups: 1) the control group which received a standard (STD) maintenance rodent diet, and 2) the diet-induced insulin-resistant group fed a high fat, high sugar (HFHS) cafeteria diet for a period of nine months. The STD diet consisted of patties formulated to comply with standard nutrient requirements, as well as tap water while the HFHS diet consisted of patties with increased fat content, plus water supplemented with 15% (w/v) fructose and sucrose (Table 4.1) (Pheiffer et al., 2016).

Both diets were formulated at the PUDAC (SAMRC) and animals in all groups received food and water *ad libitum* for the entire duration of the study. To evaluate the potential of an Afriplex GRT™ extract to modulate diet-induced alterations, each group was further divided into two subgroups (n=10) receiving daily treatment with either a placebo or the Afriplex GRT™ extract throughout the study (Figure 4.1). Feed intake and water consumption was monitored two times a week, while body weight was assessed on a weekly basis. Ethical clearance for the use of animals in this study was granted by the SAMRC Ethics Committee for Research on Animals (ECRA no. 10/17), and the Ethics Committee: Animal Care and Use of Stellenbosch University (reference number AUC-2020-14614).

Table 4.1. Diet compositions as determined by Microchem Specialized Lab Services (Pty) Ltd.

Component	STD diet	HFHS diet
Carbohydrates %	24.2	22.3
Fat %	4.37	10.61
Saturated Fat (g/100g)	1.58	5.73
Mono-Unsaturated Fat (g/100g)	2.11	3.63
Poly-Unsaturated Fat (g/100g)	0.69	1.25
Omega 3 Fatty Acids (g/100g)	0.087	0.111
Omega 6 Fatty acids (g/100g)	0.60	1.1
Cholesterol (mg/100g)	35	88
Protein %	7.7	9.1
Dietary Fibre %	5.9	5.3
Energy (kJ/100g)	650	878

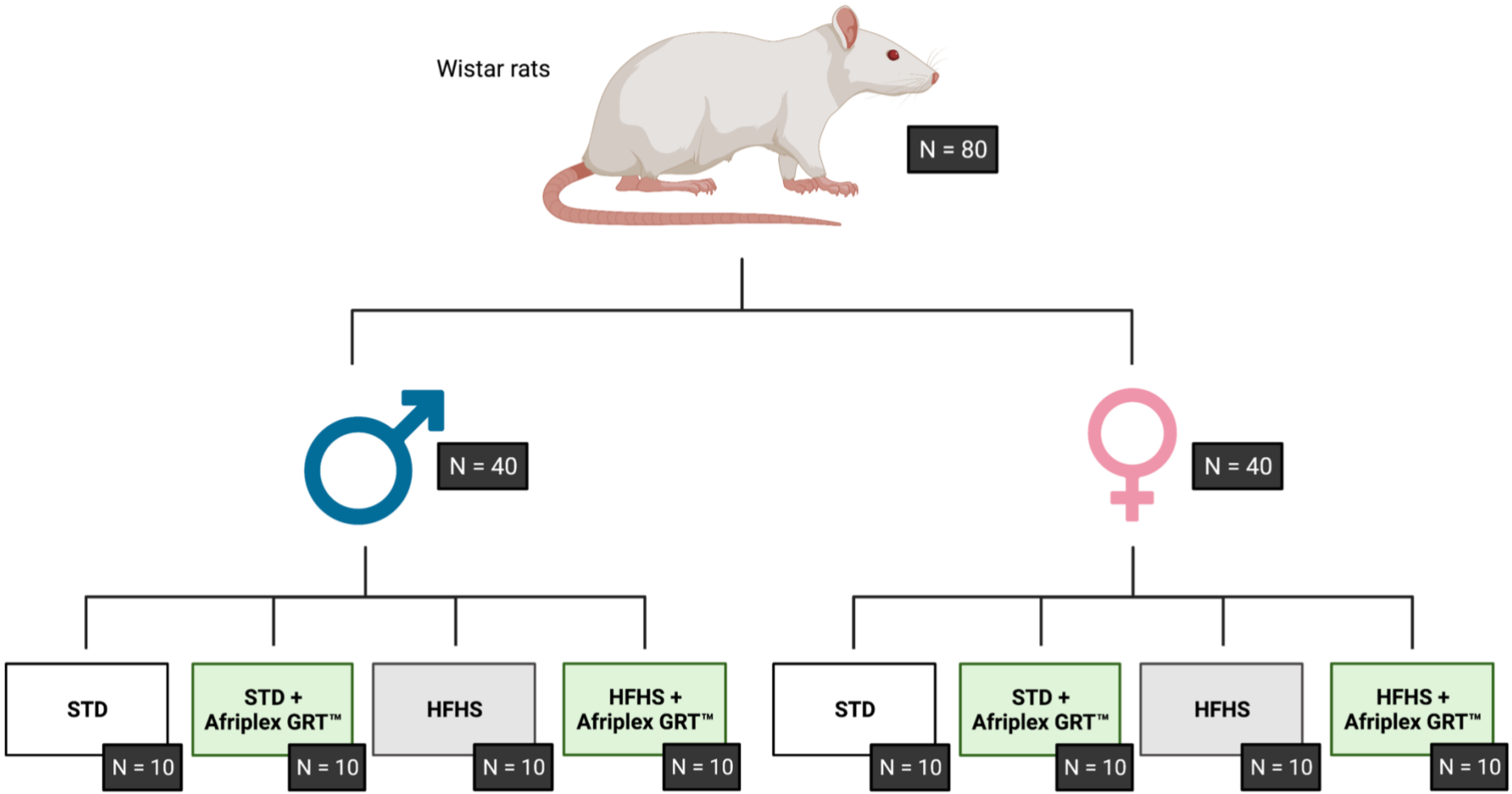

Figure 4.1. Experimental design for animal study**.** A total of 80 Wistar rats (n = 40 **males** and n = 40 f**emales**) were randomised into appropriate groups *to* evaluate the use of Afriplex GRT™ extract as a preventative for diet-induced CVD. Rats were either fed a standard maintenance rodent diet (STD) or a high fat, high sugar cafeteria diet (HFHS), with or without Afriplex GRT™ treatment throughout the study. Upon termination at 9 months, blood and heart tissue samples were collected to perform the necessary biochemical assays, mRNA expression analysis and DNA methylation profiling. Schematic constructed with Biorender.com (2021).

4.2.2 Treatment

The Aspalathin-rich green rooibos extract, Afriplex GRT™, was prepared according to good manufacturing practices and obtained from Afriplex® Pharmaceuticals (Paarl, South Africa). This solvent-based extract has previously been shown to have anti-diabetic and cardio-protective properties (Mazibuko et al., 2013; Muller et al., 2012). As the stability of Aspalathin, a major phenolic compound in the extract (Table 4.2), decreases after 12 hours in an aqueous solution, working stock solutions of the extract were prepared on the day of administration and subsequently added to jelly cubes (de Beer et al., 2015). The rats received a jelly cube containing the Afriplex GRT™ extract (60 mg/kg BW) or the vehicle only (jelly cubes without the Afriplex GRT™), once a day throughout the treatment period (nine months). In all instances, jelly cubes were fed to rats individually using a sterile laboratory spatula and observation was made to ensure the complete consumption of the entire jelly cube.

Table 4.2. Active phenolic compounds present in the Afriplex GRT™ extract as determined by the SAMRC.

Compound	g/100g Soluble solids
Aspalathin	12.78
Nothofagin	1.97
Isoorientin	1.43
Orientin	1.26
Q-3-ROB	1.04
Isoquercitrin	0.57
Rutin	0.50
PPAG	0.42
Hyperoside 0.40	0.40
Vitexin	0.39
Isovitexin	0.30

4.2.3 Fasting blood glucose (FBG) measurements and oral glucose tolerance tests (OGTTs)

Along with the assessment of the general well-being of the animals as well as phenotypic traits, fasting blood glucose (FBG) concentrations were measured monthly by tail prick after a 16 hour, overnight fast. Furthermore, oral glucose tolerance tests (OGTTs) were conducted every 3 months, as well as 14 days prior to the termination of the study. Briefly,

rats were subjected to a 16 hour fast and plasma glucose levels were determined by tail prick at time intervals of 0, 30, 60, and 120 min following the administration of glucose (2 g/kg BW) by oral gavage according to the SOPs of PUDAC (SAMRC). To maintain a constant treatment period between animals in all groups, experiments were staggered to facilitate logistics when conducting OGTTs. Relative glucose tolerance was determined by comparing the difference in blood glucose for each time point between groups, and by comparing the area under curve (AUC) of each individual rat tolerance test between groups.

4.2.4 Harvesting biological samples

At the completion of the study, all animals were anaesthetised with an Oxygen flowmeter (± 0.9 L/min) and Isoflurane vaporizer (approximately 3.5-5% for induction and 2% for maintenance) followed by termination by exsanguination. Blood samples were collected from the vena cava into SST gel tubes as well as EDTA tubes, post-mortem. The hearts were excised and subsequently cut into pieces using transverse sections along the horizontal plane and immersed in formalin for histological analysis, snap-frozen in liquid nitrogen for methylation profiling, and stored in 1 ml of RNAlater™ for expression analysis. All samples were stored at either -20°C or room temperature (RT) depending on the downstream applications.

4.2.5 Blood biochemistry analysis

Serum was isolated from peripheral blood collected into the SST gel tubes and used to assess metabolic marker concentrations in order to confirm the disease phenotype. To this end, one half of the isolated serum was used for lipid profiling (total cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL)) and liver enzyme (Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)) analysis at PathCare Medical Diagnostic Laboratories (Cape Town, RSA); while the other half was stored at -80ºC for subsequent serum insulin analysis. Rat insulin levels were determined post termination using the Rat/Mouse Insulin ELISA from Millipore® according to the manufacturer's instructions (Cat. # EZRMI-13K, Merck KGaA, Darmstadt, Germany). This ELISA based assay relies on the combinatory use of monoclonal anti-rat insulin and biotinylated polyclonal antibodies, followed by the enzymatic action of horseradish peroxidase (HRP) to fluorometrically determine insulin concentrations using a series of standards. To this end, 10 µL of each standard, sample, control, and blank was used as input and loaded in

duplicate into the respective wells of the coated 96-well microtiter plate. The assay buffer supplied was used as the blank, while the standards used represented 0.2, 0.5, 1, 2, 5 and 10 ng/mL insulin. All reagents and samples were allowed to equilibrate to room temperature prior to performing the assay. Following completion of the assay, the absorbance at 450 nm and 590 nm was read within 5 min using a BioTek® FLx800 plate reader and Gen 5® software (BioTek Instruments Inc., Winooski, VT, USA). Insulin concentrations for all samples were inferred using the relative curve method and converted to pmol/L using 1 µIU/mL = 6.00 pmol/L (Knopp, Holder-Pearson, & Chase, 2019). The homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated using the formula: HOMA-IR = glucose concentration (mmol/L) X insulin concentration (U/mL) / 22.5, using fasting values (Matthews et al., 1985; Salgado et al., 2010).

4.2.6 Histology

The cardiac tissues collected in formalin were examined for indicators of cardiovascular disease such as structural changes in the nuclei of cardiomyocytes, the arrangement of cardio myofibers, as well as the collagen content. For this purpose, cardiac tissues were embedded in paraffin wax, dissected longitudinally, and stained with Haematoxylin and Eosin (H&E), and Mayson's Trichome (MT) using optimised in-house protocols (Page et al., 2004; Thent et al., 2012). Stained sections were visualized using an Olympus BX50 light microscope (Olympus Optical Co., LTD, Tokyo, Japan), and indications of cardiac structural abnormalities were evaluated with the NIS-Elements BR imaging software version 3.0 (Nikon Inc., Tokyo, Japan). As an additional assessment of the effect of diet on glucolipotoxicity, liver tissues were stained using H&E, as previously described.

4.2.7 Statistical analysis

Data is expressed as either the geometric mean ± standard deviation (SD) or the mean ± standard error of the mean (SEM), where appropriate. The geometric mean was selected as opposed to the arithmetic mean to take into account the confounding effect. For each group, a total of 10 samples were used as independent biological replicates. The distribution of the groups was evaluated for normality and differences in variance with Shapiro-Wilk tests prior to performing any statistical comparisons. In the instances where data sets were normally distributed, they were assessed for statistical significance using ANOVA and Student t-tests, while the non-parametric Kruskal-Wallis test was used to analyse data sets

deviating from normal distribution. To allow for the direct comparison of significance, multiple testing correction was performed using either a Bonferroni or Dunn's post-hoc test, depending on the distribution of the data. Statistical analyses were performed using Microsoft Excel and GraphPad Prism software version 7.00 (GraphPad Software, Inc., La Jolla, CA, USA). In all instances, a statistical significance was inferred for a p-value less than, or equal to 0.05. Additionally, regression analysis was conducted, where applicable, to explore the relationship between independent and dependent variables, and account for time effects.

4.3 Results and Discussion

4.3.1 Physiological parameters

4.3.1.1 Food and water consumption

To evaluate the potential contribution of diet on weight gain, feed intake of all the animals in the different diet groups was assessed twice a week, from the start of diet and/or treatment administration, until termination of the study (Figure 4.2). Overall, both male (33.42 ± 1.47 vs 20.15 ± 0.35; $p < 0.0001$) and female (26.03 ± 1.31 vs 13.96 ± 0.43; $p < 0.0001$) animals in the HFHS groups, consumed significantly more food throughout the study when compared to animals maintained on the STD diet (Figure 4.2). This finding is concurrent with previous in-house and external studies reporting increased food consumption in Wistar rats fed a similar high fed diet, ultimately leading to an increase in body mass, as well as serum insulin and lipid levels (Kamau, 2018; Nduhirabandi et al., 2017; Roux, 2011). Treatment with Afriplex GRT™ did not significantly influence the average daily food consumption when compared to animals in untreated groups maintained on either diet, regardless of the sex (Figure 4.2). Several studies have implicated contradictory evidence regarding the ability of rooibos to both increase as well as have an inhibitory effect on appetite (Morton, 1983; Schloms et al., 2014; Yau & Potenza, 2013). However, these previously described effects were not observed in the current study with respect to the Afriplex GRT™.

Furthermore, no significant differences were observed regarding water intake for rodents fed the HFHS diet when compared to their control counterparts, for both male (28.37 ± 1.26 vs 27.68 ± 0.76) and female (26.02 ± 0.88 vs 24.05 ± 0.70) animals throughout the study (Figure 4.3). Although a slight increase in the amount of water consumed daily by animals fed the STD diet and receiving Afriplex GRT™ treatment, this increase was not significant

when compared to the untreated control group. At termination of the study (9 months), females in the HFHS + Afriplex GRT™ group drank significantly more water than those in the STD group (30.03 ± 1.23; p = 0.0004) (Figure 4.3). As this phenomenon was not a constant occurrence throughout the study, it is uncertain whether this can be attributed to effects introduced by the obesogenic diet; the Afriplex GRT™ extract; or a combination between the two variables. Moreover, this study does not provide any substantial evidence of the HFHS diet or the Afriplex GRT™ extract having a negative effect on hydration.

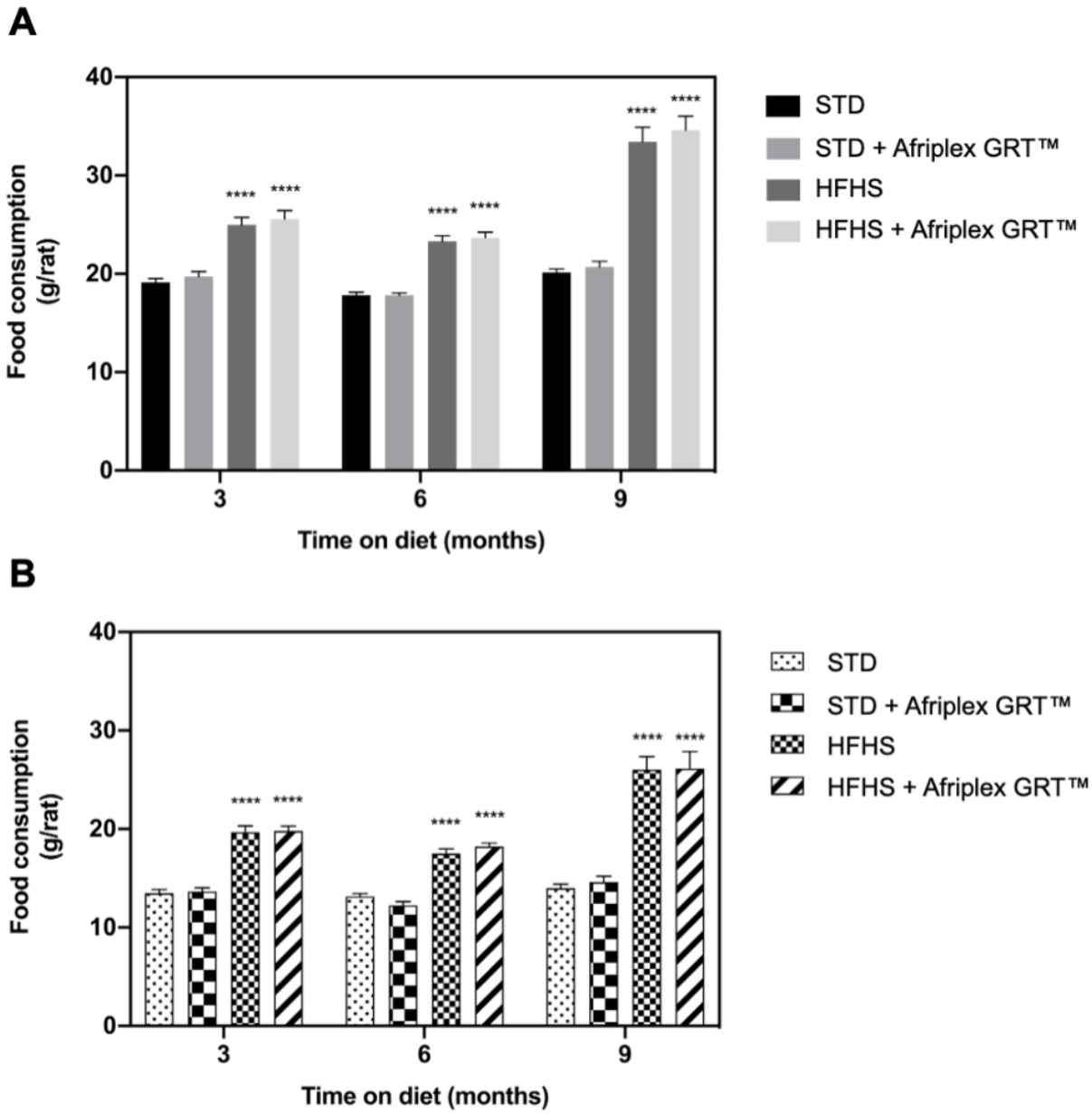

Figure 4.2. Average daily food consumption per rat for **males (A)** and **females (B)** throughout the study. Values are expressed as mean ± SEM. **A)** Food consumption at 3, 6 and 9 months: HFHS vs STD, ***** p < 0.0001 and HFHS diet + Afriplex GRT™ vs STD diet, ***** p < 0.0001. **B)** Food consumption at 3, 6 and 9 months: HFHS vs STD, ***** p < 0.0001 and HFHS diet + Afriplex GRT™ vs STD diet, ***** p < 0.0001.

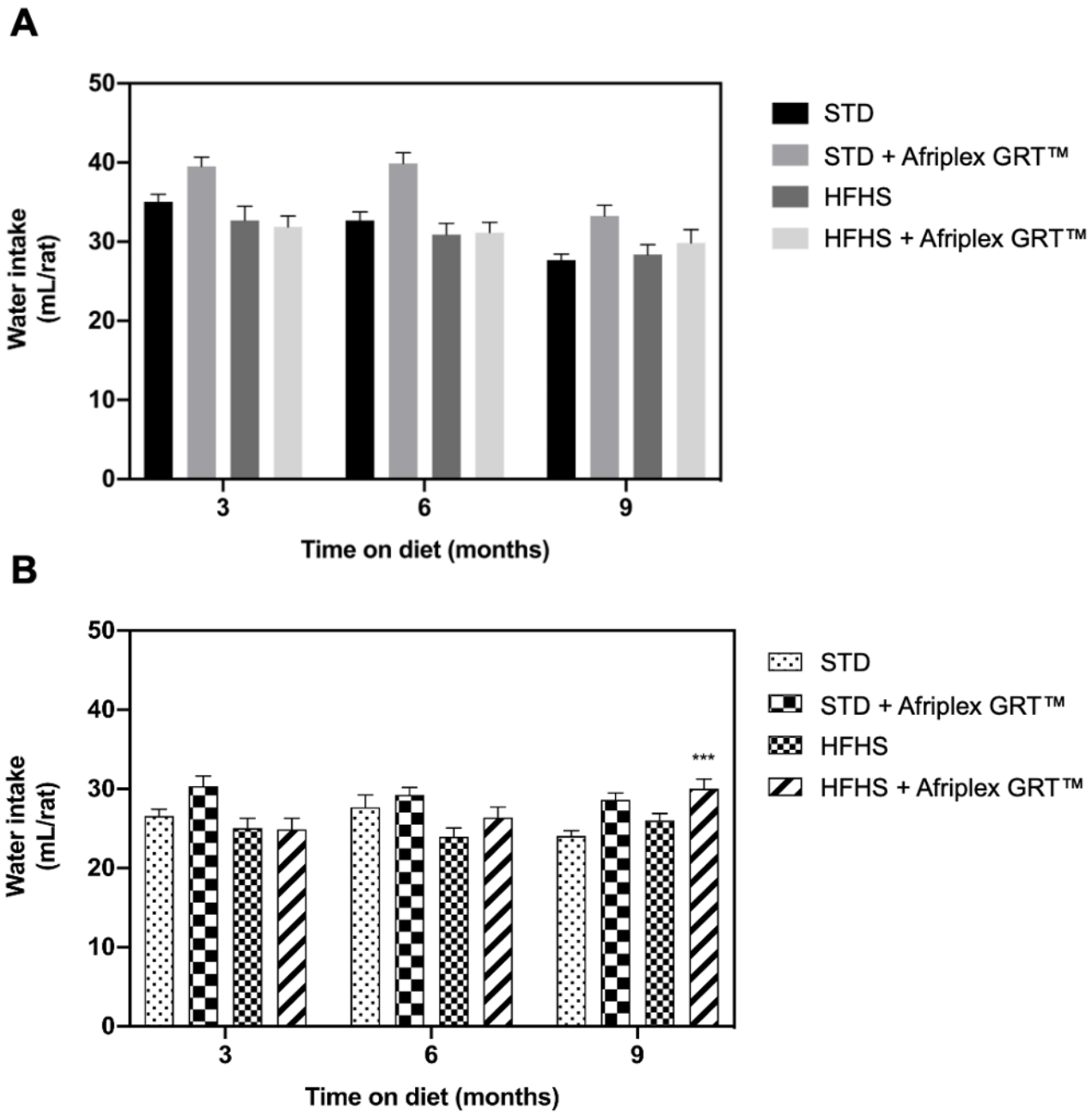

Figure 4.3. Average daily water intake per rat for **males (A)** and **females (B)** throughout the study. Values are expressed as mean ± SEM. **B)** Water intake at 9 months: HFHS diet + Afriplex GRT™ vs STD diet, **** p = 0.0004.

4.3.1.2 Body weight (BW) gain and obesity

As obesity negatively affects multiple systems of the body and remains one of the major risk factors of diabetes and its associated cardiovascular complications, the body weight of the animals was monitored on a weekly basis throughout the study. Male Wistar rats in all four groups gained weight at a steady pace throughout the study, with rats in the HFHS diet fed group being significantly heavier than those in the STD diet fed group after 9 months of feeding (Table 4.3). This significant increase was maintained for rats on the HFHS diet, in the experimental group receiving Afriplex GRT™ when compared to those on the STD diet (Table 4.3).

Table 4.3. Comparison between body weight (g) of **male** rats throughout the study.

	STD diet	**STD + Afriplex GRT™**	**HFHS diet**	**HFHS + Afriplex GRT™**
3 months	304.05 ± 7.07	304.32 ± 6.97	327.97 ± 6.24	324.97 ± 10.51
6 months	401.99 ± 9.48	391.15 ± 10.72	444.90 ± 11.17	440.92 ± 15.46
9 months	443.50 ± 9.49	429.81 ± 11.23	**509.40 ± 13.28***	**509.01 ± 16.47***

Values are expressed as mean ± SEM. Each group consists of n = 10 Wistar rats as independent biological replicates. Bold entries represent instances where a significant difference was observed. Body weight at 9 months: HFHS diet vs STD diet, * p = 0.0141 and HFHS diet + Afriplex GRT™ vs STD diet, * p = 0.0193.

Table 4.4. Comparison between body weight (g) of **female** rats throughout the study

	STD diet	**STD + Afriplex GRT™**	**HFHS diet**	**HFHS + Afriplex GRT™**
3 months	193.51 ± 4.39	196.25 ± 3.41	206.45 ± 6.77	**207.13 ± 2.38***
6 months	237.01 ± 5.24	237.22 ± 4.87	255.25 ± 9.62	**257.88 ± 4.48***
9 months	255.38 ± 5.43	254.26 ± 6.01	280.18 ± 11.73	**284.17 ± 5.47***

Values are expressed as mean ± SEM. Each group consists of n = 10 Wistar rats as independent biological replicates. Bold entries represent instances where a significant difference was observed. Body weight at 3 months: HFHS diet + Afriplex GRT™ vs STD diet, * p = 0.0232; at 6 months: HFHS diet + Afriplex GRT™ vs STD diet, * p = 0.0498 and 9 months: HFHS diet + Afriplex GRT™ vs STD diet, * p = 0.0187.

Although not significant, a similar trend of increase in BW was observed for female rats receiving the HFHS diet compared to those on the STD diet, throughout the study. Interestingly, female rats in the HFHS treatment group (HFHS diet + Afriplex GRT™) weighed significantly more than their control counterparts receiving the STD diet, which equates to an average gain of approximately 9% body weight (Table 4.4). Treatment with Afriplex GRT™ had no significant effect on the BW of control rats maintained on the STD diet in both male and female groups, suggesting the possibility of the absence of toxic effects. Furthermore, no significant differences were observed between groups on the same diet, with or without Afriplex GRT™.

In the past, there has been a general trend of over-reliance on male rodents in diet-induced physiological studies however, several studies have highlighted the significance of including female animals in preclinical research, especially in the instances of non-communicable diseases (Pektaş et al., 2015). One such study by Taraschenko et al. (2011) found a similar trend in weight gain between male and female Sprague-Dawley rats maintained on a high sucrose diet. However, when fed a high fat diet, male rats were able to gain weight excessively while female rats did not. Suggesting a differential response to high fat induced obesity between the two different genders (Taraschenko et al., 2011). In the present study male rats in all groups appeared almost twice as heavy as the female rats, which can in part be attributed to their increased food and water consumption, resulting in increased energy intake (Figure 4.4). However, even though male rats (STD diet: 36.01 ± 0.62; HFHS diet: 93.80 ± 3.27) consumed 1.5 times more calories per day than female rats (STD diet: 24.95 ± 0.78; HFHS diet: 75.34 ± 3.25), the greater intake does not explain the variance in body weight gain in its entirety (Figure 4.4). Therefore, the possible influence of female cyclic hormones on body weight cannot be entirely excluded.

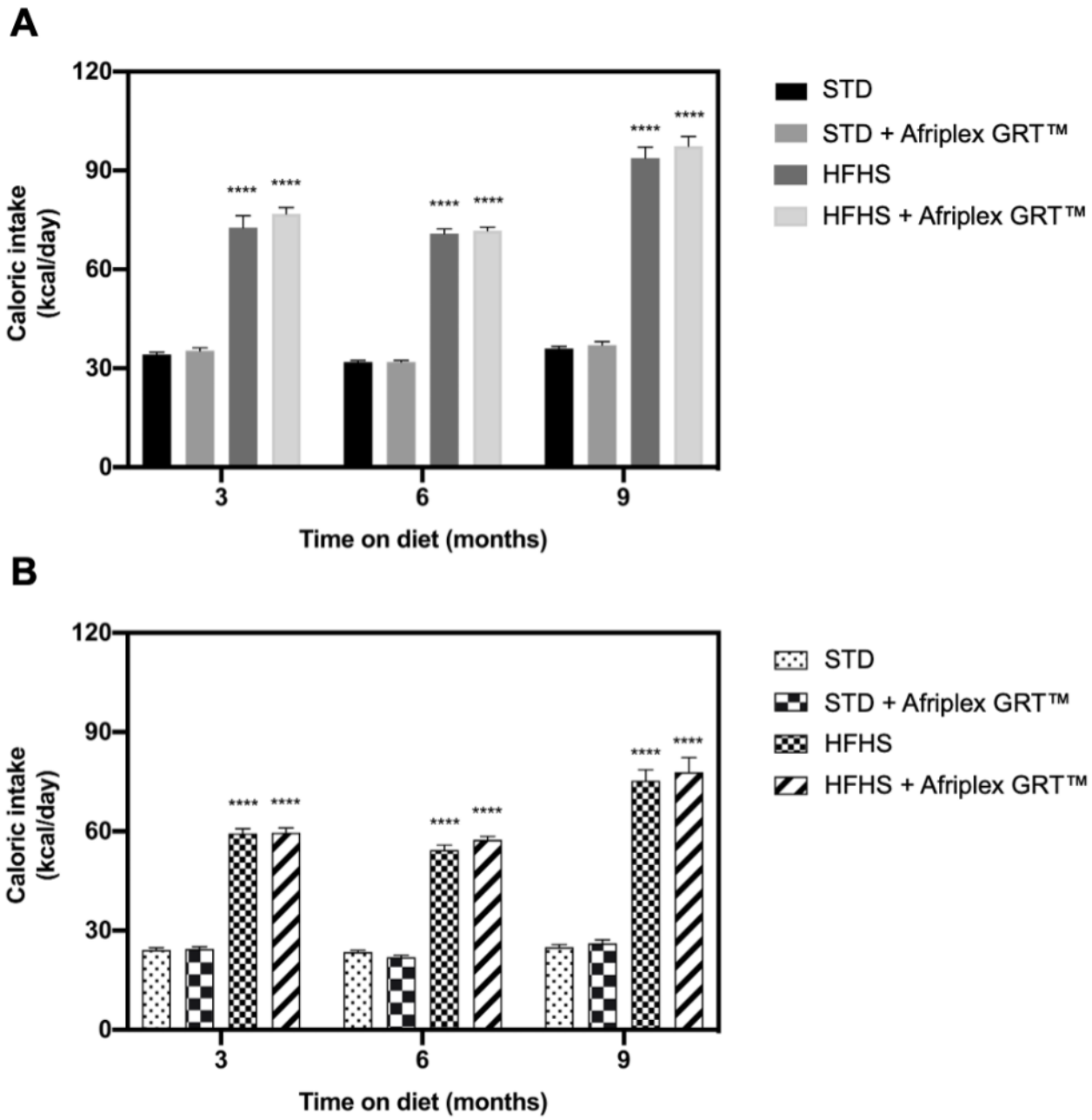

Figure 4.4. Average daily caloric intake for **male (A)** and **female (B)** rats throughout the study. Calories from both food and water intake for HFHS diet fed animals were taken into consideration. Based on nutritional analysis conducted, the STD diet comprised of 1.79 kcal/g while the HFHS diet contained 2.32 kcal/g supplemented with an additional 0.58 kcal/g from the water. Values are expressed as mean ± SEM. **A)** Caloric intake at 3, 6 and 9 months: HFHS vs STD, ***** $p < 0.0001$ and HFHS + Afriplex GRT™ vs STD, ***** $p < 0.0001$. **B)** Caloric intake at 3, 6 and 9 months: HFHS vs STD, ***** $p < 0.0001$ and HFHS + Afriplex GRT™ vs STD, ***** $p < 0.0001$.

The inferences made through BW or excessive weight gain alone cannot be used as an indication of the occurrence of obesity. As such, this study utilised retroperitoneal fat (RF) as a measurement of adiposity, and to estimate the degree of obesity (de Freitas Marthias et al., 2007; Tekus et al., 2018). The measurement of visceral adipose tissue such as RF has previously been found to be significantly correlated with the occurrence of metabolic syndrome and proves to be a valuable addition in cardio-metabolic studies (Hung et al., 2014; Yano et al., 2016). During terminations, the RF pad was excised, weighed, and normalised to the body weight of the animal. After 9 months, both male (104%) and female (100%) rats in the HFHS group displayed a significant increase in RF mass relative to body weight when compared to their gender matched controls on the STD diet (Table 4.5). For male rats specifically, the previously described weight gain observed with the administration of HFHS diet feeding directly translates into to an increase in body fat however, this was not observed for the female animals. Treatment with Afriplex GRT™ did not significantly affect the accumulation of body fat between groups on the same diet, irrespective of the gender. However, male (80%) and female (123%) rats in the HFHS + Afriplex GRT™ groups also presented with a significant increase in RF weight/body weight ratio when compared to those in the STD group (Table 4.5). This result contradicts the finding of a previous study conducted by Sanderson et al. (2014), demonstrating the ability of rooibos extracts (approximately 7 times less potent than the dose used in the present study) to significantly inhibit adipogenesis and confer potential weight loss benefits. However, as this was an *in vitro* study, these findings cannot be directly compared to the current study findings.

Table 4.5. Comparison of RF weight relative to body weight (mg/g) for both **male** and **female** animals.

	STD diet	**STD + Afriplex GRT™**	**HFHS diet**	**HFHS + Afriplex GRT™**
Males	16.24 ± 1.50	14.31 ± 1.50	**33.14 ± 3.85****	**29.31 ± 2.12*****
Females	8.10 ± 0.73	8.35 ± 0.72	**16.17 ± 1.53****	**18.09 ± 1.53*****

Values are expressed as mean ± SEM. Each group consists of n = 10 Wistar rats as independent biological replicates. Bold entries represent instances where a significant difference was observed. Males: HFHS diet vs STD diet, ** p = 0.0085 and HFHS diet + Afriplex GRT™ vs STD diet, *** p = 0.0007. Females: HFHS diet vs STD diet, ** p = 0.0021 and HFHS diet + Afriplex GRT™ vs STD diet, *** 0.0003.

4.3.1.3 Heart weight

Evaluating the effect of diet or treatments on cardiac remodelling often presenting as alterations in heart mass and function provides valuable insight into disease development and progression (Azevedo et al., 2016; Kumar et al., 2014). Upon completion of the study, the hearts of all animals were removed, and the weights were assessed prior to dissection and storage of the tissue for respective downstream analyses. The average absolute heart weight remained relatively uniform between male animals in all the respective groups (Figure 4.5. A), with no significant differences being observed between HFHS and STD animals (1.08 ± 0.04 vs 1.12 ± 0.04). As expected, this lack of variation was further maintained when normalising heart weight to body weight with the heart weight of all males remaining directly proportional to total body weight (Figure 4.5. B). The aforementioned trend also applied to females, with no differences being observed between absolute heart weight (HFHS vs STD: 0.74 ± 0.01 vs 0.75 ± 0.02) or the relative heart weight/body weight ratio (Figure 4.6. A and B). Moreover, the administration of Afriplex GRT™ had no impact on heart mass in male, as well as female animals. While these findings conform with what has previously been reported for diets with the same composition, increased caloric intake associated with high fat diets consisting of elevated palmitate and oleate content specifically have been associated with increased cardiac mass (Ouwens et al., 2005; Salie et al., 2014; Smit, 2019). This further supports the multifarious metabolic outcomes observed with the different types of fat used in high fat diets.

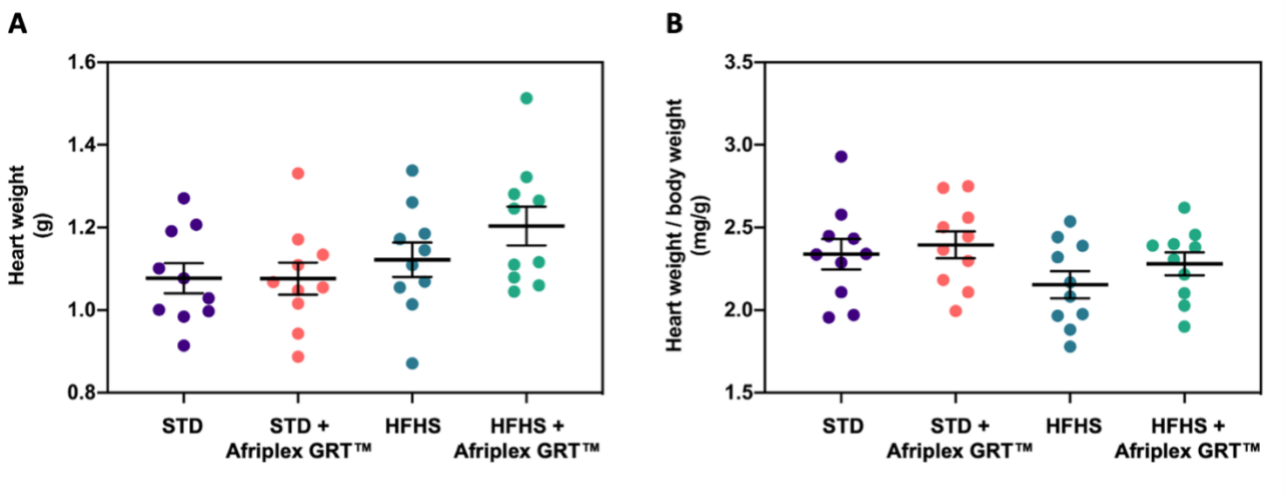

Figure 4.5. Overall distribution of absolute heart weight **(A)** and normalised heart weight relative to body weight **(B)** for **male** animals at termination. Scatter dot plots representing individual values accompanied with solid lines expressing the mean ± SEM.

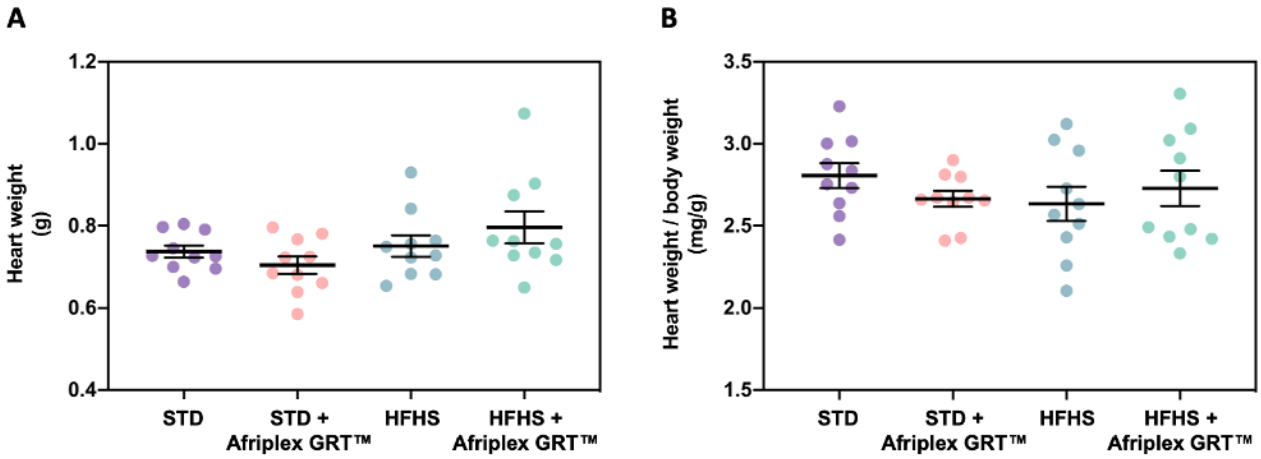

Figure 4.6. Overall distribution of absolute heart weight **(A)** and normalised heart weight relative to body weight **(B)** for **females** at termination. Scatter dot plots representing individual values accompanied with solid lines expressing the mean ± SEM.

4.3.1.4 Hyperglycaemia

During the development of metabolic syndrome and the onset of insulin resistance (IR), the body's ability to regulate and maintain blood glucose concentrations within the physiologically accepted range, becomes severely diminished (Norris & Rich, 2012). In the current study, this phenomenon was evaluated by measuring fasting blood glucose (FBG) concentrations and conducting OGTTs throughout the study. Male rats maintained on the HFHS diet were able to compensate for the diabetogenic effects of the diet throughout the study, as there were no significant differences in FBG concentrations between the groups (Figure 4.7 A). Although a general trend of increase in FBG concentration was observed for rats in the HFHS groups, this was only significant for the HFHS diet fed animals receiving Afriplex GRT™ treatment when compared to those in the STD group at 9 months (5.27 ± 0.15 vs 4.75 ± 0.11; $p = 0.0198$). Similarly, no significant differences, as well as no consistent trends were observed between FBG concentrations of female rodents maintained on the HFHFs diet when compared to their control counter parts throughout the study (Figure 4.7 B). As is the case with weight gain, no differences were observed between animals receiving the vehicle and those receiving treatment with Afriplex GRT™ for both male and female, irrespective of the diet (Figure 4.7 A and B). The ability to maintain normoglycemia, while exhibiting a mildly obese phenotype is not uncommon and can be as a result of excessive insulin production occurring in the pancreas (Sesti, 2006).

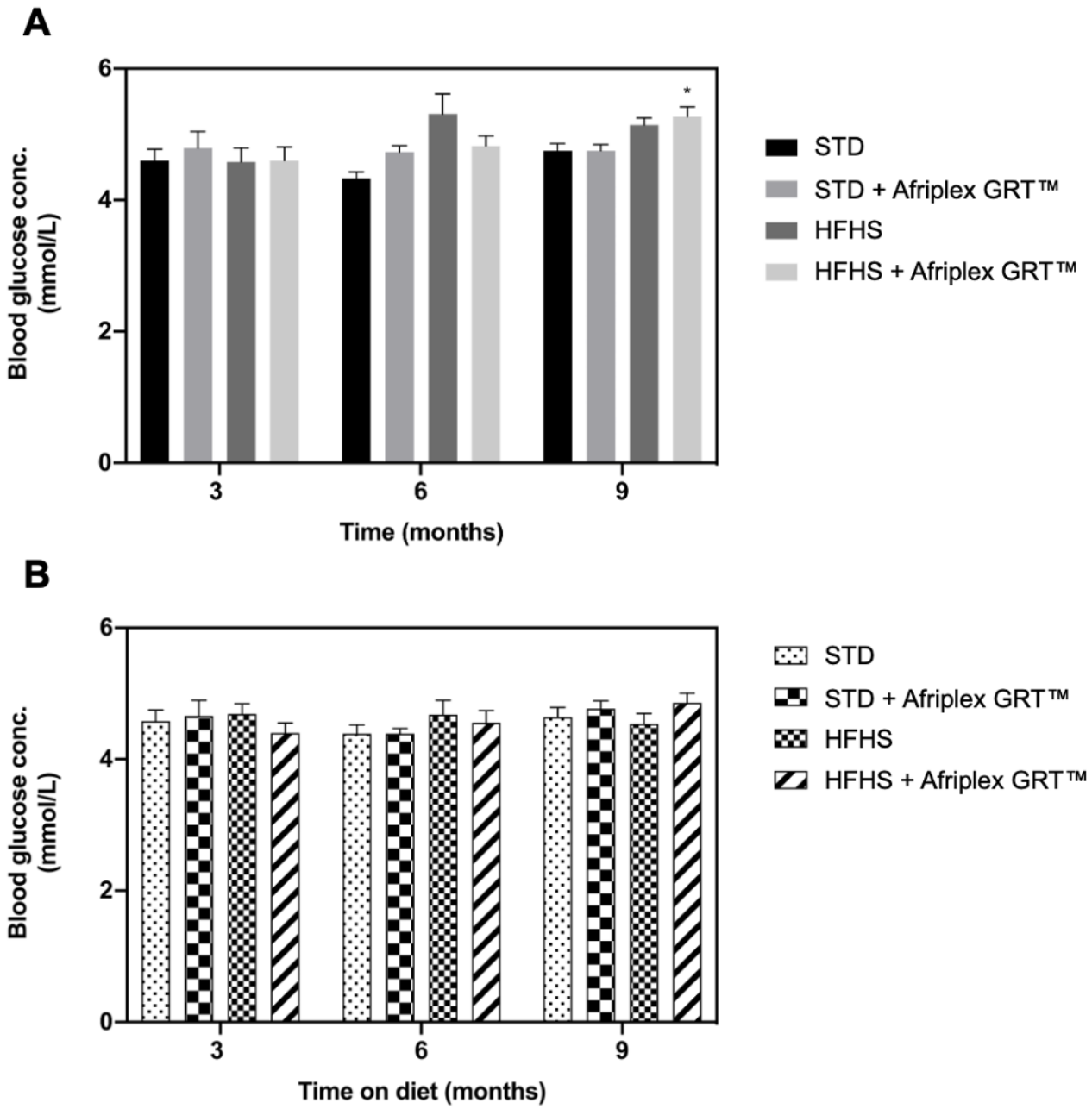

Figure 4.7. Fasting blood glucose (FBG) concentrations of **male (A)** and **female (B)** rats throughout the study. Values are expressed as mean ± SE. **A)** FBG at 9 months: HFHS + GRT vs STD, *** p<0.001.

Furthermore, as is convention with animal studies, OGTT's were performed on these animals to assess glucose concentrations in the blood after its administration. At three months, a significant difference was observed in the glucose concentration, 15 min after oral glucose stimulation between the male HFHS rats and those on the STD diet (9.03 ± 0.66 vs 7.63 ± 0.28; p = 0.0343) (Figure 4.8 A). Significant differences were also observed between male rats in the HFHS + Afriplex GRT™ group when compared to those in the STD group at both 15- (9.21 ± 0.72 vs 7.63 ± 0.28; p = 0.0112) and 30 min (8.86 ± 0.37 vs 7.39 ± 0.25;

p = 0.0218) time points (Figure 4.8 A). However, this result was not maintained, and blood glucose returned to baseline levels at the clinically relevant two-hour assessment time point. At six months, HFHS diet fed male rats displayed a significant increase in glucose concentrations, 15- (8.61 ± 0.35 vs 6.67 ± 0.14; p < 0.0001) and 30 min (7.47 ± 0.25 vs 5.95 ± 0.21; p = 0.0021) after oral stimulation when compared to those on the STD diet (Figure 4.8 B). A similar increase was displayed by male rats in the HFHS + Afriplex GRT™ group at the 15- (7.82 ± 0.32 vs 6.67 ± 0.14; p = 0.0387) and 30 min (7.99 ± 0.46 vs 5.95 ± 0.21; p < 0.0001) interval when compared to the STD group. These results translated to that obtained from OGTTs performed at nine months (Figure 4.8 C). Interestingly, a significant increase was also observed in male rats in the STD + Afriplex GRT™ group when compared to those in the STD group at six months (8.39 ± 0.36 vs 6.67 ± 0.14; p = 0.0003) (Figure 4.8 B). This increase, however, did not occur at nine months (Figure 4.8 C). Moreover, AUC analysis of each individual OGTT using the trapezoidal rule, revealed significantly increased AUC in HFHS (927.20 ± 9.68 vs 787.40 ± 14.12; p = 0.0009) and HFHS + Afriplex GRT™ (923.30 ± 16.34 vs 787.40 ± 14.12; p = 0.0012) groups compared to the STD group (Figure 4.8 D). Overall, male rats in the HFHS groups, displayed signs of diminished ability to clear glucose from the blood after its administration after nine months of feeding/treatment.

Similarly, female rats maintained on the HFHS diet displayed similar significant differences in glucose concentrations, 15 min after oral glucose stimulation when compared to those on the STD diet at three months (10.65 ± 0.79 vs 8.43 ± 0.92; p = 0.0024) (Figure 4.9 A). While this increase was observed 15 (8.28 ± 0.52 vs 6.46 ± 0.23; p = 0.0003) and 30 min (8.08 ± 0.51 vs 6.87 ± 0.30; p = 0.0384) six months after feeding, it was not maintained throughout the remainder of the study, with no significant differences being observed between the respective groups at completion of the study (9 months) (Figure 4.9 B and C). Additionally, treatment with Afriplex GRT™ had no significant effect on the glucose clearance ability of female rats however, a significant difference in glucose levels was observed between HFHS + GRT and STD animals, 30 min after administration following six months of feeding (8.79 ± 0.39 vs 6.87 ± 0.30; p = 0.0001). Overall, female rats in the HFHS group, did not display significant signs of diminished ability to clear glucose from the blood following its administration after nine months of feeding, and instances of increase can be considered as contingent. This is further supported by the absence of differences observed in AUC analysis of each individual OGTT throughout the study (Figure 4.9 D). Possibly suggesting that females are better equipped able to handle the dietary insult, as could also be observed in the current study (Pektas et al., 2015).

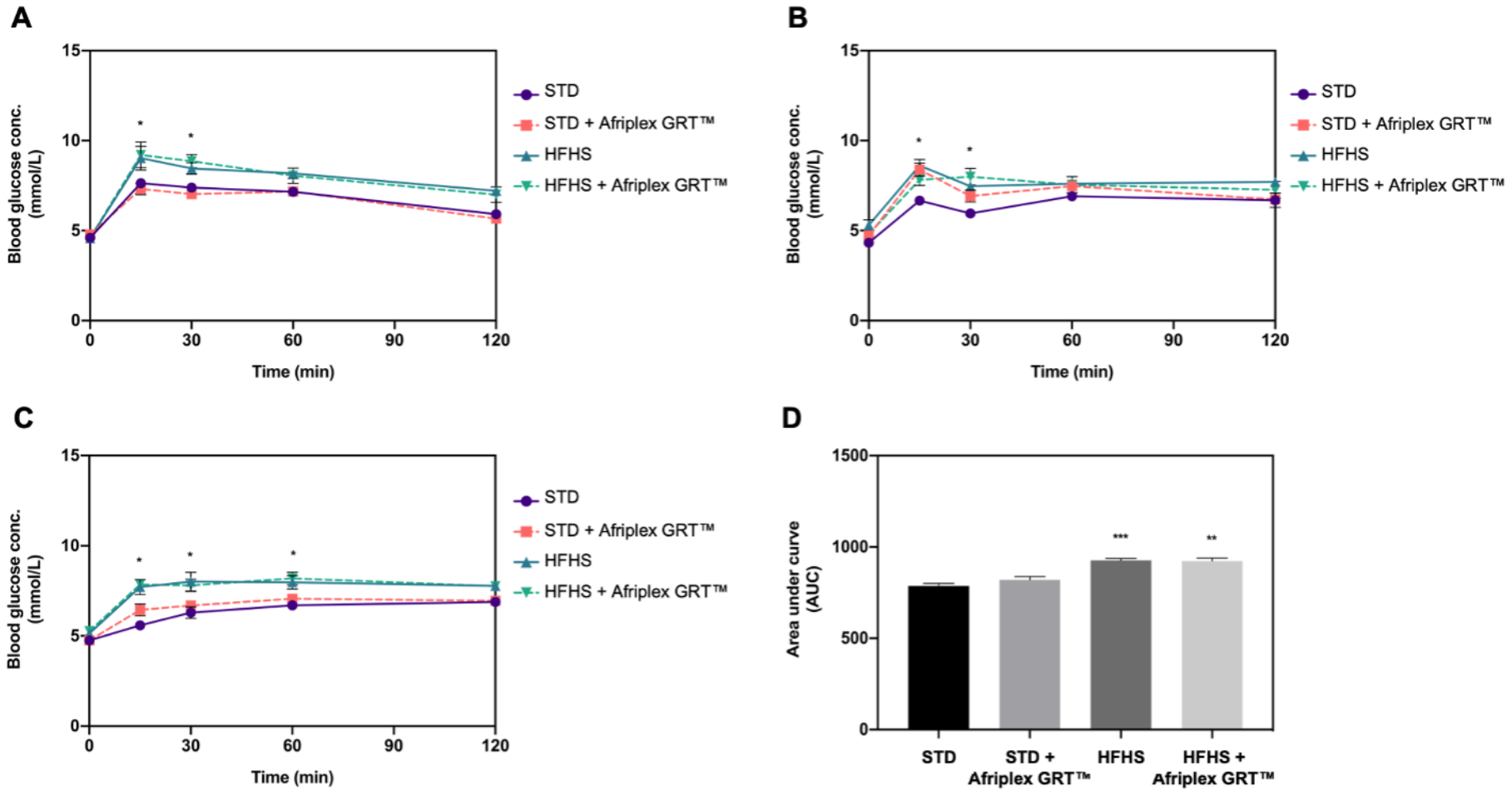

Figure 4.8. Oral glucose tolerance tests (OGTT's) at **three (A)**, **six (B)** and **nine (C)** months for **male** rats. Values are expressed as mean ± SE. **A)** 15 min: HFHS vs STD, * p = 0.0343 and Afriplex GRT™ vs STD, * p = 0.0112. 30 min: HFHS + Afriplex GRT™ vs STD, * p = 0.0218. **B)** 15 min: HFHS vs STD, **** p < 0.0001 and HFHS + Afriplex GRT™ vs STD, * p = 0.0387. 30 min: HFHS vs STD, ** p = 0.0021 and HFHS + Afriplex GRT™ vs STD, **** p < 0.0001. **C)** 15 min: HFHS vs STD, **** p < 0.0001 and HFHS + Afriplex GRT™ vs STD, **** p < 0.0001. 30 min: HFHS vs STD, *** p = 0.0001 and HFHS + Afriplex GRT™ vs STD, *** p = 0.0009. 60 min: HFHS vs STD, ** p = 0.0089 and HFHS + Afriplex GRT™ vs STD, ** p = 0,0013. **D)** Area under the curve (AUC) analysis of individual OGTT tests: HFHS vs STD, *** p = 0.0009 and HFHS + Afriplex GRT™ vs STD, ** p = 0.0012.

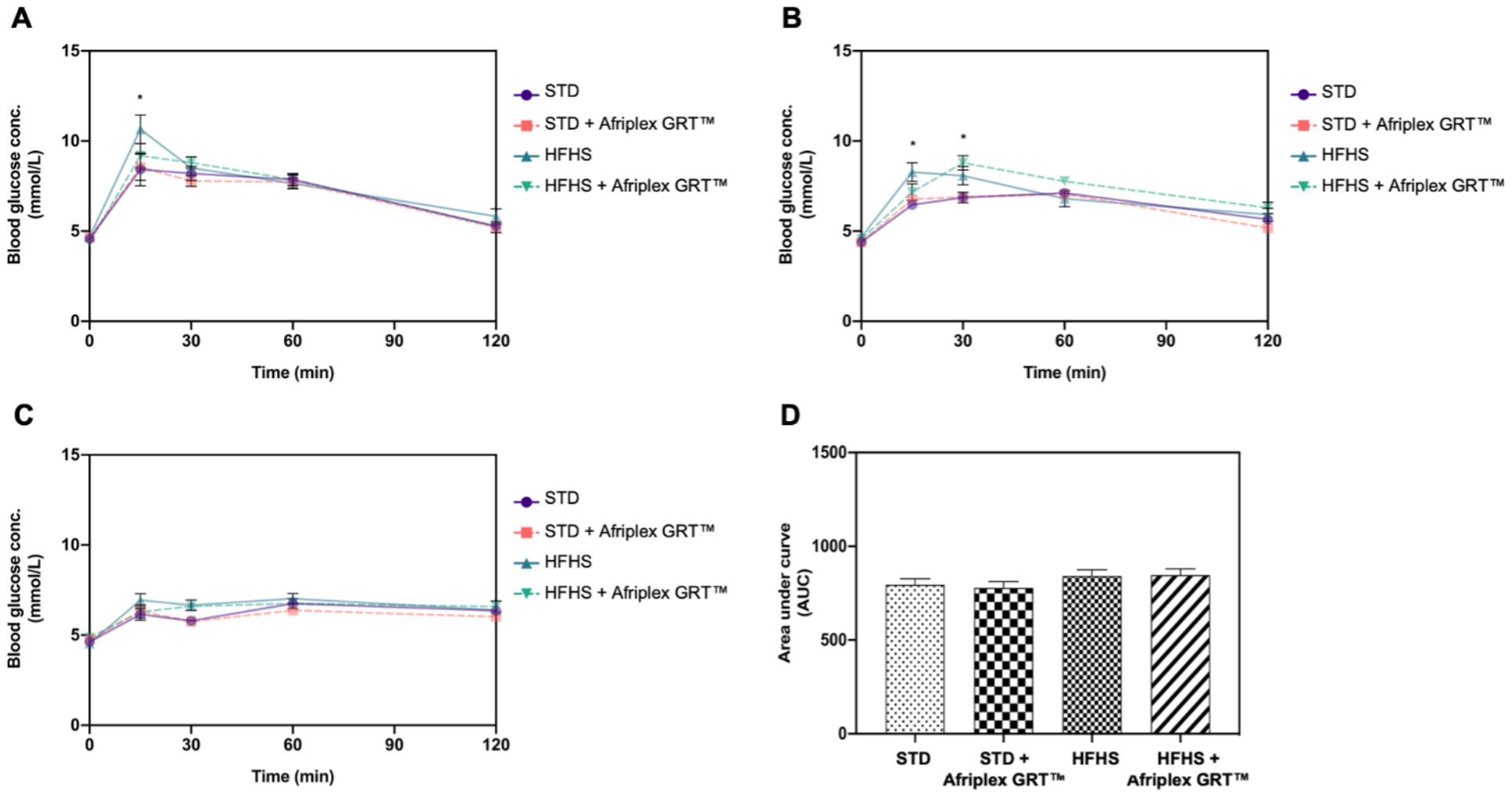

Figure 4.9. Oral glucose tolerance tests (OGTT's) at **three (A)**, **six (B)** and **nine (C)** months for **female** rats. Values are expressed as mean ± SE. **A)** 15 min: HFHS vs STD, ** p = 0.0024. **B)** 15 min: HFHS vs STD, *** p = 0.0003. 30 min: HFHS vs STD, * p = 0.0384 and HFHS + Afriplex GRT™ vs STD, *** p = 0.0001. **D)** Area under the curve (AUC) analysis of individual OGTT tests.

4.3.2 CVD risk predictors

Insulin resistance (IR), often considered as the driving force behind T2DM, results in the activation of damaging pathways, ultimately leading to diabetes-induced CVD (Bian et al., 2008; Frankel et al., 2008; Laakso, 2010; Soinio et al., 2006; Voelter-Maholknecht, 2016). Furthermore, IR has also been shown to be independently associated with increased CVD risk, even in non-diabetic subjects, and therefore remains a valuable predictor (Grant, 2007; Laakso, 2010; Libman et al., 2010; Natali et al., 2006). Considering this, serum insulin concentrations were measured upon termination of the study, accompanied by the application of the HOMA-IR model for the assessment of IR. The previously mentioned model has been widely used and validated for inferences in Wistar rats (Antunes et al., 2016). No significant differences, as well as no consistent trends in serum insulin concentrations were observed for both male and female rats after nine months of HFHS diet feeding when compared to their STD diet-fed counterparts (Table 4.6 and 4.7).

Table 4.6. Summary of serum insulin, lipid profiling and liver enzyme analyses for **male** animals.

	STD diet	**STD diet + GRT**	**HFHS diet**	**HFHS diet + GRT**
Insulin (pmol/L)	5.84 ± 1.63	8.61 ± 1.65	4.20 ± 0.73	9.15 ± 2.32
HOMA-IR index	0.21 ± 0.06	0.30 ± 0.06	0.16 ± 0.03	0.35 ± 0.09
Cholesterol				
Total (mmol/L)	1.54 ± 0.10	1.43 ± 0.09	1.63 ± 0.10	2.07 ± 0.14
LDL (mmol/L)	0.28 ± 0.00	0.28 ± 0.00	0.31 ± 0.02	0.31 ± 0.02
HDL (mmol/L)	0.97 ± 0.05	1.00 ± 0.06	0.80 ± 0.03	0.85 ± 0.04
Triglycerides (mmol/L)	1.23 ± 0.11	1.13 ± 0.13	**3.10 ± 0.34****	**3.55 ± 0.38*****
TG:HDL ratio	2.95 ± 0.29	2.54 ± 0.23	**9.02 ± 1.08****	**9.65 ± 0.10*****
Liver enzymes				
ALT (IU/L)	37.30 ± 4.08	28.60 ± 2.03	24.10 ± 1.88	**34.00 ± 2.31$^{\#}$**
AST (IU/L)	58.56 ± 2.98	53.10 ± 3.50	46.40 ± 3.65	**59.33 ± 3.06$^{\#}$**
AST:ALT ratio	1.94 ± 0.18	1.91 ± 0.11	1.97 ± 0.15	1.78 ± 0.08

Values are expressed as mean ± SEM. Each group consists of n = 10 Wistar rats as independent biological replicates. Bold entries represent instances where a significant difference was observed. **Triglycerides:** HFHS diet vs STD diet, ** p = 0.0015 and HFHS diet + Afriplex GRT™ vs STD diet, *** p = 0.0007. **TG:HDL ratio:** HFHS diet vs STD diet, ** p = 0.0017 and HFHS diet + GRT vs STD diet, *** p = 0.0004. **Liver enzymes ALT:** HFHS diet + Afriplex GRT™ vs HFHS diet, $^{\#}$ p = 0.0231 and **AST:** HFHS diet + Afriplex GRT™ vs HFHS diet, $^{\#}$ p = 0.0458.

As expected, this equated to no significant differences in HOMA-IR values between the respective groups for both male and female rats, indicating the absence of an IR phenotype (Table 4.6 and 4.7). Previous studies using similar diets reported on increased serum insulin and HOMA-IR values after three and 12 months of feeding (Huisamen et al., 2011; Nduhirabandi et al., 2017; Roux, 2011; Smit et al., 2018). However, these studies also reported significantly increased FBG levels, which was not observed in the present study. During the establishment of a pre-diabetic model, the absence of increased FBG levels could be a result of hyperinsulinemia. However, as this is not the case in the present study, the contribution of environmental or genetic predispositions towards the lack of IR phenotype cannot be excluded. Supplementation with Afriplex GRT™ had no significant effect on insulin serum levels or IR in both male and female rodents, irrespective of the diet (Table 4.6 and 4.7). Although an increase in serum insulin concentrations and HOMA-IR values were observed for male rodents in the STD + Afriplex GRT™ and HFHS + Afriplex GRT™ groups when compared to their placebo receiving counterparts, this increase was not significant (Table 4.6). This was unexpected as both rooibos and its major polyphenolic compound, Aspalathin, have been implicated to play a role in insulin sensitization (Kawano et al., 2009; Mazibuko et al., 2013; Smit et al., 2018).

Table 4.7. Summary of serum insulin, lipid profiling and liver enzyme analyses for **female** animals.

	STD diet	**STD diet + GRT**	**HFHS diet**	**HFHS diet + GRT**
Insulin (pmol/L)	2.06 ± 0.27	2.36 ± 0.48	2.36 ± 0.23	2.77 ± 0.64
HOMA-IR index	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.00	0.10 ± 0.03
Cholesterol				
Total (mmol/L)	1.57 ± 0.08	1.49 ± 0.09	1.90 ± 0.12	**1.98 ± 0.10***
LDL (mmol/L)	0.28 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.28 ± 0.01
HDL (mmol/L)	1.12 ± 0.06	1.07 ± 0.07	1.19 ± 0.06	1.24 ± 0.06
Triglycerides (mmol/L)	0.72 ± 0.12	0.77 ± 0.09	**1.81 ± 0.29***	**2.05 ± 0.22****
TG:HDL ratio	1.73 ± 0.30	1.67 ± 0.20	3.48 ± 0.62	**3.86 ± 0.48****
Liver enzymes				
ALT (IU/L)	41.30 ± 5.02	35.40 ± 2.93	**24.80 ± 11.51***	28.80 ± 1.47
AST (IU/L)	80.10 ± 9.20	73.80 ± 4.74	56.30 ± 3.24	53.80 ± 4.98
AST:ALT ratio	1.86 ± 0.08	2.17 ± 0.15	2.33 ± 0.18	1.89 ± 0.17

Values are expressed as mean ± SEM. Each group consists of n = 10 Wistar rats as independent biological replicates. Bold entries represent instances where a significant difference was observed. **Total cholesterol:** HFHS diet + Afriplex GRT™ vs STD diet, * p = 0.0375. **Triglycerides:** HFHS diet vs STD diet, * p = 0.0132 and HFHS diet + Afriplex GRT™ vs STD diet, ** p = 0.0012. **TG:HDL ratio:** HFHS diet + GRT vs STD diet, ** p = 0.0098. **Liver enzyme ALT:** HFHS diet vs STD diet, * p = 0.0495.

Amongst the pathological mechanisms proposed to underline T2D and its associated cardiovascular complications, lipid toxicity has been frequently implicated (Duncan, 2011; Goldberg et al., 2012; Marnewick et al., 2011; Sharma et al., 2004). This study therefore utilised serum isolated from peripheral blood, to perform total lipid profiling in order to provide a comprehensive overview of CVD risk in male (Table 4.6) and female (Table 4.7) Wistar rats maintained on the obesogenic diet. While no differences were observed in total cholesterol, HDL and LDL levels for both male and female rats in the HFHS group, triglyceride levels were significantly elevated compared to their control counterparts (STD). Interestingly, this increase in triglycerides was maintained for male and female rats on the HFHS diet, in the experimental group receiving the extract (HFHS + Afriplex GRT™) when compared to those on the STD diet (Table 4.6 and 4.7). Additionally, total cholesterol was significantly increased for HFHS diet fed female rats receiving GRT treatment, compared to those in the STD group (Table 4.7). Interestingly, the ability of rodents to regulate cholesterol transport differs from humans due to the diminished activity of cholesteryl ester transport protein (CETP), an enzyme responsible for facilitating the transfer of cholesteryl ester from HDL to LDL and very-low-density lipoprotein (VLDL) (Russell & Proctor, 2006; Tsutsumi et al., 2001; Yin et al., 2011). The characteristically increased HDL and decreased LDL levels often observed in rats with a moderate disease phenotype could be explained by the diminished activity of CETP. However, this was not displayed in the present study in both male and female animals.

Though the independent evaluation of HDL is indicative of the liver's ability to produce beneficial HDL cholesterol, decreased HDL levels are often accompanied with an increase in triglyceride levels during a state of metabolic stress (Bang et al., 2008; Cordero et al., 2008). Conversely, when transitioning towards a healthier metabolism, triglyceride levels generally tend to decrease while HDL cholesterol levels increase substantially. Given this apparent indirect proportionality observed between TG and HDL cholesterol levels, it is not unexpected that several studies have provided evidence supporting the use of the TG/HDL ratio as a powerful predictor of metabolic syndrome and numerous forms of CVD, such as atherosclerosis and coronary artery disease (CAD) (Boizel et al., 2000; Cordero & Alegria-Ezquerra, 2009; da Luz et al., 2008; Kim-Domer et al., 2009; Nam et al., 2019). This ratio is specifically used as an estimator of LDL particle size, which is closely associated with vascular diseases and remains unfeasible to analyse in clinical practice (Boizel et al., 2000; Cordero et al., 2009; da Luz et al., 2008). Furthermore, normal HDL cholesterol levels in patients with T2D does exclude the possibility of reduced LDL particle size, making the use

of this prediction ratio particularly useful. Evaluation of the TG/HDL ratio in this study revealed a significant increase in male rodents receiving the HFHS diet, and once more, this increase was maintained for male rats in the HFHS + Afriplex GRT™ group compared to rats in the control group (Table 4.6). Female rodents on the other hand, displayed a significant increase in TG/HDL ratio for rats in the HFHS group, receiving Afriplex GRT™ treatment only, when compared to those in the STD group (Table 4.7). However, despite the occurrence of significant increase, both male and female rats in the HFHS group displayed an TG/HDL ratio higher than 3.0, indicating atherogenic lipid profiles and significant IR and CVD risk (da Luz et al., 2008; Li et al., 2016).

In addition to the assessment of metabolic marker concentrations, liver enzymes ALT and AST were also evaluated due to its association with an increased risk of developing T2DM and liver damage. While male rats maintained on the obesogenic diet (HFHS) displayed no differences in both ALT and AST levels (Table 4.6), female rats in the HFHS group demonstrated significantly reduced ALT levels when compared to the control counterparts (Table 4.7). Intriguingly, both ALT and AST levels were increased in the male rats on the HFHS diet + Afriplex GRT™ compared to those on the HFHS diet treated with the vehicle control (Table 4.6). While this suggests a possible damaging effect on the liver introduced by GRT administration, this phenomenon was not consistent in the assessment of other metabolic markers, further investigation is required.

4.3.3 Histological assessments of cardiac tissue

Microscopic structural changes in the nuclei of cardiomyocytes were visualised using H&E staining of heart tissue collected for both male (Figure 4.10) and female rats (Figure 4.11), at termination of the study. To this end, nuclei are visible in blue while the cytoplasm and myofibres are depicted in various degrees of pink/red (Wick, 2019). Overall, single, oval, prominent and centrally located nuclei in cardiomyocytes can be observed under the light microscope for animals in all the respective groups. Comparing the experimental group (HFHS) to the control animals (STD), no significant signs of injuries, including disarrangement of cardiac myofibres and deformation in size and shape of nuclei, could be identified for both male (Figure 4.10) and female (Figure 4.11) rodents. Treatment with Afriplex GRT™ in neither the STD diet fed or HFHS diet fed animals had any effect on cardiac muscle fibres or myocyte structure (Figure 4.10 and 4.11).

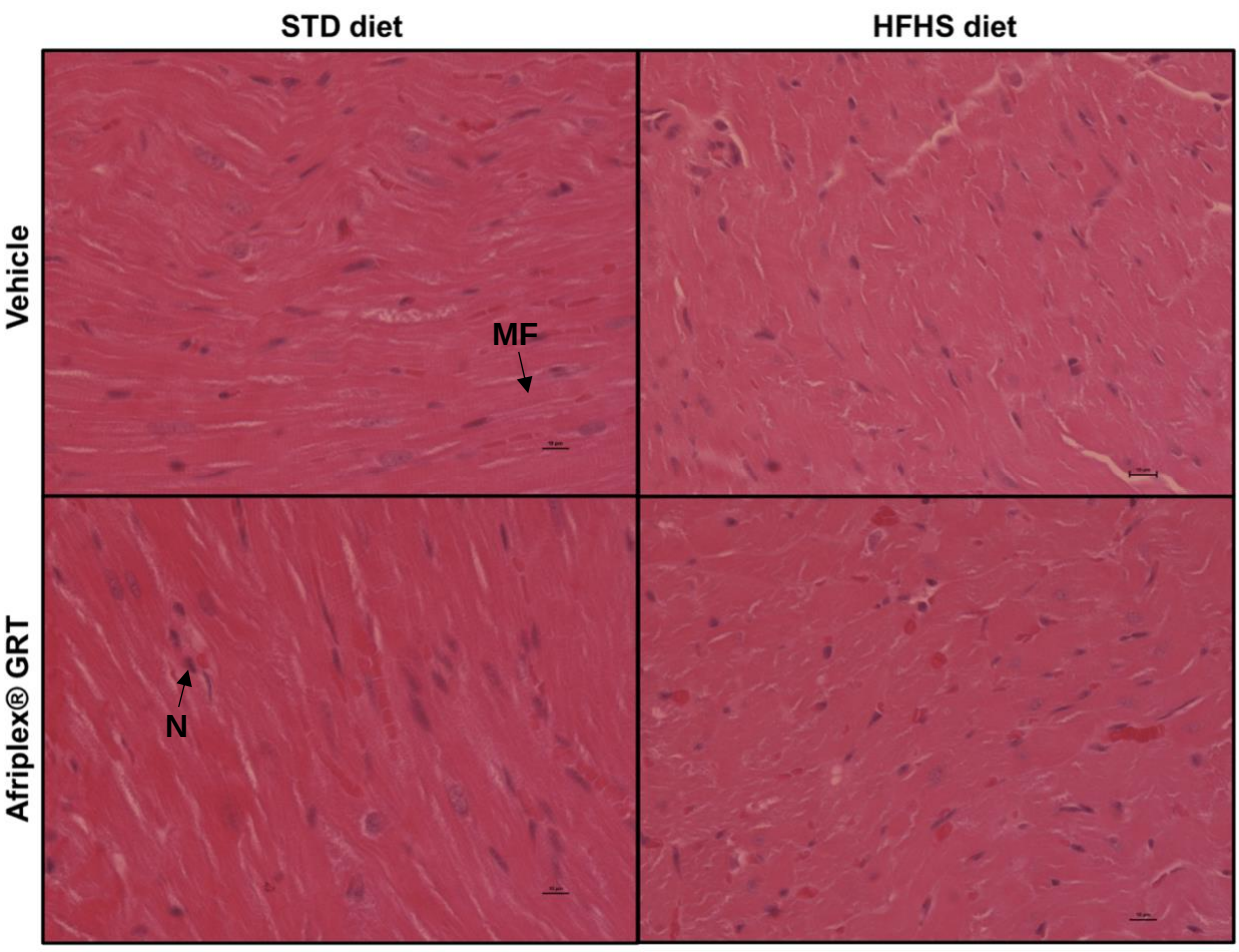

Figure 4.10. Photomicrograph showing longitudinal sections of cardiac tissue from a representative **male** animal in each group. Where **N**: nuclei of cardiomyocytes, **MF**: myofibres under Haematoxylin and Eosin (H&E) stain (LM x40).

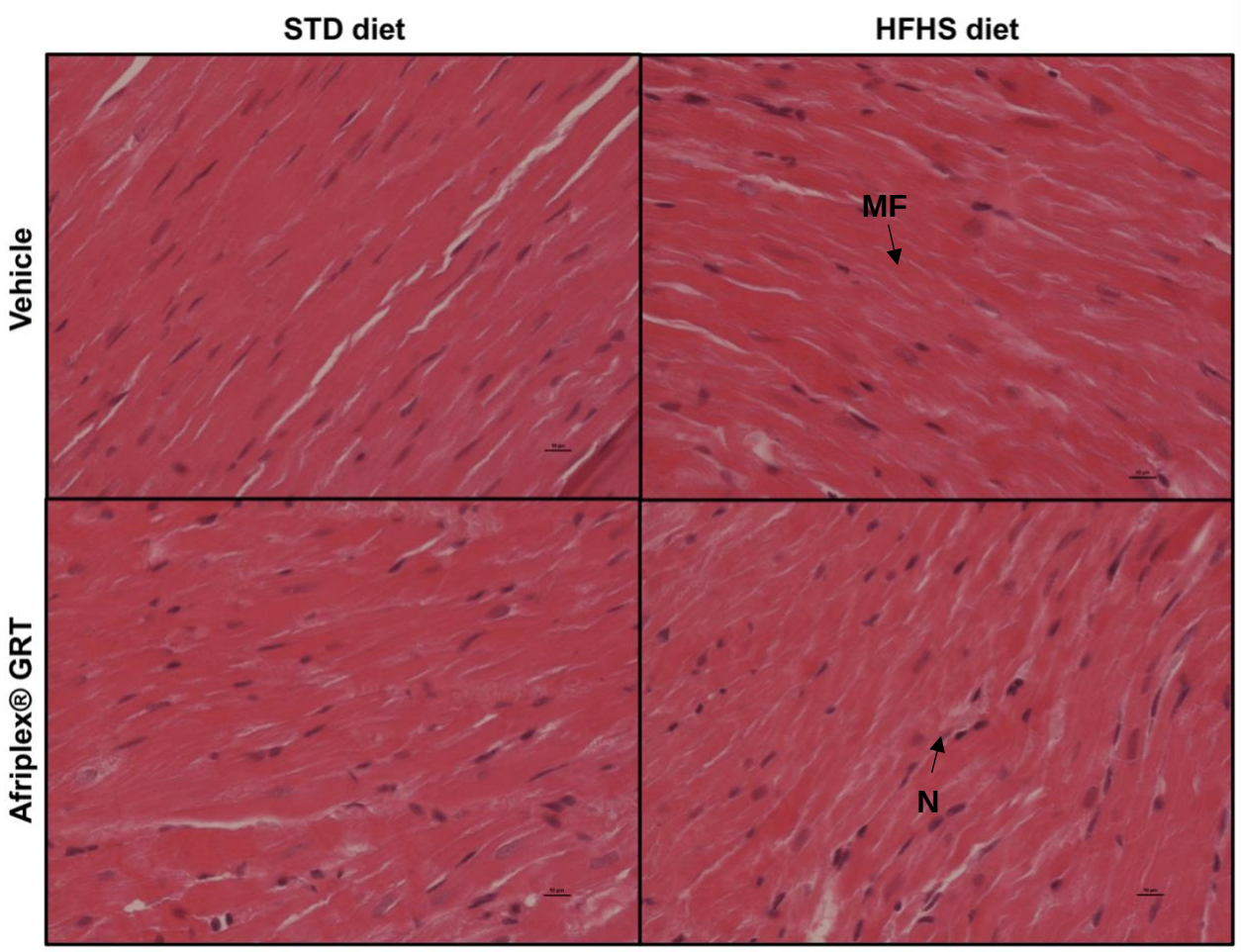

Figure 4.11. Photomicrograph showing longitudinal sections of cardiac tissue from a representative **female** animal in each group. Where **N**: nuclei of cardiomyocytes, **MF**: myofibres under Haematoxylin and Eosin (H&E) stain (LM x40).

Additionally, MT staining was used to observe the occurrence of connective tissue deposits with light microscopy. Contradictory to H&E staining, MT staining allows for the differentiation of morphological changes associated with injury and regeneration (Assaw, 2012). Briefly, heart tissue obtained from male (Figure 4.12) and female rodents (Figure 4.13) were stained with a combination of three dyes during which the cell nuclei can be observed in black, collagen fibre in blue while cardio myofibres and cytoplasm is stained in red (Assaw, 2012; Flint et al., 1975; Rieppo et al., 2019). Taken together, a similar amount of collagen fibres can be observed between animals in all the respective groups. No signs of reactive interstitial fibrosis in the form of connective tissue deposits can be observed in the animals fed the HFHS diet when compared to their control counterparts for both male (Figure 4.12) and female (Figure 4.13) Wistar rats. The administration of Afriplex GRT™ had no effect on the overall structure of cardio myofibres, collagen content, or connective tissue deposition (Figure 4.12 and 4.13).

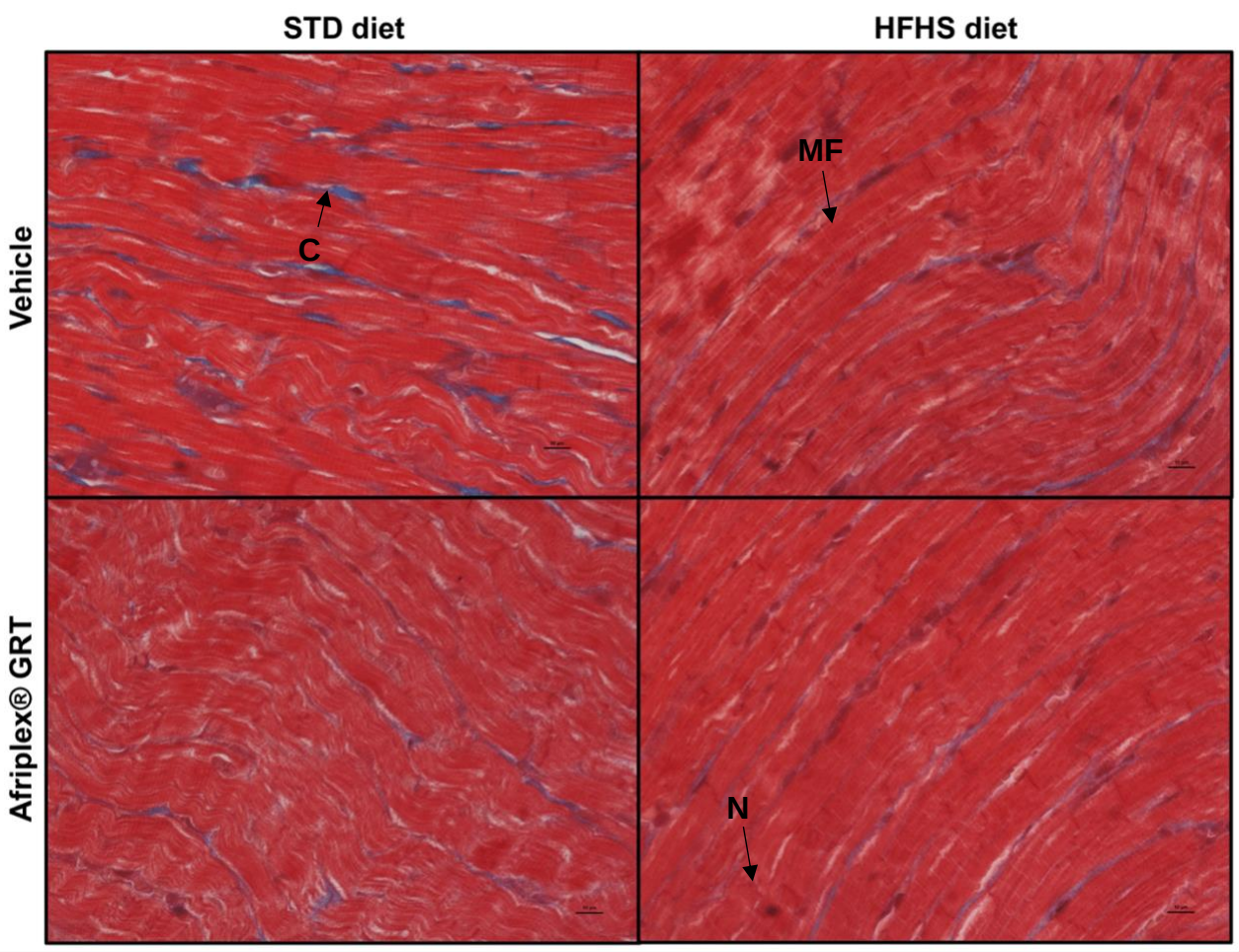

Figure 4.12. Photomicrograph showing longitudinal sections of cardiac tissue from a representative **male** animal in each group. Where **N**: nuclei of cardiomyocytes, **MF**: myofibres, and **C**: collagen under Masson's Trichome (MT) stain (LM x40).

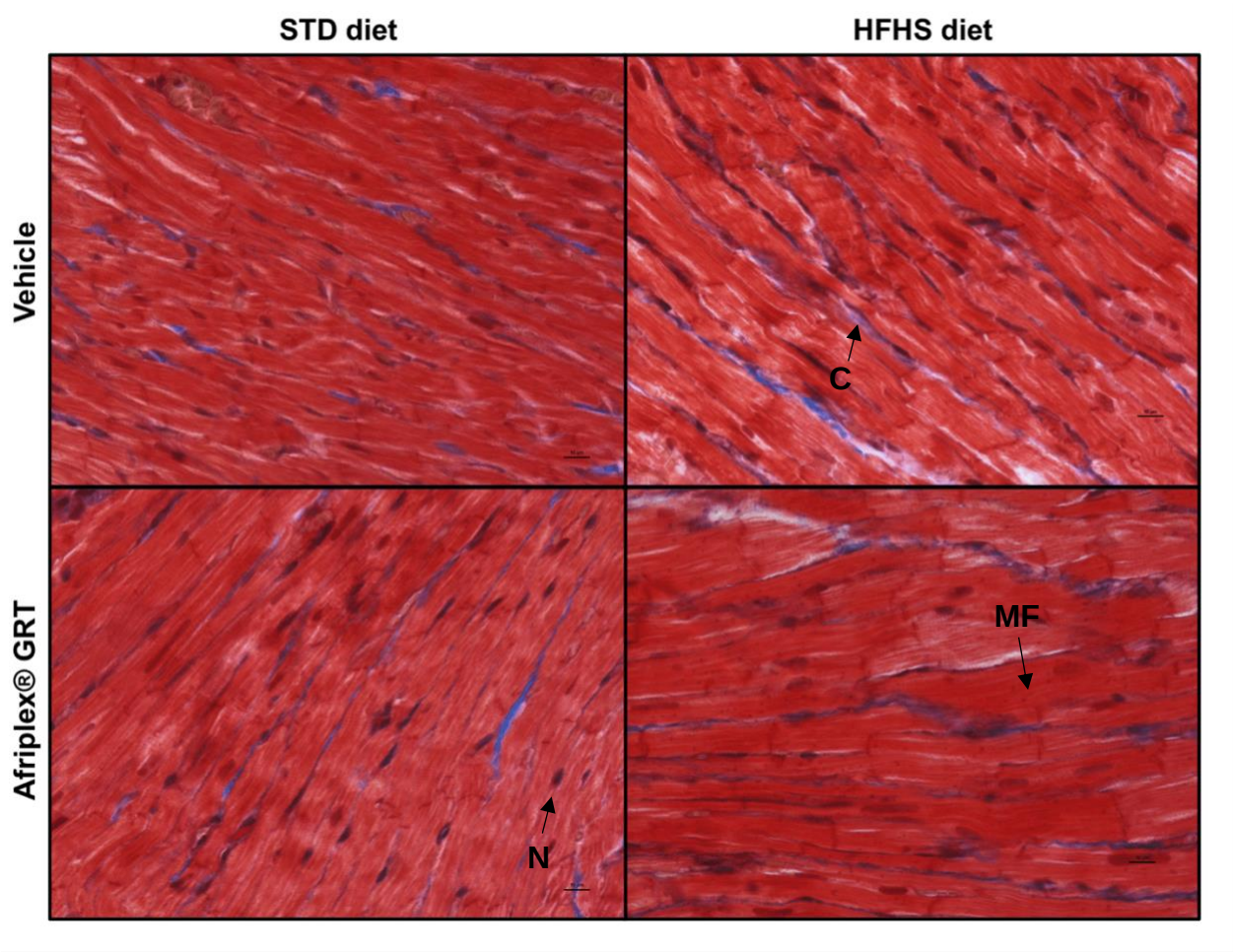

Figure 4.13. Photomicrograph showing longitudinal sections of cardiac tissue from a representative **female** animal in each group. Where **N**: nuclei of cardiomyocytes, **MF**: myofibres, and **C**: collagen under Masson's Trichome (MT) stain (LM x40).

4.3.4 Histological assessment of liver tissue

Similar to cardiac tissue, liver specimens from both male and female rodents were stained with H&E. Accordingly, the nuclei can therefore be visualised in dark blue, while the cytoplasm and extracellular matrix can be observed in different shades of pink. Under light microscopy, hepatic steatosis can be identified by either the presence of large fat droplets, together with dislocation of the nuclei to the periphery of the cell (macro vesicular steatosis); or small lipid droplets and the absence of nuclei displacement (micro vesicular steatosis). Male rats maintained on the STD diet, receiving treatment with either the placebo or the Afriplex GRT™ extract, displayed normal liver architecture and cellular integrity, characterised by the presence of centrally situated nuclei and intact cell walls (Figure 4.14). Complementary findings were observed for the female STD diet fed rats in both the experimental (STD + Afriplex GRT™) and control groups (Figure 4.15).

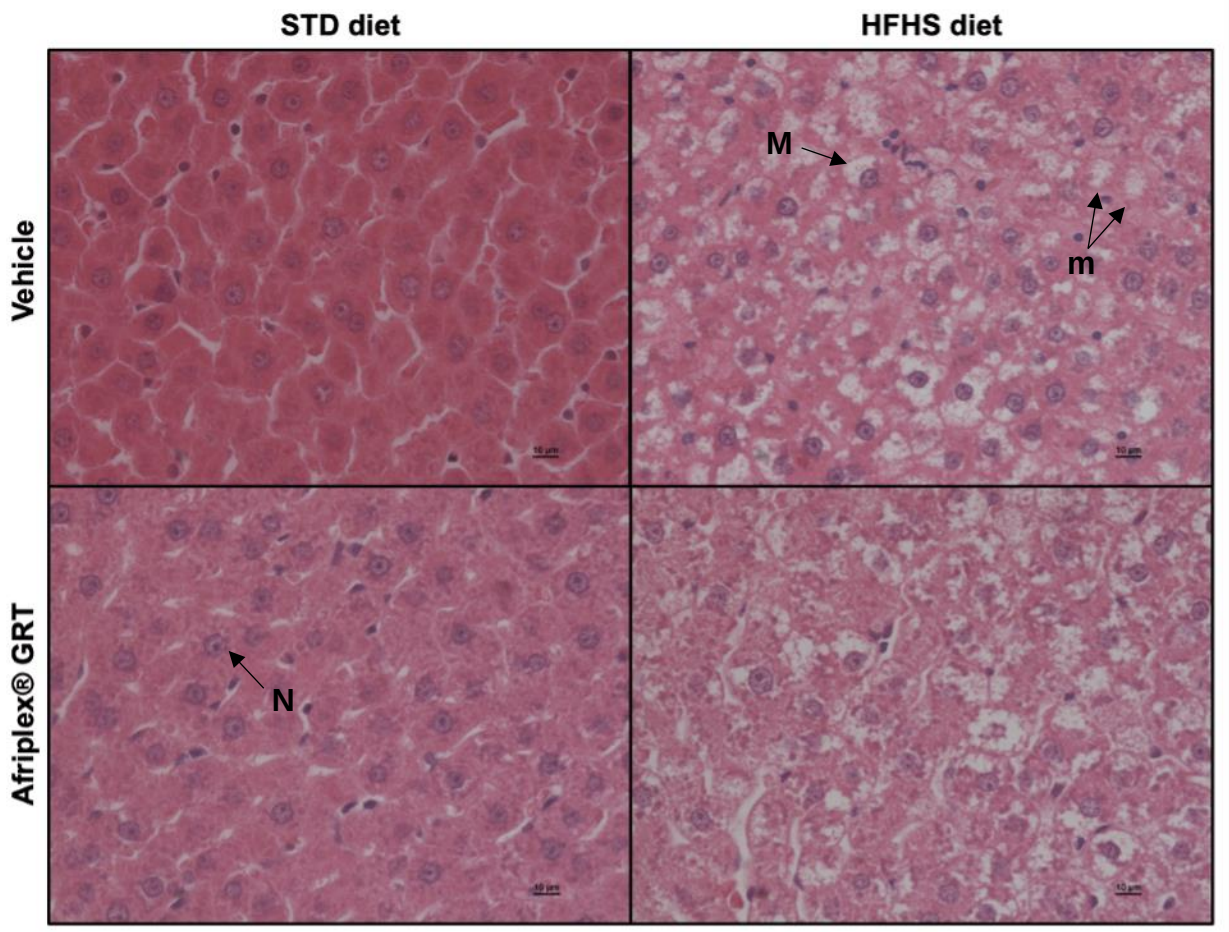

Figure 4.14. Photomicrograph showing longitudinal sections of liver tissue from a representative **male** animal in each group. Where **N**: nuclei of hepatocytes, **M**: Macro vesicular dislocation of nuclei to the periphery, and **m**: micro vesicular degeneration presented by lipid droplets under Haematoxylin and Eosin (H&E) stain (LM x10).

Liver samples obtained from male rats in the HFHS group revealed increased signs of steatosis in 30% of the animals, when compared to their control counter parts (Figure 4.14). Overall, a substantial increase in lipid accumulation and minor indication of nuclei displacement can be observed in the HFHS animals. Micro vesicular complications were also detected in a similar percentage (30%) of female rats receiving the HFHS diet, compared to those maintained on the STD diet (Figure 4.15). Interestingly, although the administration of GRT for both male and female animals on the HFHS diet had no significant effect in reducing the degree of fat accumulation in the liver, it also did not exacerbate the existing signs of lipo-toxicity (Figure 4.14 and Figure 4.15).

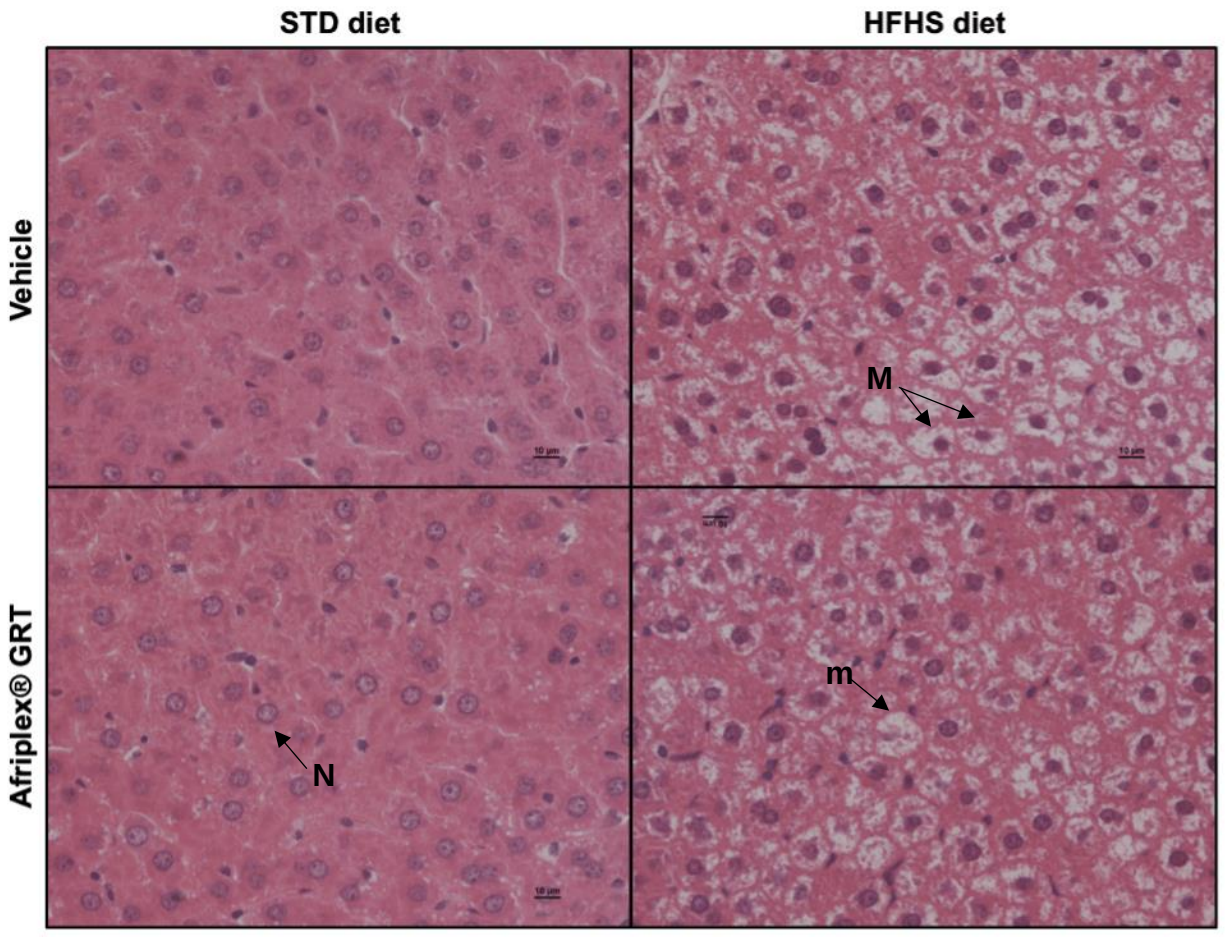

Figure 4.15. Photomicrograph showing longitudinal sections of liver tissue from a representative **female** animal in each group. Where **N**: nuclei of hepatocytes, **M**: Macro vesicular dislocation of nuclei to the periphery, and **m**: micro vesicular degeneration presented by lipid droplets under Haematoxylin and Eosin (H&E) stain (LM x10).

4.4 Conclusions

While the use of male rats has been favoured in biomedical research predominantly due to intrinsic variabilities associated with female cyclical reproductive hormones, the relevance of including female animals in pre-clinical models cannot be overstated in the instance of metabolic syndromes. Furthermore, meta-analysis of population data revealed that the rate of obesity in South African women are amongst the highest worldwide and as this trajectory continues to escalate, so too does the prevalence of obesity-related diseases such as T2D and CVD, despite the protective effects of oestrogen prior to menopause (Damasceno, et al., 2007; Joubert et al., 2007; Kucharska-Newton et al., 2010; Ng et al., 2014; Zhou, 2017). Thus, the inclusion of female rodents in this model enables extrapolation of the current study findings to the clinic.

This study demonstrated that high fat, high sugar diet feeding for a period of 9 months introduced increased food and water consumption, significant weight gain as well as elevated body mass indicative of a significant increase in RF weight in both male and female animals. Furthermore, the OGTTs performed demonstrated diminished glucose clearance ability of rodents maintained on the obesogenic diet, even in the absence of increased fasting serum insulin levels and HOMA-IR values. Taken together with the elevated triglyceride levels and TG/HDL ratio, these results confirm that the HFHS diet was successful in establishing a pre-clinical, risk prediction model of capable of being used to assess molecular mechanisms underlying CVD pathophysiology. Of interest to this study is the ability of the HFHS diet to introduce more prominent effects on the biometric parameters assessed in male Wistar rats, compared to females. Interestingly, GRT supplementation in the present study, had no significant beneficial or negative effect on the parameters assessed in both male and female animals.

Histological findings of the cardiac tissue in the present study showed no disturbances in the structural organisation of cardiac tissues or inflammation indicated by myocardial injury. However, histological assessment of the liver tissue showed significant increase in lipid droplet accumulation and mild displacement of nuclei in lipocytes towards the cell's periphery resulting from HFHS feeding. The absence of a structural phenotype in cardiomyocytes does not demolish the existing severe CVD risk demonstrated from serum biomarker analysis, further supporting the notion that the onset of CVD is acute and asymptomatic in nature. Although functional assessment of the heart was not conducted in this study due to limited access to echocardiogram infrastructure, studies have

demonstrated the ability of a diet high in fat and sugar to introduce functional abnormalities in the heart in the form of reduced ejection fraction and left ventricular dysfunction, even in the absence of molecular and structural differences (Smit, 2019; Johnson et al., 2020).

4.5 References

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Chapter 5: Transcriptome profiling of diet-induced CVD in Wistar rats through high-throughput mRNA sequencing

5.1 Introduction

For years, cardiovascular disease (CVD) has been recognised as the primary cause of premature death, claiming more lives globally than all the different forms of cancer combined (GBD, 2018; WHO, 2021). Although scientific research has made significant contributions towards understanding CVDs, its diverse nature complicates the elucidation of the underlying mechanisms involved in disease development and progression (Kim et al., 2010; Ramos et al., 2016). Despite endorsed lifestyle interventions aimed at the prevention and treatment of CVD, the associated deaths continue to escalate at an alarming rate (Roth et al., 2017). Thus, driving the need for continued research towards understanding CVD pathophysiology to potentially aid in identifying novel therapeutic and prognostic targets (Chamnan et al., 2009; Ruwanpathirana et al, 2015; Voelter-Mahlknecht, 2016).

The ability to accurately identify individuals at risk of developing a cardiac event allows for the implementation of early intervention therapies, while simultaneously increasing life expectancy (Bovet et al., 2015; Damen et al., 2016). Numerous mathematical prediction models such as Framingham, SCORE, and QRISK have been developed to estimate cardiovascular risk based on existing risk factors including diabetes, hypertension, and smoking (Antman et al., 2000; Chamnan et al., 2009; Frankel et al., 2008). Furthermore, molecular markers such c-reactive protein (CRP), glycated haemoglobin (HbA1c), N-terminal pro b-type natriuretic peptide (NT-pro-BNP) and serum inflammation markers have been extensively used in the exploration of disease pathology (Arnold et al., 2016; Bay et al., 2003; Miller et al., 2007; Panagopoulou et al., 2015; Yang et al., 2016). However, as these tools remain limited in their early onset CVD prediction ability and differentiate between stages of disease progression, the identification of biomarkers and the molecular mechanism involved is strongly encouraged (Ge & Wang, 2012; Khot et al., 2003; van Holten et al., 2013).

Moreover, the use of plant-derived polyphenols as an adjuvant to current pharmaceutical treatment options for CVD has gained considerable interest in the last decade (Bahadoran et al., 2013; Chellan et al., 2014; Gollucke et al., 2013; Pandey & Rizvi, 2009; Parks & Booyse, 2002). Of interest to the current study is the Afriplex GRT™ extract derived from the plant commonly known as rooibos (*Aspalathus linearis*), rich in polyphenols with known

health promoting and cardio-protective properties. Both *in vitro* and *in vivo* studies have demonstrated the ability of rooibos extracts and its polyphenolic compounds to beneficially impact functional cardiac injury as well as reduce metabolic risk factors associated with the development of diabetes induced cardiovascular complications (Dludla et al., 2014; Johnson et al., 2016; Kawano et al., 2009; Marnewick et al., 2011; Mazibuko et al., 2015; Muller et al., 2012; Pantsi et al., 2011; Smit, 2016; Ulicná et al., 2006). However, to characterise plant polyphenols such as the Afriplex GRT™ extract regarding CVD prevention, research providing a better understanding of the disease pathophysiology and the molecular mechanisms involved in the previously observed cardio-protective effects of Afriplex GRT™ is necessary.

Recent advances in genomics and transcriptomics over the past decade have aided in identifying the biological and pathological processes involved in disease development. Several studies have utilised microarray profiling and next generation sequencing (NGS) technologies to screen and identify altered gene expression associated with CVD. Exploration of mRNA expression and the correlation with CVD not only adds to an understanding of CVD pathology, but also provides possible new therapeutic targets. This study aims to profile differential gene expression associated with the development of diet-induced CVD using transcriptome sequencing and to ascertain whether treatment with the Afriplex GRT™ extract has the potential to alter the observed transcriptomic profile.

5.2 Materials and Methods

5.2.1 Study design and sample selection

A total of 30 male and 30 female Wistar rat (*Rattus norvegicuss*) cardiac tissue samples from the animal study described in Chapter 3 were used in the present study. The sample selection criteria were based on the above-mentioned objectives and involved the inclusion of both male and female rats from three groups namely: 1) the control group which received a standard (STD) maintenance rodent diet (n=10), 2) the high fat, high sugar (HFHS) group receiving a cafeteria diet (n=10), and 3) the HFHS group receiving treatment with the Afriplex GRT™ extract (n=10).

5.2.2 RNA extraction, assessment, and pooling

Total RNA was isolated from the cardiac tissues stored in RNAlater using the RNeasy® Fibrous Tissue Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and treated with the TURBO DNA-free kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to eliminate genomic DNA contamination. Briefly, approximately 25 – 30 mg of tissue was submerged in a lysis buffer supplemented with β-mercaptoethanol (β-ME) and homogenised using a TissueLyser (Qiagen, Hilden, Germany). The resulting lysates were then subjected to enzymatic digestion using proteinase K to facilitate the removal of contractile proteins, connective tissue, and collagen. Following centrifugation, the supernatant was attained and combined with 0.5 volumes absolute ethanol to enable the precipitation of RNA. The solution was then subjected to a series of column-based wash steps, prior to elution of RNA with 60 µL nuclease-free water as per the manufacturer's guidelines. The yield and purity of the RNA was determined using fluorometry (Qubit™ RNA HS Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) and spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA), while the Agilent 2100 bioanalyser (Agilent Technologies, CA, USA) was used to assess the RNA integrity. Upon completion of quality control (QC) assessment, highly intact RNA from individual samples with a RIN value ≥ 7 were pooled in duplicate to generate 5 biological replicates of each group (Figure 5.1). To this end each pooled sample consisted of an equal amount of DNase treated RNA (10 µg) from the two individual rats involved.

A

Rat IDs	Pooled RNA ID	Group
R1 + R2	M1	
R3 + R4	M2	
R5 + R6	M3	**STD**
R7 + R8	M4	
R9 + R10	M5	
R11 + R12	M6	
R13 + R14	M7	
R15 + R16	M8	**HFHS**
R17 + R18	M9	
R19 + R20	M10	
R21 + R22	M11	
R23 + R24	M12	
R25 + R26	M13	**HFHS + Afriplex GRT™**
R27 + R28	M14	
R29 + R30	M15	

B

Rat IDs	Pooled RNA ID	Group
R31 + R32	F1	
R33 + R34	F2	
R35 + R36	F3	**STD**
R37 + R38	F4	
R39 + R40	F5	
R41 + R42	F6	
R43 + R44	F7	
R45 + R46	F8	**HFHS**
R47 + R48	F9	
R49 + R50	F10	
R51 + R52	F11	
R53 + R54	F12	
R55 + R56	F13	**HFHS + Afriplex GRT™**
R57 + R58	F14	
R59 + R60	F15	

Figure 5.1. RNA pooling of **A) male** (n = 30) and **B) female** (n = 30) samples to generate five biological replicates per group, for sequencing and subsequent expression analysis.

5.2.3 RNA library preparation and sequencing

The pooled RNA samples (200 ng) were subjected to magnetic bead-based mRNA enrichment using the Dynabeads™ mRNA Purification Kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA), according to the protocol described in the MGIEasy RNA Library Prep Set User Manual prior to proceeding with library construction. Library preparation was performed with the entire component of mRNA for each sample using the MGIEasy RNA Library Prep Kit (MGI, Shenzhen, China), according to the manufacturer's instructions. Concisely, mRNA was fragmented to obtain 250 base pair (bp) inserts using a highly concentrated buffer at 87°C and subsequently reverse transcribed to generate copy DNA (cDNA) products. The cDNA product of each sample was purified using MGIEasy DNA Clean Beads (MGI, Shenzhen, China) and subjected to an end-repair and a-tailing (ERAT) reaction. The end-repaired products were ligated to 10 X diluted MGIEasy DNA Adapters as per the manufacturer's guidelines, purified using the previously described magnetic beads, and amplified. Following purification, the PCR products were quantified with fluorometry using the Qubit dsDNA HS Assay kit according to the manufacturer's instructions. Furthermore, the fragment size distribution of purified PCR products was assessed using gel electrophoresis.

To allow for the generation of a minimum of 30 million reads per transcriptome, samples were combined in an equal molar manner to allow for the pooling of eight uniquely labelled samples per lane. Single-stranded circular DNA libraries were generated from 1 pmol of pooled PCR products, followed by purification and quantification with MGIEasy DNA Clean Beads and the ssDNA HS Assay kit (Qubit), respectively. The MGILD-200 automatic loader was used to load pooled libraries onto the MGISEQ-2000 FCL flow cells. Massively parallel sequencing was performed using DNA nanoball-based technology on the MGISEQ-2000 (BGI, Shenzhen China) with the appropriate reagents supplied in the MGISeq-2000RS High-Throughput Sequencing Kit. A paired-end sequencing strategy was employed, with a read length of 100 bp (PE100).

5.2.4 Bioinformatic analysis

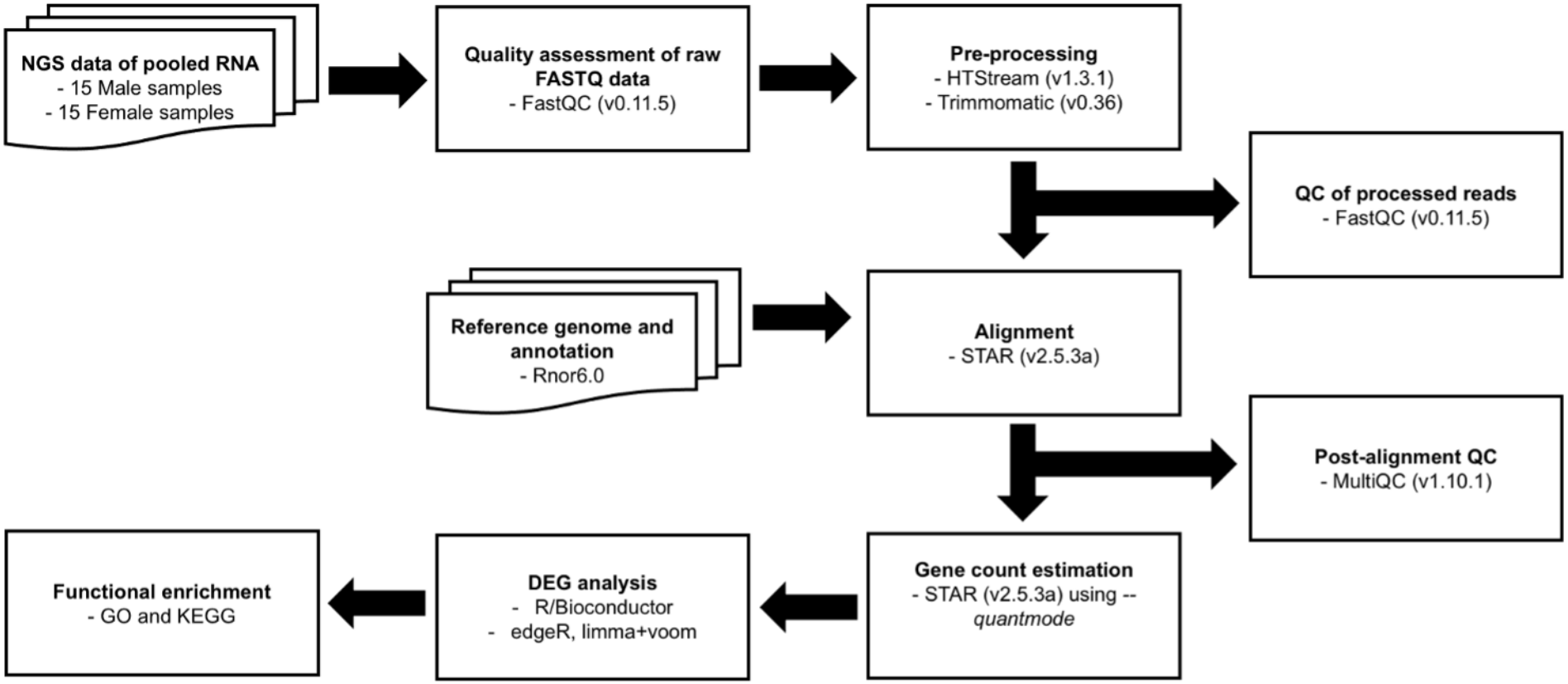

Figure 5.2. Schematic workflow describing the bioinformatic pipeline used for the analysis of RNA sequencing data. The workflow steps until the completion of "gene count estimation" was implemented for the 30 sequence datasets individually, while differential expression (DEG) analysis was performed separately for male (n = 15) and female (n = 15) datasets.

5.2.4.1 Pre-processing

The raw transcriptome sequence reads were obtained from the MGISEQ-2000 in the universal FASTQ format and processed using the computational infrastructure available at the Centre for High Performance Computing (CHPC). Measurements of quality control and sample complexity were assessed and visualised using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Read files were processed individually to identify and remove possible unwanted contaminants, while retaining high quality bases to improve downstream analyses. In brief, *hts_SeqScreener* from the HTStream suite version 1.3.1 was used to count the level of ribosomal RNA (rRNA) present in the samples using default parameters and the collection of all rRNA FASTA files for *Rattus norvegicus* available on NCBI (Street, 2017). Subsequently, Trimmomatic version 0.36 was used to remove MGI adapter sequences, bases with a Phred quality score below Q20 from the 5' end of the sequence reads using a sliding window of 3 nucleotides (nts), as well as reads with a minimum length less than 20 nts (Bolger et al., 2014). Following completion of all pre-processing steps, comparisons between trimmed and raw read files were conducted in the context of the previously described QC metrices using FastQC.

5.2.4.2 Alignment and count estimation

Gene- or transcript-level counts were generated as a proxy for gene expression by employing a conventional alignment-based strategy. To this end, “cleaned” reads from each pooled sample were aligned to the *Rattus norvegicus* reference genome version 6.0 (ftp://ftp.ensembl.org/pub/release-100/fasta/rattus_norvegicus/dna/Rattus_norvegicus.Rnor_6.0.dna.toplevel.fa.gz) using the “splice-aware” aligner, STAR version 2.5.3a (Dobin et al., 2013). The latter was selected based on its ability to compensate for aligning intron-spanning reads. The alignment was further coordinated with the corresponding reference annotation (ftp://ftp.ensembl.org/pub/release-100/gtf/rattus_norvegicus/Rattus_norvegicus.Rnor_6.0.100.gtf.gz) and executed using default parameters, with the addition of the *--quantmode GeneCounts* function in order to compute the number of reads associated with host genes. Additionally, the summary statistics obtained upon completion of alignment steps were assessed using SAMtools version 1.10 and collated with MultiQC version 1.10.1 for quality assurance purposes (Li et al., 2009; Ewels et al., 2016).

5.2.4.3 Differential expression (DE) analysis

Differential gene expression analysis has been shown to be predominantly influenced by the methodology of identifying differentially expressed genes (DEGs) as opposed to the adopted method of mapping or quantification of reads (Costa Silva et al., 2017). In light of this, DE analysis was performed in R version 4.0.3, in combination with a variety of add-on packages from Bioconductor (Figure 5.4) (Gentleman et al., 2004; R Core Team, 2020). Briefly, raw counts data were adjusted for differences in sequencing depth between libraries using the *calcNormFactors* function from the edgeR package. The latter employs a weighted trimmed means of M-values (TMM) scaling method for normalisation (Robinson et al., 2010; McCarthy et al., 2012). Following the removal of genes with a normalised read count below 3 counts per million (CPM), log transformed normalised data (log2 CPM) and mean-variance estimates were obtained using the *voom* function from the limma package, to determine the weight of each observation made initially by a linear model (Ritchie et al., 2015).

The *lmFIT* function from the previously mentioned package, was then used to fit a linear model to the *voom* transformed data using the weighted least squares (WLS) for each gene. The log2 fold changes for all the genes in the reference annotation were obtained as *contrasts* of the fitted models between two distinct comparisons for male and female animals respectively namely: 1) the HFHS diet fed group versus the STD diet fed control group and 2) the HFHS group receiving treatment with Afriplex GRT™ versus the HFHS diet fed group. Due to the large number of genes being analysed simultaneously, Empirical Bayes (EB) smoothing of standard errors, as well as multiple testing correction using the False Discovery Rate (FDR) method were applied (Benjamini & Hochberg, 1995). In all instances, a gene was considered to be significantly differentially expressed between the respective groups if it had an adjusted p-value ≤ 0.05. The information of significantly DEGs resulting from each comparison, for male and female animals respectively, was exported and used in downstream analyses.

5.2.4.4 Functional annotation

To perform biological functional enrichment of the DEGs, the R Bioconductor tool was used to identify enriched gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (Ashburner et al., 2000; Kanehisa & Goto, 2000; Yu et al., 2012). Briefly, GO terms were retrieved from the Bioconductor database org.Rn.eg.db and assigned to up- and down-regulated DEGs obtained from the previously described comparisons using the topGO package. The Kolmogorov-Smirnov test was used to identify significantly enriched terms due to its application of a weighted algorithm and subsequent usefulness in datasets with varying number of significant genes. In all instances, an adjusted probability value of less than, or equal to 0.05 was deemed biologically significant. Significant GO enrichments identified in up- and down-regulated mRNAs were categorised in to the three controlled vocabularies namely: biological processes (BP), cellular components (CC) and molecular function (MF). Furthermore, KEGG pathway enrichment testing was conducted for the identified DEGs using the KEGGREST package and subsequent rat specific (rno) pathway assignments. Significant pathways were identified using the Wilcoxon rank-sum test, which is the nonparametric analogue of the two-sample t-test.

5.3 Results and Discussion

5.3.1 RNA isolation and library preparation

The isolation of total RNA from male (n = 30) and female (n = 30) cardiac tissue resulted in concentrations ranging from 336 to 1218 and 324 to 1302 ng/µL, respectively. Further assessment of the RNA quality using a bioanalyzer computed average RIN values of 9.2 and 9.6 for RNA isolated from male and female samples. Both male and female samples yielded RNA of sufficient quantity (> 200 ng) and quality (RIN value > 7), with no significant differences observed between samples from different genders or treatment groups. Following the successful completion of library preparation, 15 male and 15 female single stranded (ss), circular DNA libraries with an average yield of 6.6 and 5.7 pmol, respectively, were utilised for paired-end 100 bp (PE100) sequencing on the MGISEQ-2000. All the samples passed in-house quality control measurements throughout the library preparation and sequencing protocols.

5.3.2 Transcriptome sequencing and pre-processing

The raw datasets generated through mRNA sequencing (2 x 100 nts) on the MGISEQ-2000 platform consisted of between 86.31 and 92.64 million read pairs for male libraries while female libraries contained between 75.97 and 82.70 million read pairs. Overall, approximately 30.66 gigabases (GB) of raw data was obtained per sample, suggesting that the pooling strategy employed (i.e., eight per lane) was effective. Initial QC assessment and visualisation with FastQC illustrated bases of a Phred quality score > 20 across the read length (100 bp), over-represented kmers and an unequal proportion of bases at the 5' end of the reads. The latter is a common occurrence with RNA sequencing experiments that utilise random primers during the library preparation protocol, such as the one employed in this study (Hansen et al., 2010). Following pre-processing, an average of 0.30% and 10% of the total reads were removed because of over-represented kmers and rRNA contamination, respectively.

Additional QC metrices displayed in Table 5.1 and 5.2, revealed that all processed samples possessed reads with exceptionally high-quality bases, with approximately 91.40% of sequence bases displaying a quality score above 30 (Figure 5.3). Furthermore, the considerably low amount of read pairs (between 8.96 and 11.32%) lost during pre-processing steps can be ascribed to efficacious library preparations and subsequent sequencing. In the case of DEG analysis, as this study intends to explore, the influence of sufficient biological replicates and read depth (number of reads per samples), outweighs that of genome coverage (ENCODE, 2017; Illumina, 2020). Overall, an average of approximately 59.66 million "cleaned" read pairs per sample were retained post processing, which is in line with majority of published whole transcriptomic experiments and the guidelines stipulated in the ENCODE Bulk RNA-seq Data Standards and Processing Pipeline.

Table 5.1. Read statistics of **male** sample datasets following pre-processing.

Group	Sample	Clean reads	Clean bases	%GC content	Q20%	Q30%
	M1	61 734 249	12 346 849 800	48	98,13	91,85
	M2	64 067 075	12 813 415 000	48	98,09	91,68
STD	M3	57 597 622	11 519 524 400	47	98,00	91,39
	M4	52 313 659	10 462 731 800	48	98,07	91,65
	M5	56 714 398	11 342 879 600	48	97,54	89,68
	M6	63 696 717	12 739 343 400	47	97,31	89,13
	M7	70 993 790	14 198 758 000	47	97,96	91,15
HFHS	M8	66 084 258	13 216 851 600	47	97,93	91,08
	M9	62 306 822	12 461 364 400	47	97,77	90,99
	M10	53 761 537	10 752 307 400	47	97,78	91,11
	M11	61 656 846	12 331 369 200	47	97,82	91,21
	M12	58 607 535	11 721 507 000	47	96,45	89,37
HFHS + Afriplex GRT™	M13	66 246 502	13 249 300 400	48	97,75	90,93
	M14	53 072 740	10 614 548 000	48	97,27	89,55
	M15	61 495 619	12 299 123 800	47	96,79	89,12

Table 5.2. Read statistics of **female** sample datasets following pre-processing.

Group	Sample	Clean reads	Clean Bases	%GC content	Q20%	Q30%
	F1	59 141 630	11 828 326 000	47	98,07	92,12
	F2	57 843 581	11 568 716 200	48	98,00	91,85
STD	F3	58 139 072	11 627 814 400	47	98,26	92,85
	F4	56 085 888	11 217 177 600	47	98,13	92,36
	F5	61 813 339	12 362 667 800	47	98,05	91,99
	F6	55 970 378	11 194 075 600	46	97,36	89,86
	F7	58 521 066	11 704 213 200	47	98,01	91,86
HFHS	F8	57 427 629	11 485 525 800	47	98,21	92,65
	F9	52 614 213	10 522 842 600	47	98,24	92,44
	F10	57 440 705	11 488 141 000	47	98,29	92,64
	F11	53 357 559	10 671 511 800	47	98,28	92,60
	F12	57 062 909	11 412 581 800	47	98,33	92,82
HFHS + Afriplex GRT™	F13	62 704 044	12 540 808 800	47	98,38	92,92
	F14	65 718 748	13 143 749 600	47	97,66	90,57
	F15	65 628 069	13 125 613 800	47	98,26	92,50

Figure 5.3. Per base sequence quality scores obtained from FastQC for a representative sample of **A) male** and **B) female** processed reads. The BoxWhisker plot represents the range of Phred quality score for each base across the read length. **Red line:** median value; **yellow box:** inter-quartile range (25-75%); **upper and lower whiskers:** 10% and 90% points; **blue line:** mean quality. A consistent distribution of quality scores was observed for all male and female sequence samples. The higher the quality score, the more reliable the base call at the specific position.

5.3.3 Alignment and gene expression comparison

Upon completion of alignment of the trimmed and filtered reads to the *Rattus norvegicus* (Rnor6.0) reference using STAR, the resulting alignments was assessed with the *bamstats* command in SAMtools. Overall, an average of 85.41% of the "cleaned" reads mapped uniquely to the reference genome across all the samples, with approximately 9.28 to 15.51% mapping to multiple loci (Table 5.3 and Table 5.4). Furthermore, an average insert size of 253 bp and 261 bp was obtained for male and female libraries, respectively. The assessment of quality metrices of the individual sample alignments revealed no significant differences between or within the different experimental groups for both male and female datasets, therefore allowing for the incorporation of all sample datasets in downstream analyses.

Table 5.3. Summary of mapping statistics obtained following the alignment of processed **male** datasets to the Rattus norvegicus (Rnor6.0) reference genome.

Group	Sample	Mapping rate (%)	Duplication rate (%)	Mismatch rate (%)
	M1	87.28	9.94	0.44
	M2	86.17	10.97	0.45
STD	M3	87.56	9.50	0.47
	M4	87.36	9.80	0.45
	M5	87.04	9.28	0.52
	M6	86.75	10.49	0.52
	M7	85.72	11.28	0.47
HFHS	M8	86.66	10.36	0.48
	M9	86.39	10.50	0.51
	M10	86.37	10.51	0.52
	M11	86.31	10.83	0.53
	M12	82.58	11.68	0.50
HFHS + Afriplex GRT™	M13	82.94	13.65	0.54
	M14	82.60	14.21	0.54
	M15	86.31	10.79	0.53

Table 5.4. Summary of mapping statistics obtained following the alignment of processed **female** datasets to the Rattus norvegicus (Rnor6.0) reference genome.

Group	Sample	Mapping rate (%)	Duplication rate (%)	Mismatch rate (%)
	F1	86.27	10.61	0.49
	F2	80.98	15.51	0.50
STD	F3	83.62	13.38	0.50
	F4	85.73	11.35	0.50
	F5	85.07	11.78	0.50
	F6	83.84	13.23	0.57
	F7	86.56	10.55	0.50
HFHS	F8	86.05	11.21	0.50
	F9	85.11	11.87	0.45
	F10	84.05	13.12	0.46
	F11	84.24	12.73	0.48
	F12	84.39	12.74	0.46
HFHS + Afriplex GRT™	F13	85.67	11.52	0.46
	F14	86.52	10.80	0.49
	F15	86.26	10.95	0.47

Raw count data for all genes was extracted from the alignment outputs and used as a measure of gene expression. To determine the appropriate cut-off criteria to be applied for the removal of lowly expressed genes, the transcripts were filtered in terms of counts per million (CPM) values ranging from 1 to 3 (Figure 5.4). Among the 32 883 transcripts annotated in the *Rattus norvegicus* (Rnor6.0) assembly, 32.09% of the total transcripts were covered at a CPM of ≥ 3 in the male datasets, and 32.08% in the female datasets (Figure 5.4). As no significant differences were observed between the number of transcripts covered in male and female datasets at the different CPM thresholds, the more stringent bottom threshold value of 3 was deemed biologically significant and the genes meeting this criterion were included in DEG analysis. Additionally, as gender is considered a binary characteristic in the present study, genes located on the Y chromosomes were excluded to provide a more global view of gene expression as it relates to the disease state.

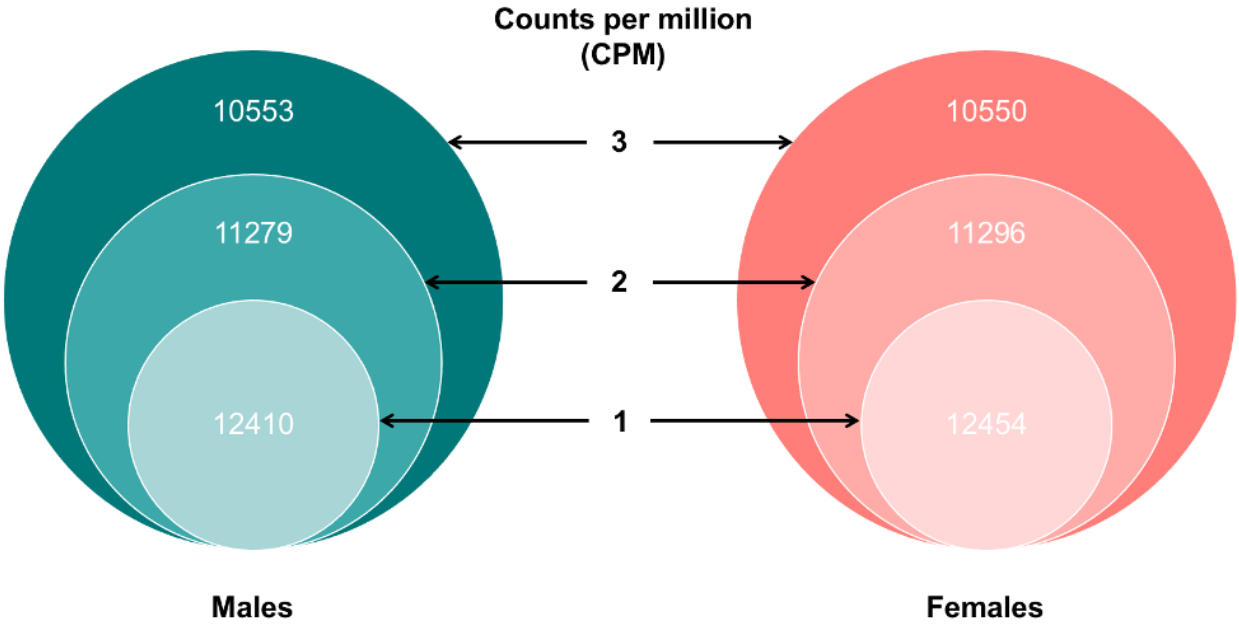

Figure 5.4. Transcript abundance levels covered at a range of 1 to 3 counts per million (CPM) for male and female samples. No significant variation was observed in the number of genes remaining after CPM filtering between male and female samples, irrespective of the cut-off value. Therefore, lowly expressed genes with a normalised read count < 3 CPM were excluded from downstream differential expression analysis.

Normalised read counts (log2CPM) of the filtered datasets were used to identify differences in transcript levels between the HFHS group and the STD control group, as well as the HFHS + Afriplex GRT™ group and the HFHS group. Initial visualisation of the characteristics between biological replicates of the different groups was conducted using the top 1 000 genes with the highest variation (mean-variance as determined by the *voom* function) across male and female samples, independently. Hierarchical clustering revealed distinct expression profiles between the HFHS group and the STD control for both male (Figure 5.5) and female datasets (Figure 5.6), with all biological replicates correctly classified to the respective groups. While previous pre-clinical and clinical studies have been able to demonstrate expression profiles unique to CVD, it is promising to be able to discriminate between the present animals in the absence of an extreme disease phenotype (Civelek et al., 2011; Heidecker et al., 2008; Liao et al., 2019; Patino et al., 2005; Seo et al., 2004). Furthermore, administration of Afriplex GRT™ did not result in a third, distinct expression profile, with biological replicates clustering closely together with those in the HFHS group for both male (Figure 5.5) and female (Figure 5.6) samples.

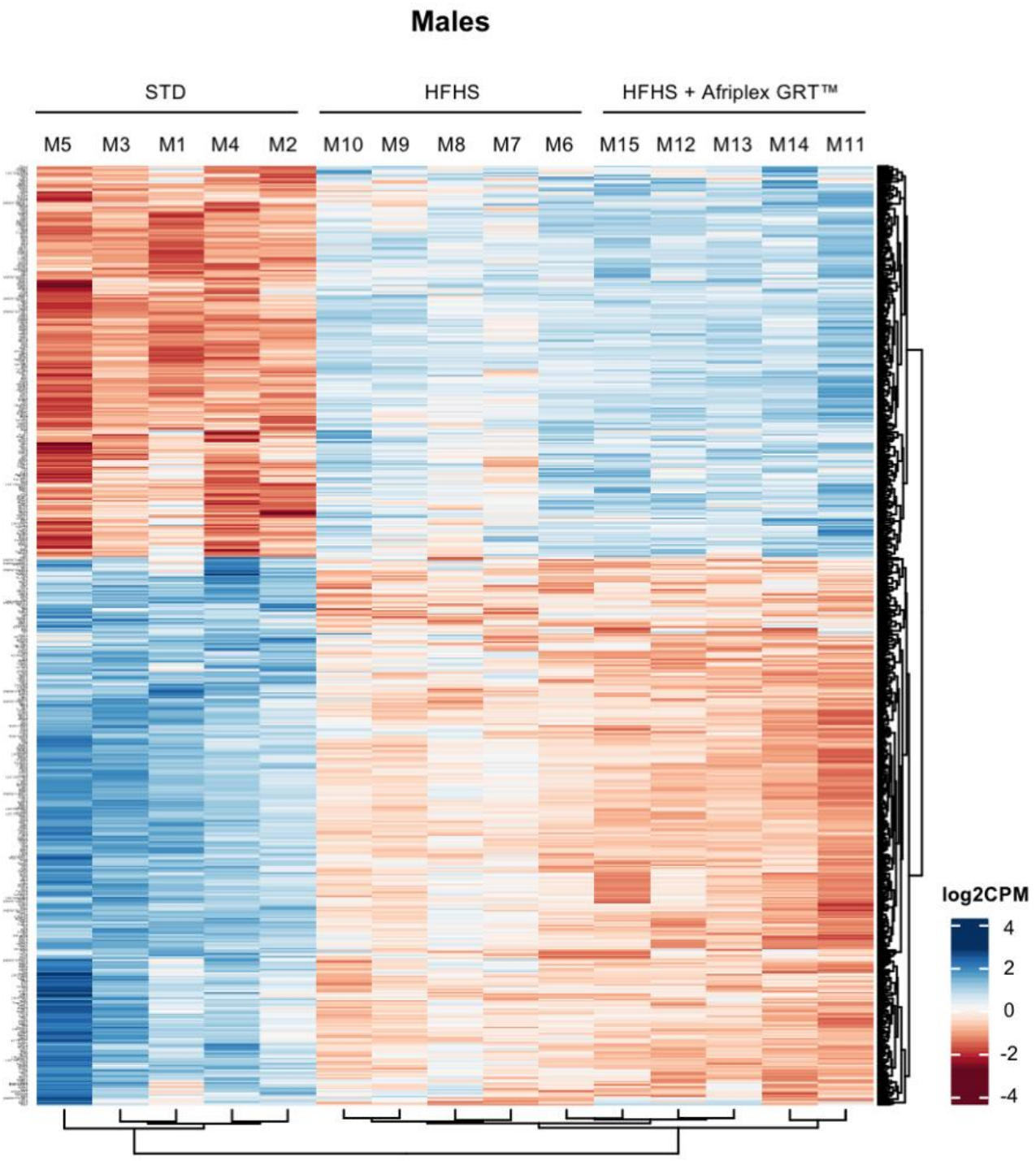

Figure 5.5. Heatmap illustrating normalised expression (log2CPM) of the 1 000 most variable genes across **male** mRNA sequencing datasets. The heatmap was constructed using the commonly applied red and blue colour scheme, where over expression is indicated in varying shades of blue and decreased expression in red. Hierarchical clustering demonstrated that the HFHS and STD biological replicates were categorised into two distinct groups based on mRNA expression. Biological replicates in the HFHS + Afriplex GRT™ group clustered closely with those in the HFHS group.

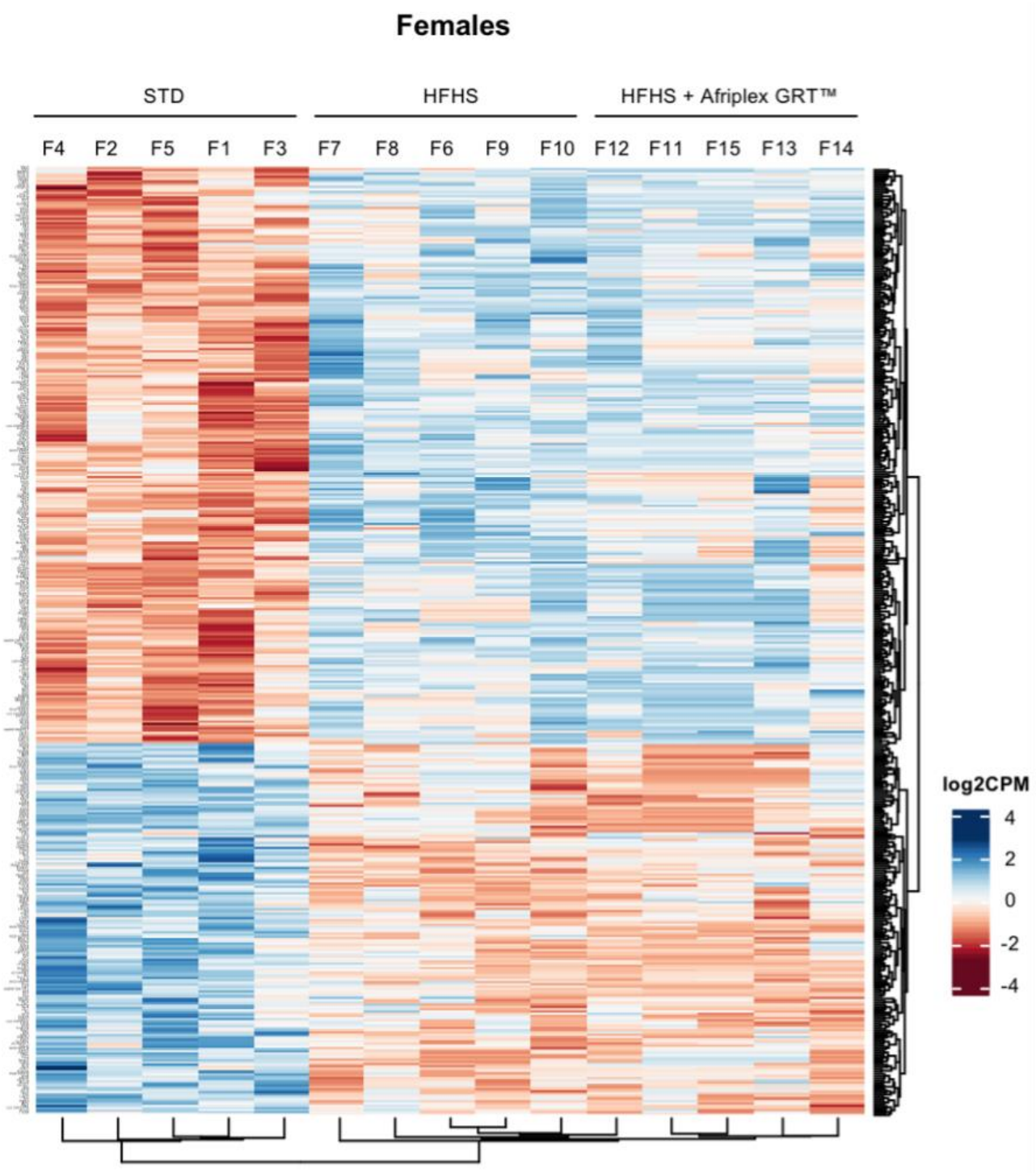

Figure 5.6. Heatmap illustrating normalised expression (log2CPM) of the 1 000 most variable genes across **female** mRNA sequencing datasets. The heatmap was constructed using the commonly applied red and blue colour scheme, where over expression is indicated in varying shades of blue and decreased expression in red. Hierarchical clustering demonstrated that the HFHS and STD biological replicates were categorised into two distinct groups based on mRNA expression. Biological replicates in the HFHS + Afriplex GRT™ group clustered closely with those in the HFHS group.

Employing the limma pipeline, including subsequent contrasts and statistical analyses described previously, a total of 571 genes were differentially expressed (FDR ≤ 0.05) in HFHS diet fed male rats compared to those fed the STD diet. Amongst these, 238 genes were upregulated, and the remaining 333 genes were downregulated in the HFHS group (Supplementary Data – Table S1). Conversely, 414 differentially expressed genes (DEGs) were identified in the HFHS fed female rats, of which 252 genes were over expressed and 162 genes under expressed in the HFHS group (Supplementary Data – Table S2). Evaluation of the DEGs identified in the HFHS group using an in-house developed python script revealed that 35 DEGs were shared amongst male and female animals maintained on the HFHS diet (Figure 5.7). Furthermore, contrast comparisons between the HFHS + Afriplex GRT™ and the HFHS group revealed 120 and 337 DEGs in male and female animals receiving treatment with the Afriplex GRT™ extract, respectively (Supplementary Data – Table S3 and S4). Interestingly, no common DEGs were observed between male and female animals in the HFHS + Afriplex GRT™ group.

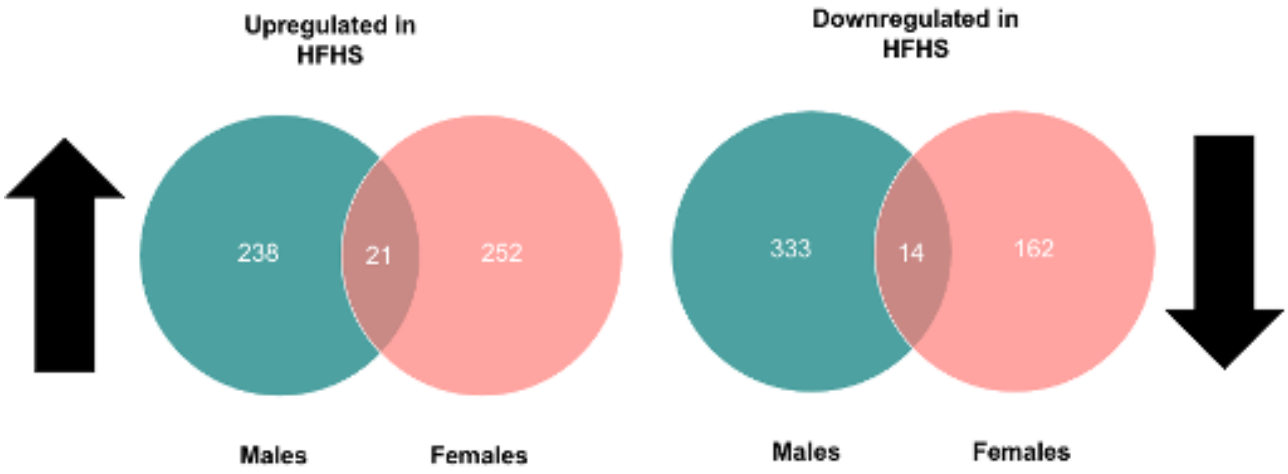

Figure 5.7. Differentially expressed genes (DEGs) in HFHS diet fed animals compared to those fed the STD diet. All DEGs had an adjusted p-value of ≤ 0.05 (with an FDR ≤ 0.05) following the bioinformatical analyses. A total of 35 DEGs were shared between male and female animals of which 21 were over expressed and 14 under expressed.

5.3.4 Functional annotation of DEGs associated with HFHS diet

To gain further insight into the biological function of the identified DEGs, gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed. To this end, up- and down-regulated mRNA obtained from the separate comparisons for both male and female animals were categorised into the three controlled GO vocabularies namely: biological processes (BP), cellular components (CC) and molecular function (MF) using the R/Bioconductor package topGO. Overall, GO terms were assigned to 508 of the 571 DEGs in the HFHS diet fed male Wistar rats. The major GO categories in upregulated mRNA included gene products involved in the regulation of transcription by RNA polymerase II and positive regulation of gene expression (biological processes: GO:0006357; GO:0010628), gene products localised in nuclear chromatin and the membrane raft (cellular component: GO:0000790; GO:0045121), as well as gene products involved in ATP- and transcription regulatory region sequence-specific DNA binding (molecular function: GO:0005524; GO:0000976) (Figure 5.8). On the other hand, the top GO enrichment terms in downregulated mRNA were gene products involved in protein localisation and vesicle mediated transport (biological processes: GO:0045184; GO:0016192), gene products localised in the protein containing complex and the perinuclear region of cytoplasm (cellular component: GO:0032991; GO:0048471), as well as gene products involved in ATP- and kinase binding (molecular functions: GO:0005524; GO:0019900) (Figure 5.9). Aside from the processes required for primary cellular function, GO enrichment analysis revealed the involvement of DEGs in cardiac muscle contraction, cardiac myofibril assembly, response to oxidative stress, inflammatory response and autophagy of the mitochondrion is of particular interest for this study (Figure 5.8 and 5.9). Furthermore, the top KEGG pathways enriched in up- and downregulated mRNAs included HIF-1 signalling pathway, adrenergic signalling in cardiomyocytes, insulin signalling pathway, MAPK signalling pathway, mitophagy, cytokine-cytokine receptor interaction and TGF-beta signalling pathway (Figure 5.10). Taken together these results illustrate the involvement of DEGs associated with a HFHS diet in molecular mechanisms and biological processes known to play a significant role in the development of CVD.

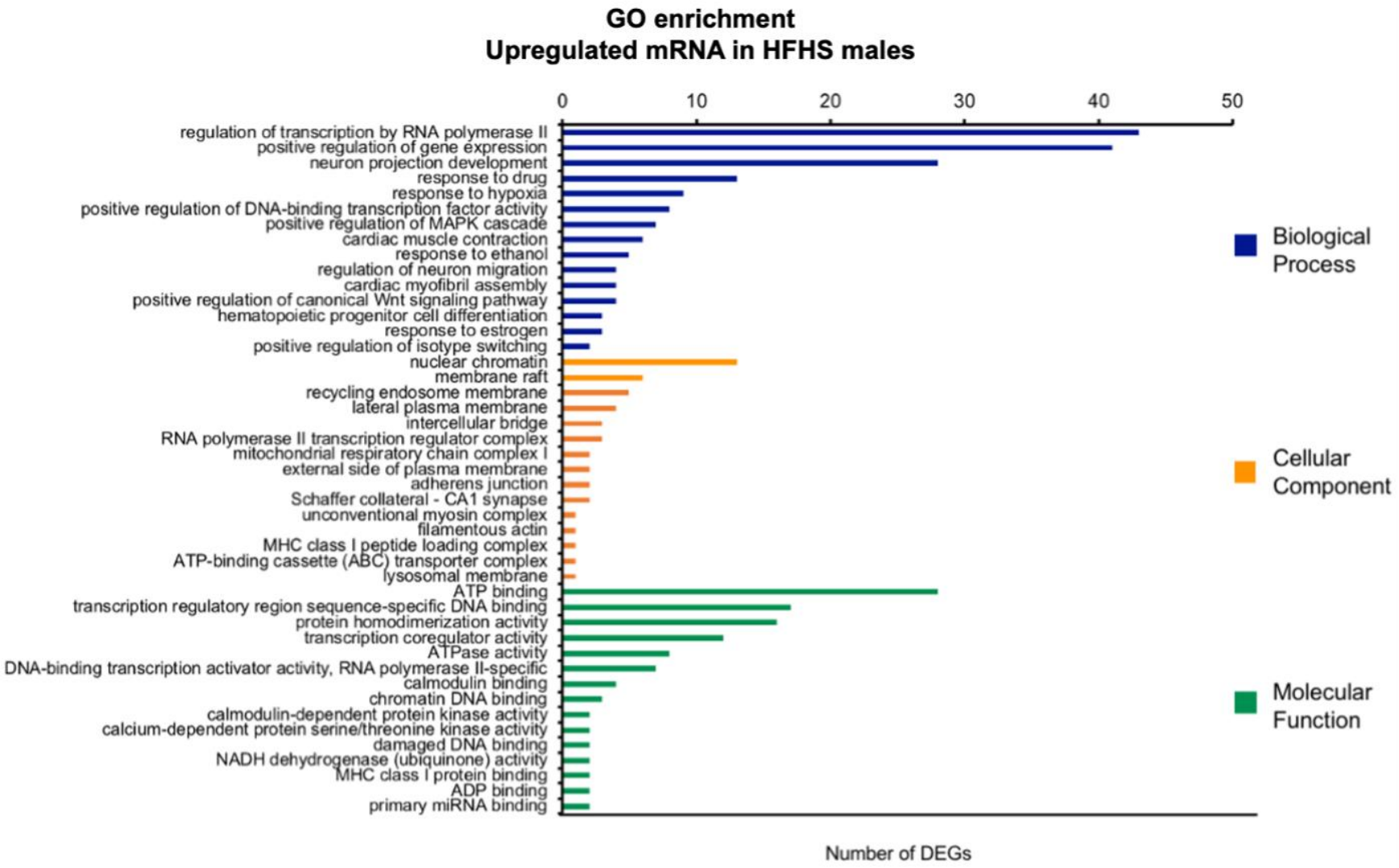

Figure 5.8. Gene ontology (GO) enrichment analysis of **upregulated** differentially expressed genes (DEGs) in high fat, high sugar (HFHS) diet fed **male** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

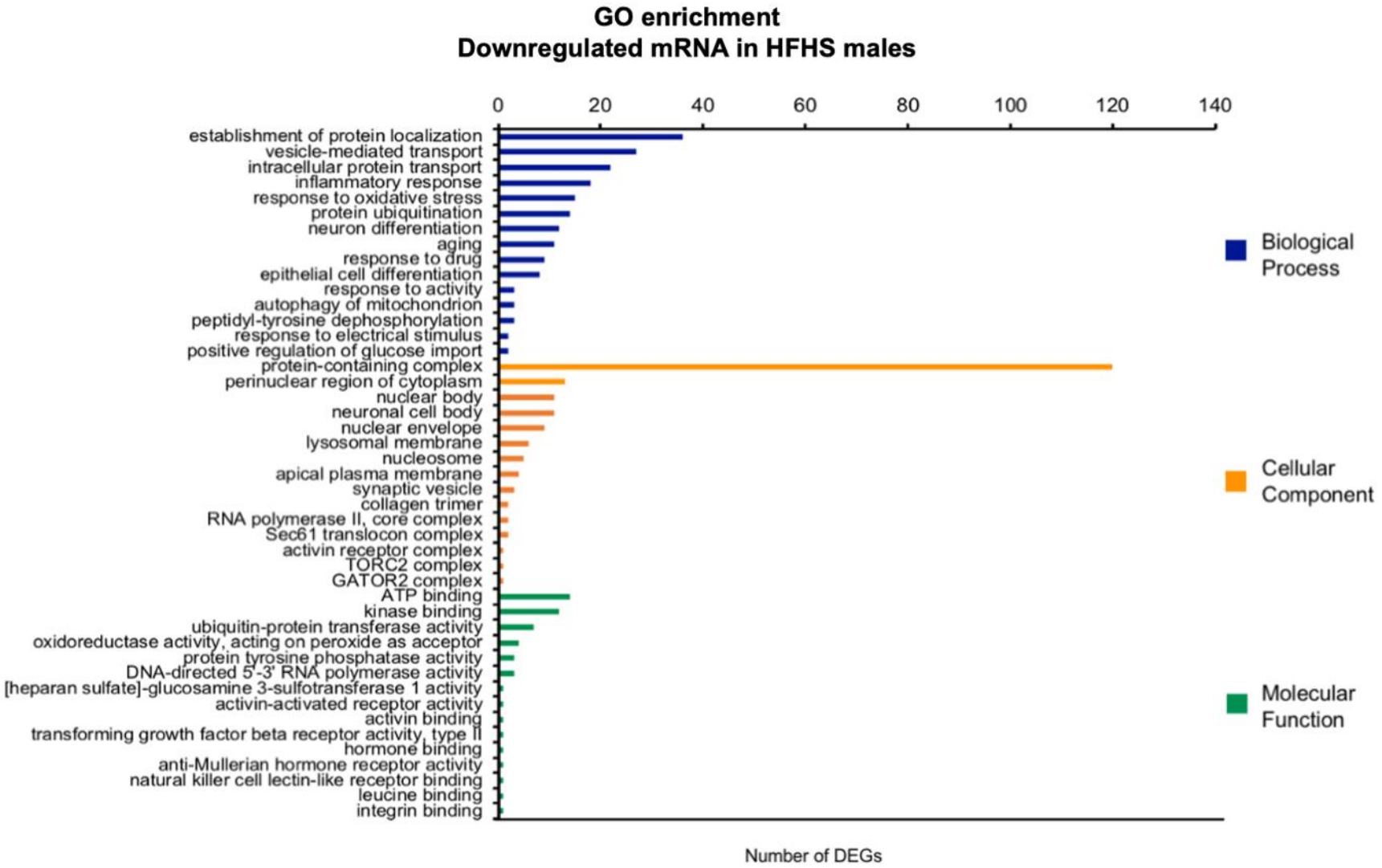

Figure 5.9. Gene ontology (GO) enrichment analysis of **downregulated** differentially expressed genes (DEGs) in high fat, high sugar (HFHS) diet fed **male** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

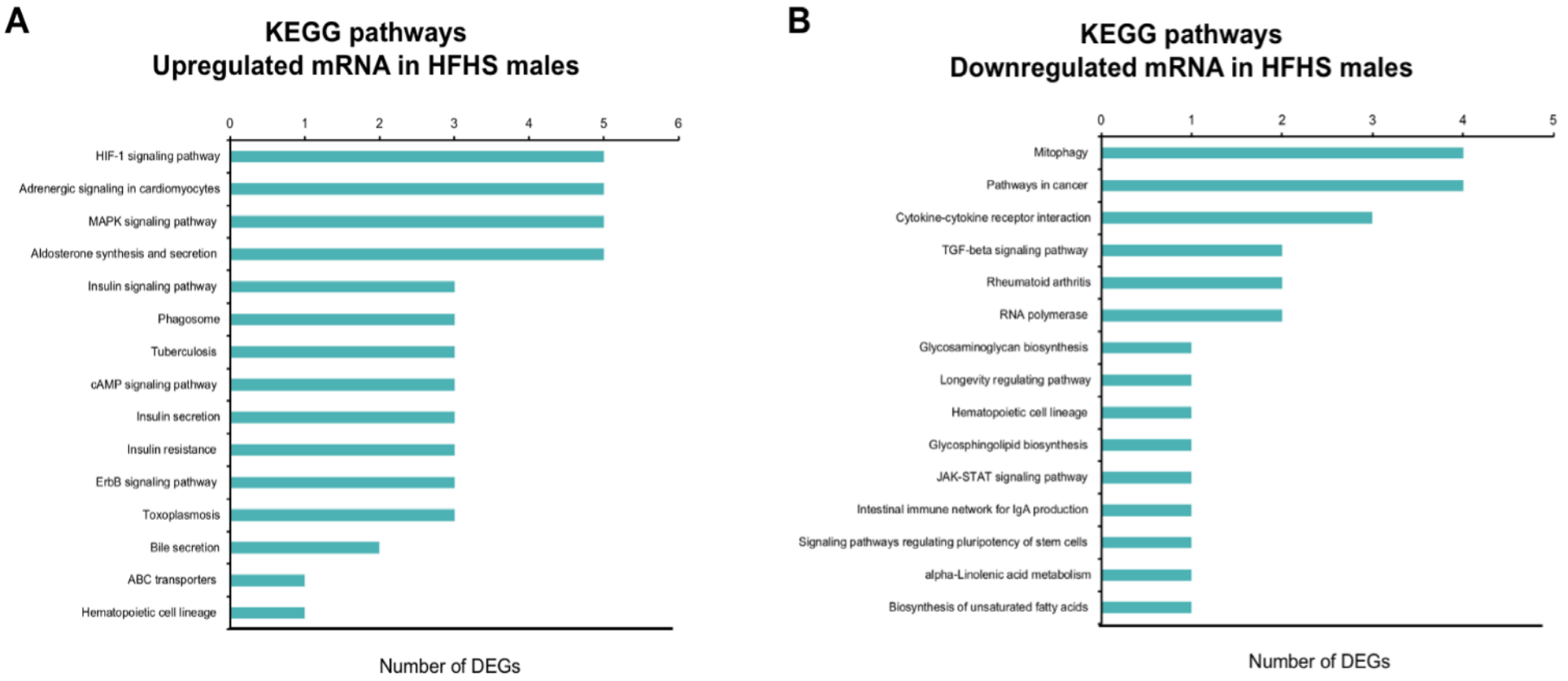

Figure 5.10. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes (DEGs) in high fat, high sugar (HFHS) diet fed **male** Wistar rats. The top KEGG enrichment items of **A) upregulated** and **B) downregulated** DEGs are listed respectively.

Of the 414 DEGs identified in female HFHS fed Wistar rats, 369 were annotated following GO enrichment analysis. The top GO terms assigned to upregulated mRNA included participation in regulation of cell population proliferation and chaperone-mediated protein folding (biological processes: GO:0042127; GO:0061077), gene products that are intrinsic components of the plasma membrane and dendrite (cellular component: GO:0031226; GO:0030425), as well as those involved in GTP binding and GTPase activity (molecular functions: GO:0005525; GO:0003924) (Figure 5.11). Moreover, the leading GO categories in downregulated mRNA included gene products implicated in the positive regulation of transcription, DNA-templated and regulation of cell population proliferation (biological processes: GO:0045893; GO:0042127), genes products that are localised in the nucleoplasm and cell junction (cellular component: GO:0005654; GO:0030054), as well as gene products involved in enzyme- and RNA polymerase II cis-regulatory region sequence-specific DNA binding (molecular functions: GO:0019899; GO:0000978) (Figure 5.12). Of particular interest to the current study, was the involvement of DEGs in response to insulin, response to glucose and Notch signalling, especially in downregulated mRNA (Figure 5.10). Additionally, pathway enrichment analysis revealed the major KEGG pathways involved were the MAPK signalling pathway, B cell receptor signalling pathway, hypertrophic cardiomyopathy, and Adrenergic signalling in cardiomyocytes, exclusively in down-regulated DEGs (Figure 13). Interestingly both MAPK signalling pathway and adrenergic signalling in cardiomyocytes were also enriched in upregulated mRNAs in HFHS males (Figure 5.9). Collectively, these results demonstrate the involvement of DEGs identified in HFHS diet-fed female rats, in the underlying mechanisms known as risk factors for CVD pathophysiology.

In order to potentially elude the possible role of observed transcriptomic interactions and CVD risk or pathophysiology, significant (FDR ≤ 0.05) GO terms were selected and discussed further. Expectedly, majority of the significant GO terms linked to CVD were identified in the HFHS males following FDR adjustment, with no significant disease related ontologies observed in the HFHS female GO analysis (Supplementary Table 2 and 3).

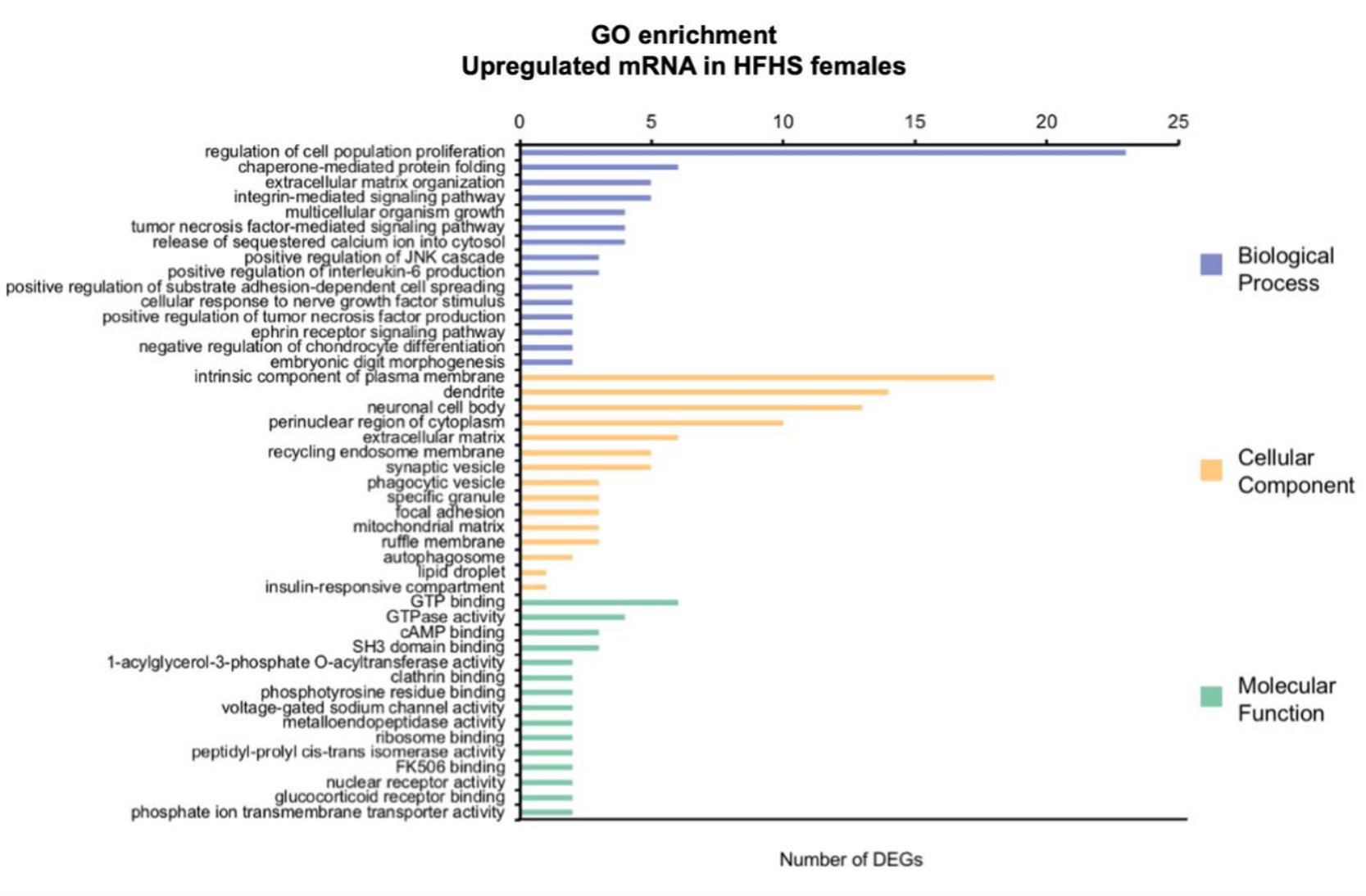

Figure 5.11. Gene ontology (GO) enrichment analysis of **upregulated** differentially expressed genes (DEGs) in high fat, high sugar (HFHS) diet fed **female** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

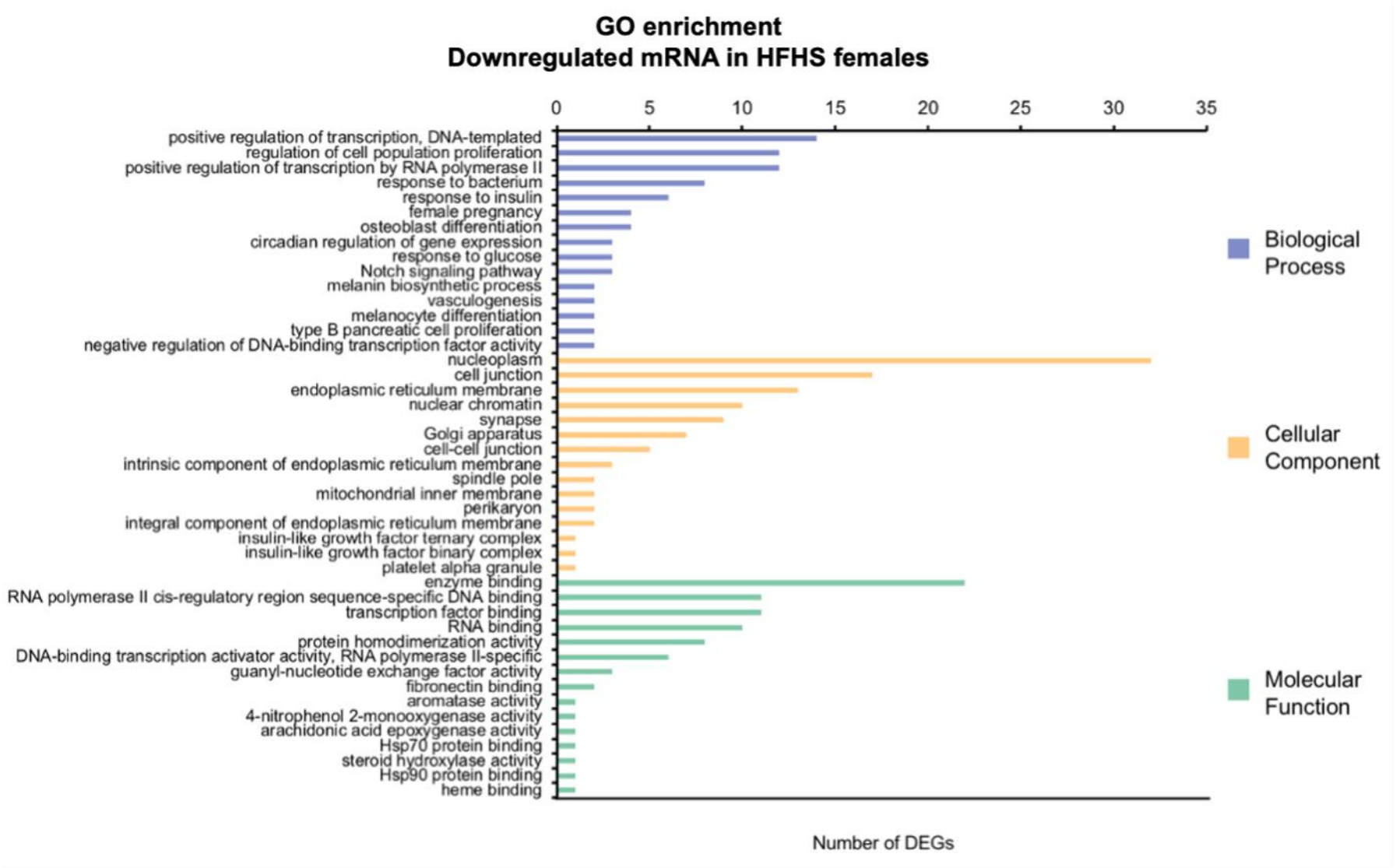

Figure 5.12. Gene ontology (GO) enrichment analysis of **downregulated** differentially expressed genes (DEGs) in high fat, high sugar (HFHS) diet fed **female** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

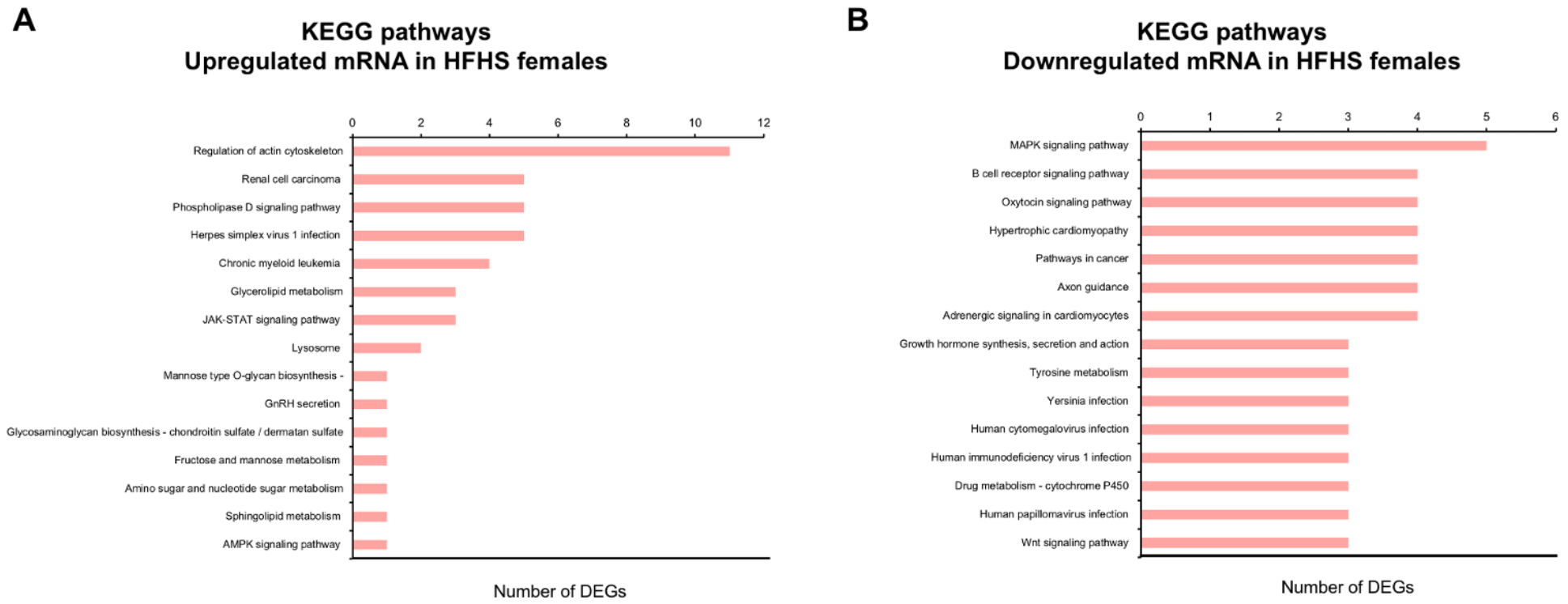

Figure 5.13. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes (DEGs) in high fat, high sugar (HFHS) diet fed **female** Wistar rats. The top KEGG enrichment items of **A) upregulated** and **B) downregulated** DEGs are listed respectively.

5.3.4.1 DEGs involved in cardiac muscle contraction and myofibril assembly

The heart continuously needs to adapt to the body's demands brought on by either physiological or pathological stimuli. These adaptations often include vascular modifications associated with cardiac remodelling such as myofibrillar assembly, hypertrophy, myopathies, and fibrosis (Machackova et al., 2004; Small et al., 2010; Xiong et al., 2019). Although these changes can be acutely beneficial under certain circumstances, they often contribute to altered contractile function over time. In this study, 11 DEGs identified in the HFHS diet fed males were associated with cardiac muscle contraction and myofibril assembly, as is evident from the GO enrichment results (Figure 5.8). In depth analysis revealed that two of the DEGs significantly increased following HFHS diet feeding, encoded transcription factors (TFs) that drive fetal programming in the heart namely GATA-4 and serum response factor (SRF) (Dirkx et al., 2013; Harvey & Leinwand, 2011). The role of GATA TFs in the developing heart cannot be understated, with GATA-4 being an important regulator of hypertrophic cardiac gene transcription (Liang & Molkentin, 2002; Pikkarainen et al., 2004). While overexpression of GATA-4 has been known to increase cardiomyocyte differentiation, inhibition of GATA-4 triggers extensive apoptosis and even embryonic fatality (Crispino et al., 2001; Garg et al., 2003; Konhilas & Leinwand, 2006; Kuo et al., 1997; Latinkic et al., 2003; Pikkarainen et al., 2003).

The second TF, SRF is widely expressed and unlike majority of TFs, has no known paralogs and therefore relies on the use of coactivators to direct cell specific gene expression profiles (Miano et al., 2004; Wang et al., 2004). The importance of SRF is highlighted by its ability to bind the CArG DNA consensus sequence which numerous cardiac muscle genes require for accurate expression (Konhilas & Leinwand, 2006; Reecy et al., 1998; Sprenkle et al., 1995). Studies involving knockout models of the SRF gene have demonstrated that it is not only essential for heart development but also cardiac homeostasis (Dirkx et al., 2013; Parlakian et al., 2004; Parlakian et al., 2015). Furthermore, the cardiac overexpression of SRF, prevents DNA binding and has been linked to several CVDs in response to cytoskeletal stress (Angelini et al., 2015; Xiong et al., 2019; Zhang et al., 2001). As both the abolition and overexpression of SRF have been linked to the pathological development of dilated cardiomyopathy and hypertrophy, the heart is required to maintain sufficient balance of SRF levels during developmental stages and adult life. The activation of SRF is often stimulated by the same signalling cascades which induce GATA-4 activation, allowing the TFs to interact with each other, possibly cooperatively, to bind DNA of interest (Lin et al., 2002; Morimoto et al., 2000; Oh et al., 2004; Sepulveda et al., 2000). Although it is evident that

both GATA-4 and SRF are activated through hypertrophic catalysts induced by high fat diet, they often require the simultaneous activation of cofactors in order to affect the expression of their dependent cardiac genes. One such cofactor capable of conveying signals to the rest of the genome, myocardin (MYOCD), has been shown to be a transcriptional coactivator of SRF and interestingly, MYOCD expression is also regulated bi-directionally through GATA-4 (Figure 5.14) (Aravind & Koonin, 2000; Chen et al., 2002; Oh et al., 2004; Wang et al., 2001). It can therefore be hypothesised that MYOCD causes the recurrence of fetal cardiac genes under regulation of GATA-4 and SRF in the pathological heart. However, since the role of MYOCD in the adult heart has not been fully elucidated, the importance of alternative regulatory mechanisms such as posttranslational modifications of the previously mentioned TFs and cofactor become apparent in target cardiac gene activation (Li et al., 2003). Furthermore, the upregulation of GATA-4 and SRF in the absence of a severe disease phenotype and structural alterations in the heart, as shown in this study, supports the asymptomatic nature of CVD onset.

Amongst the many SRF target genes, α-cardiac actin (ACTC1), the most abundant isoform in the cytoskeleton of mature cardiomyocytes, was also significantly increased in the HFHS diet fed males in the current study (Angelini et al., 2020; Boutilier et al., 2017; Ilkovski et al., 2005; Suurmeijer et al., 2003). Studies involving knockout mouse models revealed that disruption of SRF results in drastically decreased expression of ACTC1, ultimately leading to severe heart defects (Parlakian et al., 2005; Touvron et al., 2012). Moreover, ACTC1 loss of function mutations have been associated with dilated cardiomyopathy and reduced protein levels have also been detected during heart failure (Daehmlow et al., 2002; Dirkx et al., 2013; Mundia et al., 2012). Although diminished ACTC1 plays a key role during cardiomyogenesis, studies have shown that overexpression of ACTC1 mRNA can be induced following pathology, especially during regeneration of myofibers (Angelini et al., 2020; Jiang et al., 2010). Of particular interest to this study is the existing evidence supporting modulation of ACTC1 regulatory mechanisms through diet, as well as the correlation of variable ACTC1 expression with promoter methylation (Boutilier et al., 2017; Wang et al., 2016). Taken together the current study supports the conception of HFHS diet acting as a pathological stimulus with the ability to alter the expression of cardiac genes such as ACTC1 through the combinatory action of TFs (SRF and GATA-4) and coactivators (such as MYOCD), ultimately affecting hypertrophic related functional pathways. Correlation of the expression of the DEGs involved in muscle contraction and myofibril assembly,

especially ACTC1, and differential DNA methylation in Chapter 6 will therefore aid further insight into the underlying mechanisms involved.

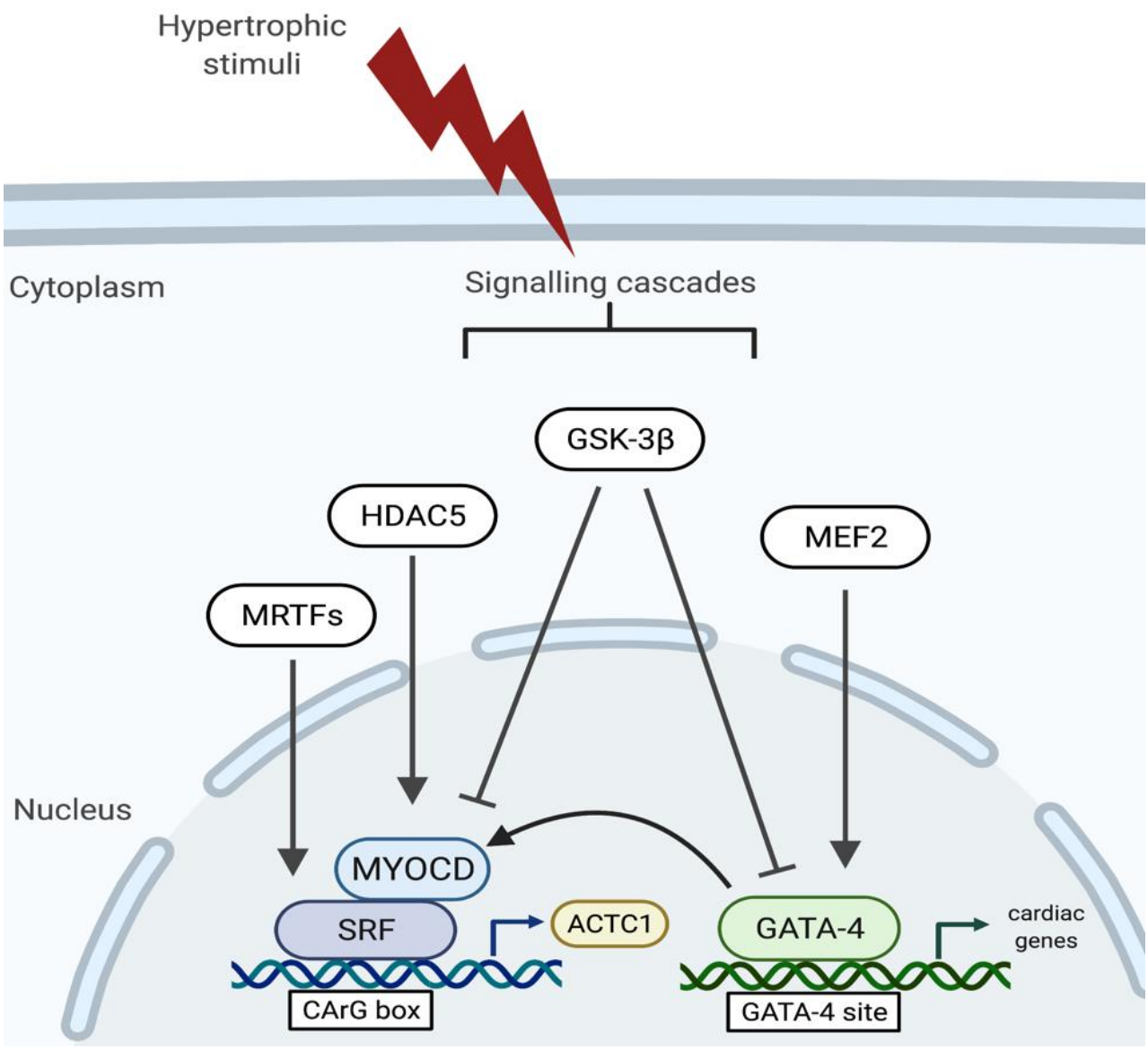

Figure 5.14. Schematic diagram of GATA-4- and serum response factor (SRF) dependent cardiac gene regulation adapted from Konhilas & Leinwand, 2006. A signalling cascade that reacts on transcriptional regulators of GATA-4 and SRF, is activated through hypertrophic stimuli in the heart. **GSK-3β,** glycogen synthase kinase 3β; **HDAC5**, histone deacetylase 5; **MRTF**, myocardin-related transcription factor; **MEF2**, myocyte enhancer factor 2; **ACTC1**, α-cardiac actin.

5.3.4.2 DEGs involved in response to oxidative stress

One of the most widely studied pathological mechanisms underlying the development of CVDs is increased oxidative stress. Oxidative stress associated with type 2 diabetes mellitus (T2DM) plays a significant role in CVD disease pathogenesis (Guldiken et al, 2009; Lapolla et al., 2007; Senoner & Dichtl, 2019; Van Campenhout et al., 2006). While basal levels of reactive oxygen species (ROS) are undoubtedly necessary for cellular function, excessive amounts have damaging effects on multiple cellular components and may eventually lead to mitochondrial dysfunction (Finkel, 2011; Holmström et al., 2014; Padmalayam, 2012). Several studies have linked abnormal ROS production to the induction of hypertrophic signalling and pro-inflammatory responses, consequently augmenting cardiac remodelling and myocardial apoptosis (Dludla et al., 2014; Ingwall, 2009; Kim & Kang, 2010; Soliman et al., 2008; Tahhan et al., 2017). These ROS mediated mechanisms have particularly been highlighted in the instance of diet induced metabolic syndrome due to the chronic hypoglycaemia and altered lipid content conditions (Boudina & Abel, 2010; Dludla et al., 2014; Ingwall, 2009; Joseph et al., 2014; Kattoor et al., 2017; Kuroda et al., 2010; Mapanga & Essop; 2016; Senoner & Dichtl, 2019; Tahhan et al., 2017; Tarquini et al., 2011; Zhou et al., 2018). Additionally, ROS have also been associated with the promotion of atherosclerotic plaque formation, hypertension, and heart failure (Baradaran et al., 2014; Kattoor et al., 2017; Lapolla et al., 2007; Tsutsui et al., 2011).

Nonetheless, the body is equipped with an endogenous antioxidant defence network, comprised of both enzymatic and non-enzymatic compounds with the potential to circumvent free radical damage (Balaban et al., 2005; Senoner & Dichtl, 2019). However, these protective measures are known to become deficient following chronic exposure to pathological stimuli such as hyperglycaemia (Bhatt et al., 2013; Marnewick et al., 2011; Padmalayam, 2012). In the present study, genes involved in the response to oxidative stress were differentially expressed in males following HFHS diet feeding for 9 months (Figure 5.9). A total of 15 genes associated with intracellular antioxidant activity and ROS neutralisation were significantly down regulated including *LIAS*, *PCNA*, *NQO1*, *PRDX1*, *OGG1*, *GPX1*, *SELENOS* and *RPS3* which may have led to an increase in ROS levels and subsequent oxidative stress.

Amongst the significantly decreased DEGs was lipoic acid synthase (*LIAS*), a gene responsible for the production of α-lipoic acid (LA) in the mitochondria (Parker et al., 1995; Xu et al., 2016). This potent antioxidant plays a central role in the body's antioxidant defence

network through its ability to scavenge several ROS and the regeneration of various other antioxidants including vitamin E, coenzyme Q10 and glutathione (Biewenga et al., 1997; Konstantinidou et al., 2013; Shay & Hagen, 2009). Furthermore, LA also serves as an essential cofactor for the α-ketoglutarate dehydrogenase complex, suggesting its potential involvement in mitochondrial energy production (Padmalayam, 2012; Reed, 1998). Due to its potent antioxidant capabilities, the efficacy of externally administered LA to improve oxidative stress in diabetes and its related complications has gained considerable interest in several animal studies and clinical trials (Koh et al., 2008; Parker & Cadenas, 2011; Salehi et al., 2019; Shay et al., 2009; Shindyapina et al., 2017; Ziegler, 2009). The reduced expression levels of LIAS observed in the current study could imply an overall reduction in the antioxidant defence network following HFHS feeding.

Furthermore, a significant reduction in the expression of genes encoding enzymes involved in the dissimilation of ROS including quinone reductase (NQO1), glutathione peroxidase (GPX1) and peroxiredoxin (PRDX1), was observed in males receiving the HFHS diet. NAD(P)H:quinone oxidoreductase (NQO1), one of the major mammalian two-electron reductases, is known to be induced in response to oxidative stress (Mondal et al., 2018; Ross & Siegel, 2017). Besides for its role in quinone detoxification, NQO1 has also been implicated in the generation of antioxidants (such as vitamin E), as well as the modulation of metabolic disorder through interacting with *SERPIN1A1* mRNA which encodes a serine protease inhibitor α-1-antitrypsin (Di Francesco et al., 2016; Hyun et al., 2006; Siegel et al., 1997; Siegel et al., 2004). Accordingly, GPX1 and PRDX1 exert their antioxidant capabilities by preventing the accumulation of hydrogen peroxide within cells through its reduction to water, under numerous physiological conditions (Antunes et al., 2002; Huang et al., 2017; Liebthal et al., 2018; Lubos et al., 2011; Rhee et al., 2018). While PRDX1 is more abundantly expressed than GPX1 and has been known to attenuate cardiovascular events through oxidative stress and apoptosis; certain isoforms are subject to inactivation at low levels of hydrogen peroxide (Forman et al., 2010; Jiang et al., 2020; Jing et al., 2011; Martinez-Pinna et al., 2011).

Despite the ongoing debate regarding the effectiveness of *GPX1* versus *PRDX1* to modulate intracellular peroxides, a reduction in the expression of both genes, as seen in this study, further supports the cumulative decrease in antioxidant activity associated with HFHS feeding. Interestingly, the master transcriptional regulator of antioxidant genes, nuclear factor erythroid-2-related factor 2 (*NRF2*) was also decreased in the HFHS diet-fed males, however this reduction in expression was not significant ($p = 0.31$) when compared to the

corresponding control group. Substantial evidence exists supporting the role of NRF2 in mediating numerous components of the body's antioxidant response including the expression of NQO1 and GPX1, both identified to be significantly reduced following HFHS feeding in the present study. The exclusion of *NRF2* from the DEGs identified in the present study, as well as the inclusion of alternative DEGs not under the regulation of NRF2, suggests the potential involvement of alternative mechanisms resulting in an ineffective antioxidant defence network following HFHS feeding.

5.3.4.3 DEGs involved in inflammatory response

The role of inflammation as a first line defence mechanism in the body has been well established and is integral in the detection and removal of detrimental stimuli (Fernández & Lamkanfi, 2015; Strauss et al., 2018). To this end multiple metabolic risk factors including age, obesity, and smoking, have been linked to the onset of inflammation through the assembly of inflammasome complexes (Sastre et al., 2011; Yu & Du, 2017). Furthermore, several studies have described the inflammatory response as a significant underlying mechanism contributing to the development of T2DM and its associated cardiovascular complications (Frangogiannis, 2012; Mann, 2015; Montecucco et al., 2017; Ridker & Luscher, 2014; Yu & Du, 2017). The chronic hyperglycaemic conditions associated with T2DM, activate pro-inflammatory transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which subsequently increases pro-inflammatory gene expression, ultimately accelerating cardiac fibrosis and apoptosis (Castello et al., 2010; Esposito et al., 2002; Lorenzo et al., 2011; Miguel-Carrasco et al., 2010). The latter is further exacerbated by an increase in the release of inflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNF-α) and monocyte chemotactic protein 1 (MCP1), respectively (Goyal et al., 2008; Inoue et al., 2008; King, 2008; Melter et al., 2001).

In the current study, 18 DEGs associated with inflammatory response were significantly under expressed in HFHS diet fed male Wistar rats, as evident from the GO enrichment results (Figure 5.9). Further analysis revealed the inclusion of mRNA encoding the inflammatory caspase, cysteine-dependent aspartate-specific protease 4 (CASP4), as well as the chemokine c-c motif ligand 24 (CCL24). Along with several other inflammatory caspases, CASP4 has been demonstrated to play a key role in inflammatory signalling responsible for the release of extracellular pro-inflammatory cytokines and activation of apoptotic pathways (Bian et al., 2009; Fernández & Lamkanfi, 2015; Hitomi et al., 2004;

McIlwain et al., 2013; Yin et al., 2008). While little is known regarding decreased expression of CASP4 and CVD, as observed in the present study, reduction in CASP4 expression has been linked to poor prognosis in cancer (Papoff et al., 2018; Shibamoto et al., 2017). The chemokine CCL24 on the other hand, is a member of a group of signalling proteins responsible for the induction of migration and activation of immune cells during inflammation (Bacon et al., 2002; Griffith et al., 2014; Mor et al., 2019; Sahin & Wasmuth, 2013). Through acting on the CCR3 receptor, increased expression of CCL24 has been shown to be involved in pro-inflammatory processes including fibrosis (Gaspar et al., 2013; Heiman et al., 2005; Kohan et al., 2010). Due to the important role of CCL24 in fibroblast activation and the migration of inflammatory cells to the inflamed area, the potential exists that a decrease in the expression of mRNA encoding CCL24, as observed in this study, could lead to alterations in the overall host immune response to pathological stimuli (Mor et al., 2019).

The previously mentioned DEGs related to antioxidant activity including *LIAS*, *GPX1* and *SELENOS* were also linked to the inflammatory response enrichment terms. The inevitable link between ROS and inflammation cannot be understated, with a decline in antioxidant defence extensively implicated in the pathogenesis of vascular inflammation, ultimately leading to cardiac dysfunction (Kamata et al., 2005; Kim et al., 2007; Sharma et al., 2016). To this end, selenoproteins such as glutathione peroxidase 1 (GPX1) and selenoprotein S (SELENOS) have been recognised as key components involved in inflammatory regulation, immunity, oxidative stress, and endoplasmic reticulum stress (Christensen et al., 2012; Fradejas et al., 2011; Gao et al., 2004; Yu & Du et al., 2017). These pathophysiological reactions usually have reciprocal causations, mutually promote one another, and participate in the occurrence and development of T2DM and cardiovascular complications (Chistiakov et al., 2014; Donath & Shoelson, 2011; Forbes & Cooper, 2013; Rani et al., 2016). Furthermore, a study conducted by Sharma et al. (2016) demonstrated that the lack of GPX1 prolonged correlated with a reduction in nitric oxide (NO) bioavailability and augmented NF-κB activity, further supporting the critical role of GPX1 in regulating pro-inflammatory pathways. Therefore, the decline in antioxidant defences observed in the present study, as evident by the decreased expression of *LIAS*, *GPX1* and *SELENOS* mRNA, could suggest the activation of pro-inflammatory pathways such as MAPK and NF-κB, ultimately facilitating the development of a cardiac insult.

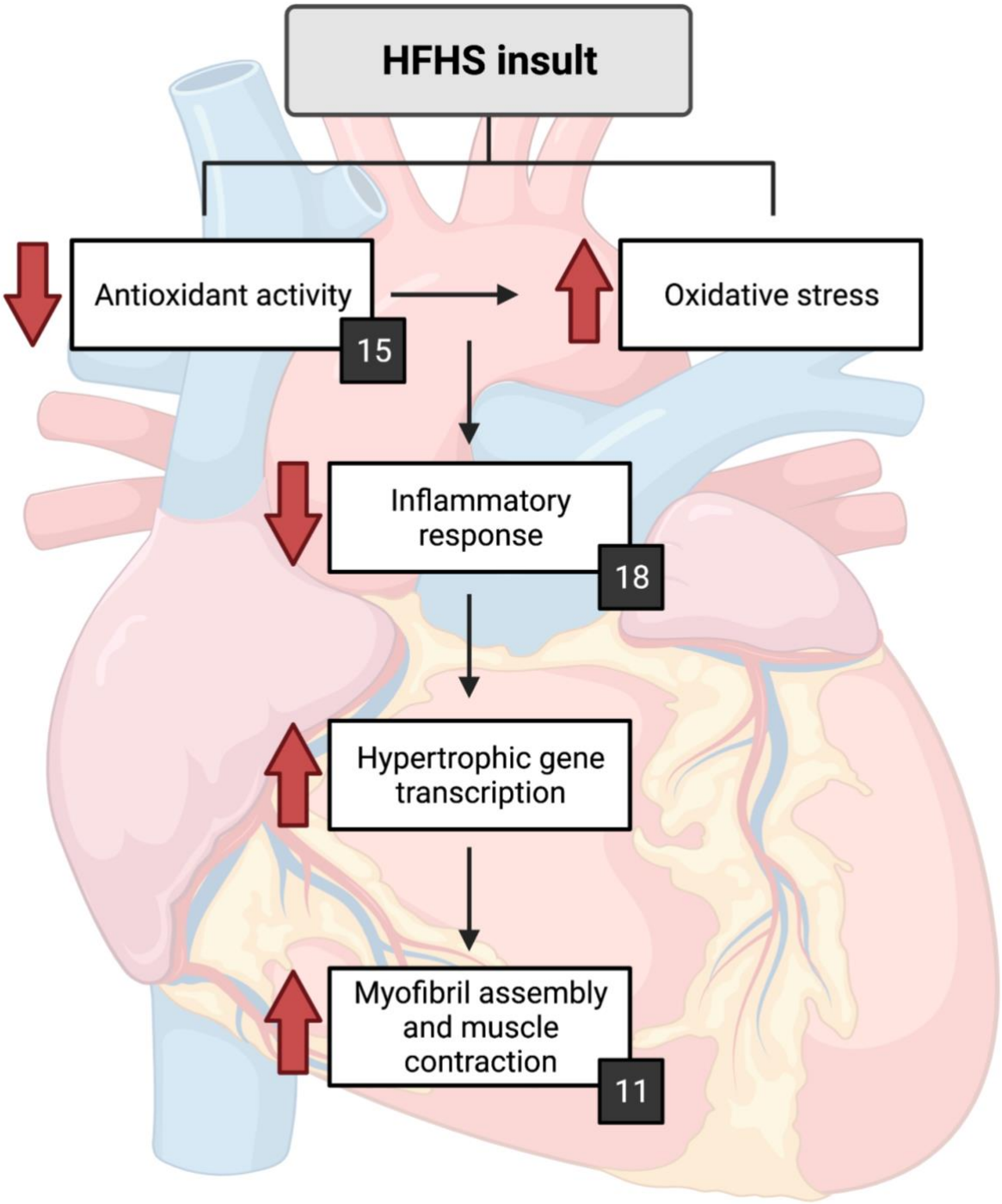

Figure 5.15. Summary of major functional enrichments involved in disease pathophysiology in HFHS diet fed male Wistar rats. Pathways labelled with a red arrow represents functional enrichments where an up-arrow indicates overexpression and a down-arrow decreased expression. The number of DEGs associated with the enriched terms are indicated in the dark grey boxes. The HFHS diet was capable of transcriptionally decreasing antioxidant activity, which could alter ROS neutralisation capabilities and increase oxidative stress, ultimately influencing host inflammatory responses. This was further accompanied by an increase hypertrophic gene expression, possibly affecting cardiac muscle functionality. Schematic constructed with Biorender.com (2021).

5.3.5 Functional annotation of DEGs associated with Afriplex GRT™ treatment

Differentially expressed mRNAs obtained from the separate comparisons for both male and female animals receiving the HFHS diet + Afriplex GRT™ treatment were subjected to GO and KEGG pathway enrichment analysis. Considering this, up- and downregulated DEGs were categorised into the three previously mentioned GO vocabularies namely: biological processes (BP), cellular components (CC) and molecular function (MF) using the R/Bioconductor package topGO. Of the 120 DEGs identified in male HFHS diet + Afriplex GRT™ Wistar rats, 101 were annotated following GO enrichment analysis. The top GO terms assigned to upregulated mRNA included participation in the regulation of DNA-templated transcription and brain development (biological processes: GO:0006355; GO:0007420), gene products that are intrinsic components of the nucleus and external side of the plasma membrane (cellular component: GO:0005634; GO:0009897), as well as those involved in DNA- and cytoskeletal protein binding (molecular functions: GO:1990837; GO:0008092) (Figure 5.15). Moreover, the leading GO categories in downregulated mRNA included gene products implicated in cell migration and the positive regulation of DNA-binding transcription factor activity (biological processes: GO:0016477; GO:0051091), genes products that are located in the mitochondrion and the apical plasma membrane (cellular component: GO:0005739; GO:0016324), as well as gene products involved in RNA- and lipid binding (molecular functions: GO:0003723; GO:0008289) (Figure 5.16).

GO terms were assigned to 296 of the 337 DEGs in the HFHS diet + Afriplex GRT™ female Wistar rats. The major GO categories in upregulated mRNA, included gene products involved in the regulation of cell differentiation and adhesion (biological processes: GO:0030154; GO:0007155), genes confined to the cytosol and extracellular space (cellular component: GO:0005829; GO:0005615), as well as those involved in cell adhesion molecule binding and RNA polymerase II-specific, DNA-binding transcription factor activity (molecular function: GO:0050839; GO:0000981) (Figure 5.15). On the other hand, the top GO enrichment terms in downregulated mRNA were gene products associated with female pregnancy and ribosomal RNA generation (biological processes: GO:0007565; GO:0000467), gene products situated in the mitochondrion and cytoplasmic vesicle (cellular component: GO:0005739; GO:0031410), as well as gene products involved in signalling receptor- and ATP binding (molecular functions: GO:0005102; GO:0005524) (Figure 5.16).

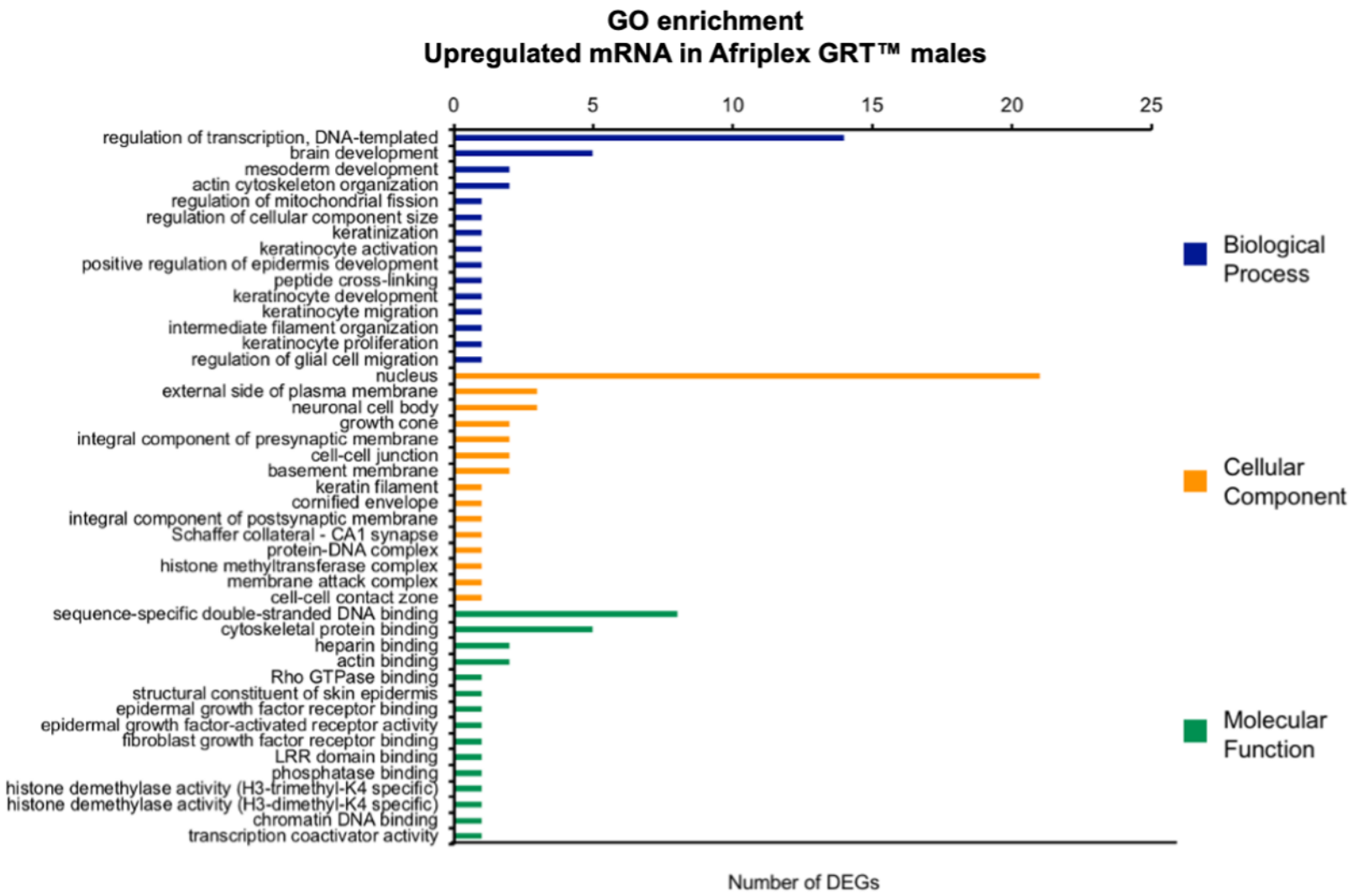

Figure 5.16. Gene ontology (GO) enrichment analysis of **upregulated** differentially expressed genes (DEGs) in HFHS + Afriplex GRT™ **male** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

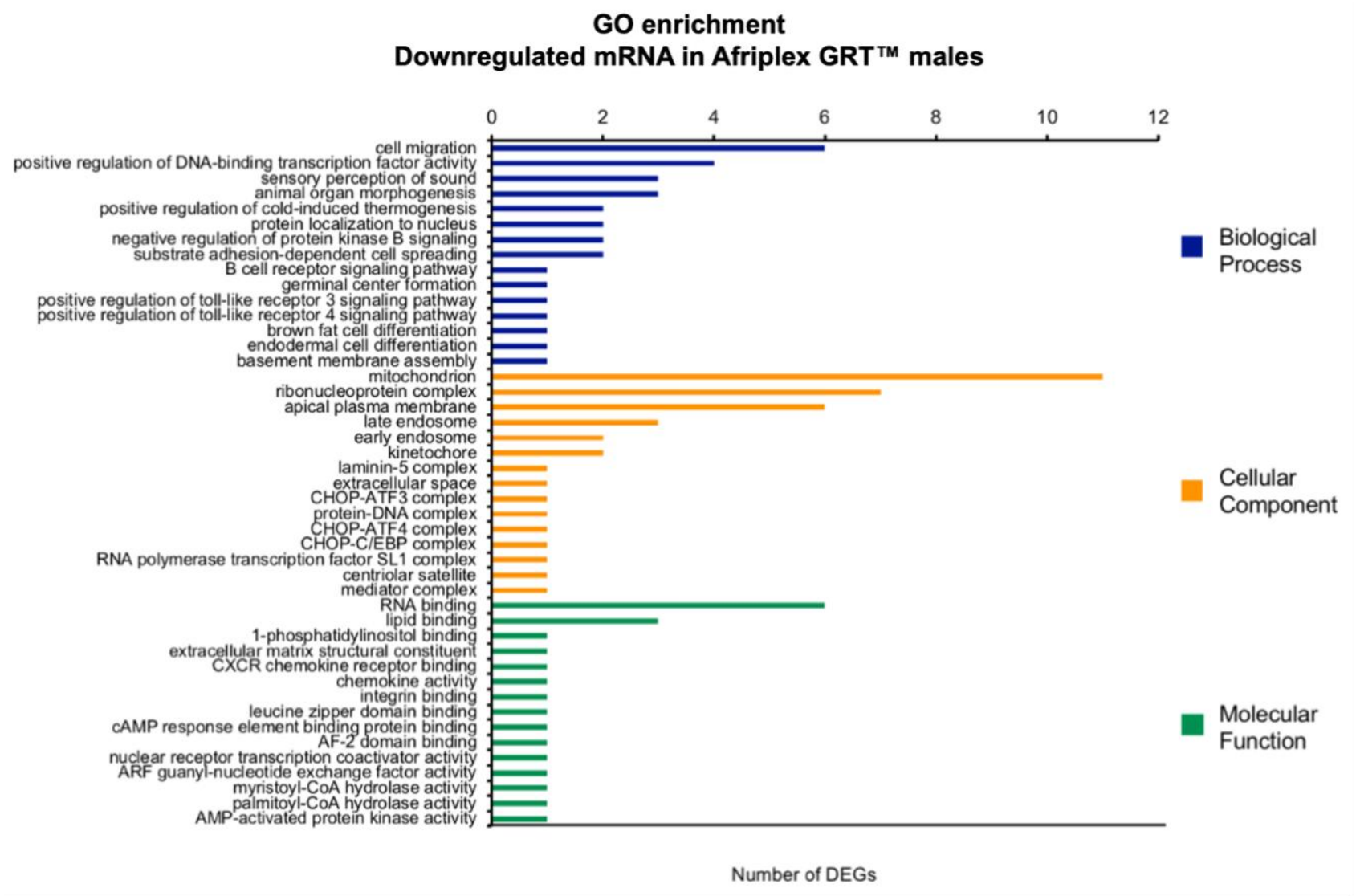

Figure 5.17. Gene ontology (GO) enrichment analysis of **downregulated** differentially expressed genes (DEGs) in HFHS + Afriplex GRT™ **male** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

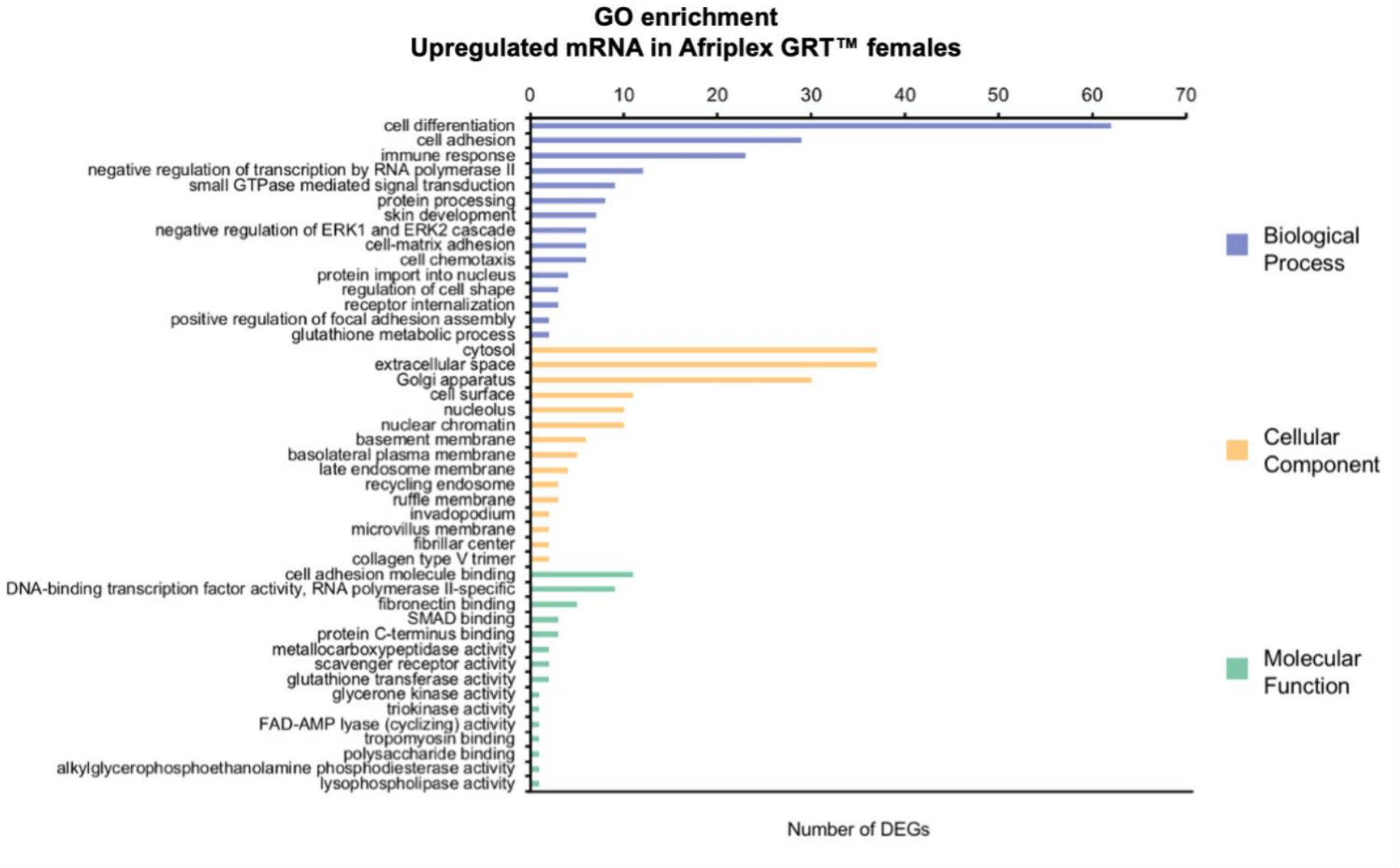

Figure 5.18. Gene ontology (GO) enrichment analysis of **upregulated** differentially expressed genes (DEGs) in HFHS + Afriplex GRT™ **female** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

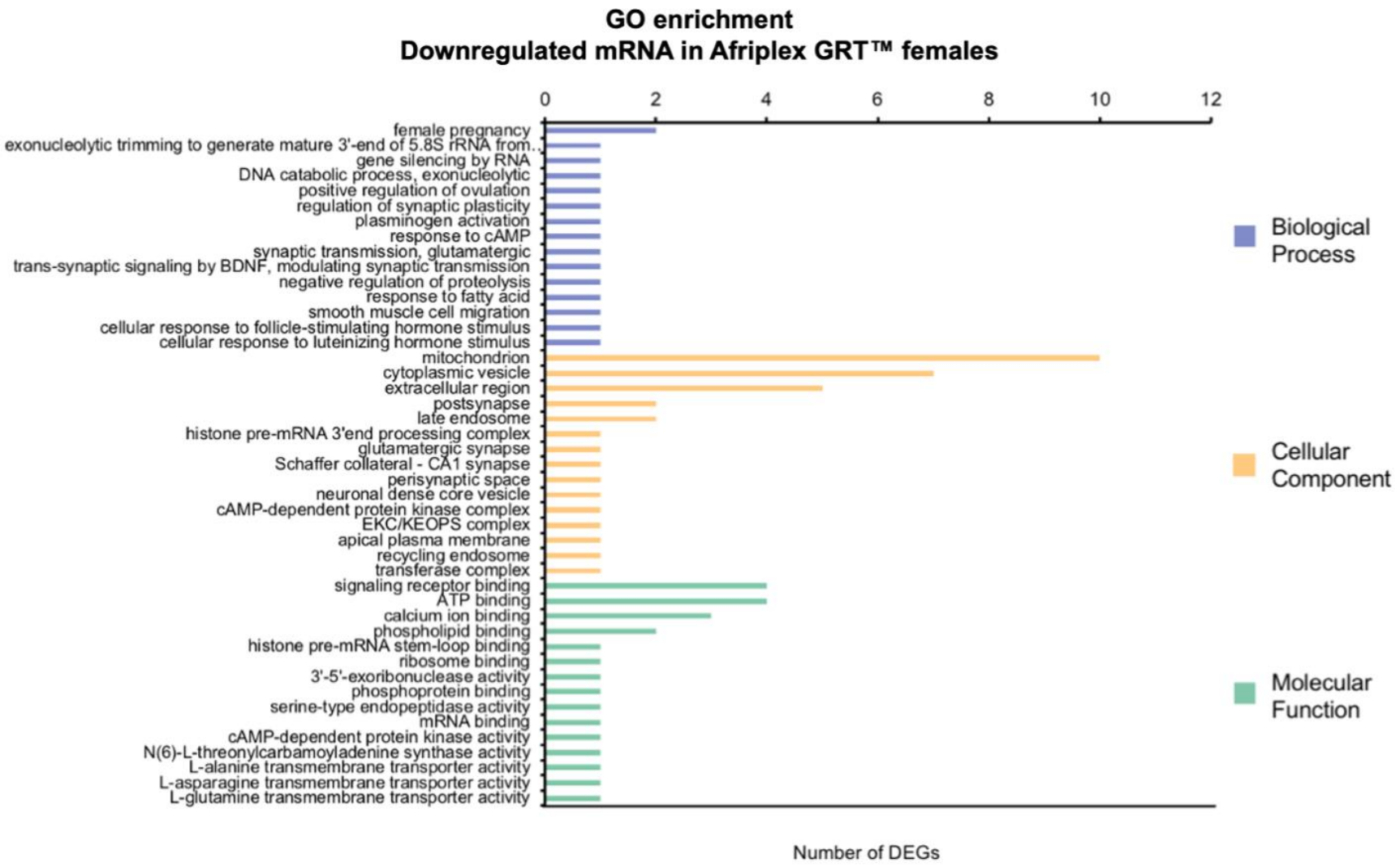

Figure 5.19. Gene ontology (GO) enrichment analysis of **downregulated** differentially expressed genes (DEGs) in HFHS + Afriplex GRT™ **female** Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

Further analysis of all GO enrichments revealed that majority of the DEGs associated with Afriplex GRT™ treatment were involved in processes required for intrinsic cellular and molecular function. Due to the lack of significantly enriched, disease related ontologies observed in males and females receiving Afriplex GRT™ treatment (Figure 5.15 to 5.18), further in-depth discussion of the ontologies identified was not of interest to this study. Additionally, pathway analysis revealed that while only one KEGG pathway, cell adhesion molecules (rno04514) was significantly enriched for in upregulated mRNAs ($p = 0.04$) of males, no significant KEGG pathways were enriched in the female data set (data not shown). Although HFHS diet + Afriplex GRT™ animals appeared to cluster closely together to those maintained on the HFHS diet in terms of normalised read counts, the DEGs identified do not display a similar coherence in terms of biological and functional assignment. This finding further signifies the importance of the pipeline used to identify DEGs between groups and the statistical methods used to correct for multiple comparisons on a genome wide scale.

Evaluation of common DEGs shared amongst animals in the HFHS group and those in the HFHS diet + Afriplex GRT™ group revealed 5 common DEGs for males and 24 for females, respectively (Supplementary Data – Table S5 and S6). Interestingly, albeit it not significant, treatment with Afriplex GRT™ was able to reverse the average expression of all common DEGs in terms of the logFC values. One of the common DEGs identified in make datasets, was the previously described antioxidant, NQO1. Although, Afriplex GRT™ had no significant effect on the expression of any other antioxidant genes or those involved in ROS production, the modulating effect on NQO1 expression provides a promising avenue for further exploration (da Costa et al., 2019; Di Francesco et al., 2020). However, as one of the aims of the current study was to evaluate the ability of Afriplex GRT™ to modulate transcriptional signatures associated with HFHS diet feeding on a genome wide scale, the alteration of the expression of an individual gene of interest does not directly fall within the scope. Overall, the lack of relevant enrichments observed in the current study suggest that Afriplex GRT™ was unable to significantly ameliorate transcriptional, disease associated modifications induced by HFHS diet (Figure 5.15 to 5.18). Therefore, animals from this group were therefore excluded from subsequent DNA methylation analysis described in Chapter 6.

5.4 Conclusions

Non-communicable diseases (NCDs) place a significant burden on the health care systems of underdeveloped countries such as South Africa. Considering this, the comprehensive application of high throughput sequencing technologies has aided in the profiling and discovery of numerous differentially expressed targets involved in NCDs including CVD. Studies have demonstrated the aberrant expression of multiple RNA species in left ventricular dysfunction, myocardial infarction, and several other cardiovascular events (Jneid et al., 2012; Liao et al., 2019; Lundmark et al., 2015).

This study provides the first large scale RNA sequencing (RNA-Seq) analysis of both male and female Wistar rats maintained on this particular diet and treated with the Afriplex GRT™ extract. To explore differentially expressed mRNA signatures in the heart, RNA from five biological replicates of rodents in the three experimental groups previously described were subjected to PE100 transcriptome sequencing. Following an extensive pre-processing pipeline, an average of 59.66 million paired-end reads were obtained per sample with approximately 85.41% mapping uniquely to the latest version of the annotated reference genome. The remaining percentage of unmapped reads could be attributed to the presence of PCR duplicates, discrepancies in the reference annotation or the stringency of the mapping criteria applied.

Rigorous bioinformatic analysis revealed that a total of 238 mRNAs were significantly upregulated and 333 mRNAs downregulated in the HFHS males, while 252 mRNAs were upregulated, and 162 mRNAs downregulated in female rats maintained on the HFHS diet when compared to the control. Furthermore, 64 mRNAs were significantly upregulated, and 56 mRNAs downregulated in the HFHS + Afriplex GRT™ males whereas 221 mRNAs were upregulated, and 116 mRNAs downregulated in female rodents in the HFHS + Afriplex GRT™ group when compared to the HFHS group. Hierarchical clustering revealed the distinct classification of DEGs by treatment groups, with animals in the HFHS + Afriplex GRT™ group more closely related to those in HFHS group, suggesting the possible use of these mRNA to distinguish the disease group from the control.

In order to determine the landscape of the molecular functions and associated pathways of the identified DEGs, GO enrichment and KEGG pathway analyses were performed for each of the separate signatures. Some of the major findings associated with the HFHS group included the overexpression of DEGs involved in cardiac muscle contraction and cardiac

myofibril assembly, as well as the decreased expression of DEGs involved in the response to oxidative stress. Additionally, the enrichment of immune related terms was observed in downregulated mRNAs including inflammatory response, cytokine-cytokine receptor interaction and the TGF-beta signalling pathway. Collectively these results demonstrate the ability of the HFHS diet to act as a pathological stimulus capable of decreasing antioxidant activity, which could alter ROS neutralisation capabilities and increase oxidative stress, ultimately activating host inflammatory responses. This is further accompanied by an increase hypertrophic gene expression, possibly affecting cardiac muscle functionality. Furthermore, albeit the lack of a severe CVD disease phenotype in these animals, the enrichment of the pathways involved in disease pathophysiology nonetheless indicates the disruption of cardiac tissue homeostasis. As expected, the previously described enrichments were more frequent in males than females, with female animals only displaying enrichment of one of the underlying pathways involved in hypertrophic signalling. Due to the limited changes in the metabolic risk parameters of females assessed in Chapter 4, the bases exist for speculating that the insult of the HFHS diet may have been insufficient to induce molecular changes in the cardiac tissue of female rodents specifically, further supporting the possible protective involvement of female hormones against metabolic disorders.

Functional analyses of DEGs associated with HFHS + Afriplex GRT™ groups yielded no significant enrichment of disease or pathology related processes and no high confidence results for the amelioration of the transcriptomic signatures resulting from HFHS diet feeding. However, this data set still provides a basis for further exploration in terms of candidates specifically associated with the Afriplex GRT™ profile that were not focussed on in the current study. Transcriptomic signatures associated with the HFHS diet identified will be compared comprehensively to the methylation analysis described in Chapter 5 and has the potential of providing insight into the underlying mechanisms associated with a HFHS dietary insult, as well as assist the development of further preventative and therapeutic studies.

5.5 Supplementary Data

Supplementary Data: Table S1 - List of DEGs in HFHS males.

Supplementary Data: Table S2 - List of DEGs in HFHS females.

Supplementary Data: Table S3 - List of DEGs in HFHS + Afriplex GRT™ males.

Supplementary Data: Table S4 - List of DEGs in HFHS + Afriplex GRT™ females.

Supplementary Data: Table S5 - List of common DEGs amongst HFHS and HFHS + Afriplex GRT™ males.

Supplementary Data: Table S6 - List of common DEGs amongst HFHS and HFHS + Afriplex GRT™ females.

Supplementary Table 2. GO enrichment analysis of overexpressed DEGs in HFHS males.

Supplementary Table 3. GO enrichment analysis of under expressed DEGs in HFHS males.

Supplementary Table 4. GO enrichment analysis of overexpressed DEGs in HFHS females.

Supplementary Table 5. GO enrichment analysis of under expressed DEGs in HFHS females.

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Chapter 6: Whole genome bisulfite sequencing to assess aberrant DNA methylation associated with diet-induced CVD in Wistar rats

6.1 Introduction

The advent of epigenetics, the interplay between genetic components and environmental influences, has provided a new dimension to pharmacogenomics (Khalil, 2014; Stenvinkel et al., 2007). Epidemiological research has shown that cardiovascular disease (CVD) has both a heritable and an environmental component, suggesting that epigenetic mechanisms may play a critical role in CVD development (Baccarelli et al., 2010; Meaney, 2014). Amongst the numerous forms of epigenetic modifications investigated, DNA methylation remains the most widely studied (Michels, 2012; Solomon et al., 2020; Zhong et al., 2016). DNA methylation is the addition of a methyl group to the 5th carbon position of the cytosine ring, occurring primarily in CpG islands in the promoter regions of genes (Bird, 2002; Deaton & Bird, 2011; Du et al., 2015). This process is catalysed by DNA methyltransferases (DNMTs) and plays an imperative role in maintaining genomic stability and regulating gene expression (Jaenisch & Bird, 2003; Kirchner et al., 2013; Kurdyukov & Bullock, 2016). Furthermore, DNA methylation also alters protein binding sites on DNA, often leading to transcriptional silencing of genes, which may affect phenotype and lead to disease development (Moore et al., 2013).

Numerous studies have implicated aberrant DNA methylation in the development of obesity, T2D, as well as biological processes underlying CVD (Baccarelli et al., 2010; Bansal & Pinney, 2017; Friso et al., 2008; Ikram et al., 2009; Muka et al., 2016; Pasquier et al., 2015; Ron & Ling, 2015; Turunen et al., 2009). Of particular interest to this study is the role of altered DNA methylation associated with hyperglycaemia and glucose intolerance resulting from increased dietary fat intake, described in fetal programming human and animal models (Cannon et al., 2014; Moody et al., 2017; Seki et al., 2017; Soubry et al., 2015; Zang et al., 2019). Furthermore, the adverse effects brought on by increased sugar intake, specifically fructose, has also been suggested to be mediated by DNA methylation (Davegårdh et al., 2018; Yamanda et al., 2017). While the previously described metabolic conditions are all risk factors contributing to the development and progression of diet-induced CVD, limited evidence exists evaluating the combinatory ability of high fat and high sugar to stimulate DNA methylation-induced transcriptional changes, ultimately resulting in the dysregulation

of several pathways involved in cardiac dysfunction such as inflammation and oxidative stress.

The advent and evolution of next generation sequencing (NGS) has considerably impacted genomic research. However, until recently, researchers were unable to access platforms capable of massive parallel sequencing on a genome-wide scale, locally and at an affordable price. Consequently, this study utilised a genome-wide sequencing approach to investigate DNA methylation associated with diet-induced CVD in rodents. The reversible nature of epigenetic phenomena allows for the modification of the transcription of critical genes associated with the development of diet-induced CVD (Crider et al., 2012; Jamaluddin et al., 2007; Nazki et al., 2014). Identifying DNA methylation signatures associated with CVD on a genome wide scale, as proposed by this study, aids in the elucidation of the pathophysiology of diet-induced CVD and the identification of possible new targets for epigenetic therapies.

6.2 Materials and Methods

6.2.1 Study design and sample selection

A total of 20 male Wistar rat (*Rattus norvegicuss*) cardiac tissue samples from the animal study described in Chapter 3 were used in the study. The sample selection criteria were based on the abovementioned objectives and involved the inclusion of male rats from two groups namely: 1) the control group which received a standard (STD) maintenance rodent diet (n=10), and 2) the high fat, high sugar (HFHS) group receiving a cafeteria diet (n=10). As a result of the limited protective metabolic (Chapter 4) and transcriptional (Chapter 5) changes previously observed with administration of Afriplex GRT™, male rodents treated with Afriplex GRT™ diet were not included in DNA methylation analysis. Similarly, female rodents were excluded from this part of the study as observational alterations in cardiovascular risk factors including increased body weight, glucose intolerance, as well as elevated triglyceride levels and TG/HDL ratio were less prominent (Chapter 4).

6.2.2 DNA extraction, assessment and pooling

Genomic DNA (gDNA) was extracted from snap frozen cardiac tissues with a modified, non-organic protocol that involved the use of highly concentrated sodium chloride (NaCl). Briefly, approximately 25 – 30 mg of tissue was submerged in a 100 µL lysis buffer (400mM NaCl, 10mM Tris-HCl pH 8, 2mM EDTA pH 8 and 10% [w/v] SDS) supplemented with 5 µL proteinase K (20 mg/µL) and homogenised using a TissueLyser (Qiagen, Hilden, Germany). The resulting homogenates were then incubated in a water bath for 1 hour at 80°C to facilitate sufficient tissue disruption and the removal of proteins. The isolation of DNA was promoted by the addition of 1/3 volume of 6 M NaCl, followed by vigorous vortexing (~15 seconds) and subsequent centrifugation at max speed for 10 min in order to pellet cell debris. Following centrifugation, the supernatant was attained and combined with equal volume ice cold isopropanol and incubated overnight at -20°C to enable the precipitation of DNA. The DNA was then precipitated with centrifugation at 13000 rpm for 10 min and the resulting pellet was subjected to a series of wash steps using 70% ethanol, before air drying and resuspension in 50 µL TE buffer. All above mentioned centrifugation steps were conducted at room temperature (RT) unless otherwise stated.

The yield and purity of the DNA was determined using fluorometry (Qubit™ DNA HS Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) and spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA), while the quality was evaluated through electrophoresis on a 1% [w/v] TBE agarose gel stained with ethidium bromide. Upon completion of quality control (QC) assessment, DNA isolated from individual samples were pooled in duplicate to generate 5 biological replicates of each group (Table 6.1). To this end each pooled sample consisted of an equal amount of high molecular weight, gDNA (10 µg) from the two individual rats involved.

Table 6.1. DNA pooling of male (n = 20) samples to generate five biological replicates per group, for sequencing and subsequent expression analysis.

Rat IDs	Pooled DNA ID	Group
R1 + R2	M1	
R3 + R4	M2	
R5 + R6	M3	**STD**
R7 + R8	M4	
R9 + R10	M5	
R11 + R12	M6	
R13 + R14	M7	
R15 + R16	M8	**HFHS**
R17 + R18	M9	
R19 + R20	M10	

6.2.3 DNA library preparation and sequencing

Pooled gDNA samples (1 μg) were fragmented with the M220 Focused-ultrasonicator (Covaris, Woburn, MA, USA), followed by size selection using MGIEasy DNA Clean Beads (MGI, Shenzhen, China) to obtain an optimal insert size of 250 base pairs (bp). Lambda phage DNA (MGI, Shenzhen, China) was used to evaluate the C to T conversion efficiency and subjected to the previously described fragmentation and size selection protocol prior to library construction. Library preparation was performed with the MGIEasy Whole Genome Bisulfite Sequencing (WGBS) Library Prep Kit V2.0 (MGI, Shenzhen, China) according to an adapted version of the manufacturer's protocol. Briefly, 20 ng size selected DNA of each sample was combined with 0.4 ng size selected lambda DNA (1:50) and subjected an end repair and A-tailing (ERAT) reaction. The end-repaired products were ligated to 5 X diluted MGIEasy DNA Adapters and subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) without the addition of pH Indicator I to Buffer PB. Bisulfite treatment and purification was performed with the EZ DNA Methylation-Gold Kit (Zymo Research, CA, USA) using the purified adapter-ligated DNA products and 200 ng unfragmented lambda DNA, as input sample material. Following amplification, PCR

products were purified using previously described magnetic beads, and quantified with fluorometry using the Qubit dsDNA HS Assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Furthermore, the fragment size distribution of purified PCR products was evaluated using gel electrophoresis.

Double-stranded (ds), circular DNA libraries were generated from 1 pmol of PCR product, followed by purification and quantification with MGIEasy DNA Clean Beads and the Qubit dsDNA HS Assay kit, respectively. In accordance with the proprietary technology unique to the MGI sequencing platform, DNA nanoballs (DNBs) were generated from the ds, circular DNA libraries using rolling circle replication (RCR). Considering the imbalanced nucleotide composition observed in WGBS libraries, the libraries were combined with whole genome sequencing (WGS) libraries constructed from the same samples using the MGIEasy Universal DNA Library Prep Kit (MGI, Shenzhen, China). Pooling occurred post DNB generation in a 2:1 ratio (300 ng WGBS DNB:150 ng WGS DNB). The MGILD-200 automatic loader was used to load pooled DNBs onto MGISEQ-2000 FCL flow cells, following a one sample per lane approach. Massively parallel sequencing was then performed on the MGISEQ-2000 (MGI, Shenzhen China) with the appropriate reagents supplied in the MGISeq-2000RS High-Throughput Sequencing Kit. A paired-end sequencing strategy was employed, with a read length of 100 bp (PE100).

6.2.4 Bioinformatic analyses

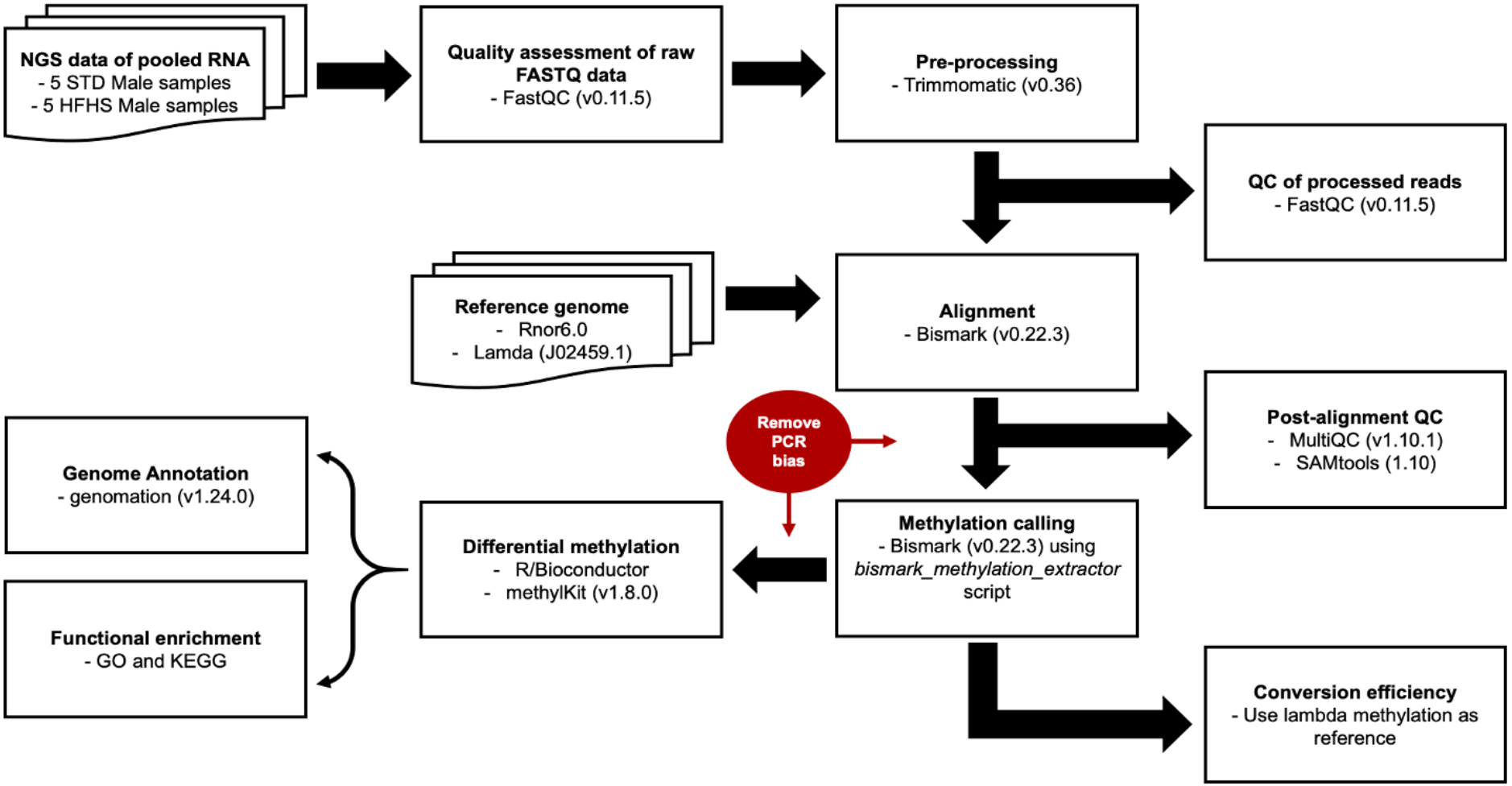

Figure 6.1. Schematic workflow describing the bioinformatic pipeline used for the analysis of whole genome bisulfite sequencing (WGBS) data. The workflow steps until the completion of "gene count estimation" was implemented for the 10 sequence datasets individually, while differential methylation analysis was performed comparing HFHS (n = 5) to STD (n = 5) datasets.

6.2.4.1 Data pre-processing

The raw WGBS datasets generated on the MGISEQ-2000 were obtained in FASTQ format and processed using the computational resources provided by the Centre for High Performance Computing (CHPC). All datasets were first individually evaluated in terms of sequencing quality and library composition using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Subsequently, quality trimming was performed with Trimmomatic version 0.36 and bases with a Phred quality score below Q20 from the 5' end of the sequence reads were removed using a sliding window of 3 nucleotides (nts) to get rid of potentially incorporated sequencing errors (Bolger et al., 2014). This led to a base calling accuracy of 99%, further improving the overall quality of datasets for subsequent analyses. Additional Trimmomatic parameters included a minimum read length of 20 bp and the removal of possible MGI adapter contamination. Following completion of all trimming and filtering steps, read file QC metrices were once again assessed with FastQC, to evaluate the efficiency of the pre-processing pipeline.

6.2.4.2 Alignment, methylation calling and conversion rate estimation

An alignment-based approach was utilised in order to infer the methylation status of every cytosine position in the processed WGBS datasets. To this end, “cleaned” reads from each sample were aligned to a custom reference genome containing the *Rattus norvegicus* reference genome version 6.0 (ftp://ftp.ensembl.org/pub/release-100/fasta/rattus_norvegicus/dna/Rattus_norvegicus.Rnor_6.0.dna.toplevel.fa.gz) and the Escherichia phage Lambda, complete genome (https://www.ncbi.nlm.nih.gov/nuccore/J02459.1?report=fasta) using Bismark version 0.22.3 (https://www.bioinformatics.babraham.ac.uk/projects/bismark). The selection of this three-letter aligner was largely based on flexibility to support the mapping of bisulfite reads generated from different sequencing technologies (Krueger & Andrews, 2011). Briefly, sequence reads were first temporarily transformed to generate complete bisulfite converted versions of the forward (C > T) and reverse (G > A) reads. These *in silico* generated read versions were then subsequently aligned to two, similarly converted versions of the custom reference genome (C > T and G > A) (Krueger & Andrews, 2011). The Bismark read alignment step was executed using default parameters with the addition of the *--bowtie2* and *--non_directional* options to perform mapping with Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) and accommodate the non-stranded nature of the

constructed WGBS libraries, respectively. Following completion of individual alignments, the deduplicate_bismark script was used to remove duplicate alignments from SAM output files possibly originating from excessive PCR amplification, prior to executing the supplementary bismark_methylation_extractor script with default parameters to obtain base pair methylation level measurements. The conversion efficiency (C > T) of the bisulfite treatment was further calculated as 100 % minus the methylation level of the lambda DNA spike-in. Furthermore, summary statistics obtained from the various alignment steps were assessed using SAMtools version 1.10 and collated with MultiQC version 1.10.1 for quality assurance purposes (Li et al., 2009; Ewels et al., 2016).

6.2.4.3 Differential methylation (DM) analysis

To take advantage of the per base resolution provided by WGBS, differential methylation was evaluated using the Bioconductor package, methylKit version 1.8.0 in R version 4.0.3 (Figure 6.1) (kalin et al., 2012; Gentleman et al., 2004; AR Core Team, 2020). Briefly, *methylRaw* objects containing per base methylation calls were generated from SAM output files sorted by both chromosome and read position. Following the removal of cytosines with sequencing depth < 10 X to account for low-coverage regions and normalisation using the *normalizeCoverage* function, samples were merged to facilitate comparative analysis (Pepin et al., 2019). Differentially methylated cytosines (DMCs) were obtained using the *calculateDifMeth* function, which employs a logistic regression test when biological replicates are present. The resulting p-values were adjusted to their corresponding q-values using the SLIM method (Wang et al., 2011). Additionally, an overdispersion correcting Chisq-test was performed to account for increased biological variability and the lack of technical replicates (Wreczycka et al., 2017). Due to the large number of cytosines being analysed simultaneously, multiple testing correction using the False Discovery Rate (FDR) method was applied and an adjusted p-value ≤ 0.05 was used to assign statistical significance (Benjamini & Hochberg, 1995). The significant DMCs resulting from the comparison between HFHS- and STD diet fed male animals was then utilised in downstream analyses.

6.2.3.4 Annotation and functional analyses

The Bioconductor package, genomation version 1.24.0 was used to annotate previously identified DMCs using the most recent BED file annotation of the *Rattus norvegicus* genome (rno6.0) obtained from the UCSC Genome Browser (Akalin et al., 2014; Kent et al., 2002). To this end, regional annotation was conducted in terms of promoters (defined as 1000 bp on up- or downstream of the TSS), introns, exons, and intergenic regions as well as CpG islands and shores. Transcripts overlapping with DMCs were regarded as differentially methylated genes (DMGs), with the mean difference in methylation of all corresponding DMCs representing the gene level difference.

Furthermore, the potential biological significance inferred by the differentially methylated genes was investigated with gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analyses (Ashburner et al., 2000; Kanehisa & Goto, 2000; Yu et al., 2012). Briefly, GO terms were retrieved from the Bioconductor database, org.Rn.eg.db, while rat specific (rno) pathway assignments were obtained from the KEGGREST package. Statistically significant enrichments were identified using either the Kolmogorov-Smirnov or Wilcoxon rank-sum test where applicable, with an adjusted probability value of less than, or equal to 0.05 deemed biologically significant. Comparative analysis was performed between differentially expressed genes (DEGs) identified in Chapter 5 and overlapping DMGs to identify potential associations between methylation status and the degree of expression.

6.3 Results and Discussion

6.3.1 DNA isolation and library preparation

For WGBS specifically, utilising DNA of high quality, purity and quantity are crucial as the bisulfite treatment may degrade up to 90% of the input sample (Ehrich et al., 2007; Grunau et al., 2001; Kint et al., 2018). While traditional organic, phenol-chloroform protocols yield highly concentrated DNA, the presence of impurities such as PCR inhibitors have been shown to influence downstream applications (Fan & Gulley, 2001; Gumińska et al., 2018; Psifidi et al., 2015). Conversely, commercial tissue extraction kits yield extremely pure DNA but less than the required concentration, often requiring repeat extraction in many cases (DNA < 50 ng/µL) (Gumińska et al., 2018; Psifidi et al., 2015). In this study, a non-organic, highly concentrated NaCl protocol was utilised and optimised to enable the extraction of high quantity DNA, with minimal co-purified contaminants, from snap frozen tissue. The adapted protocol resulted in highly intact gDNA from male (n = 20) cardiac tissue samples, with concentrations ranging between 205 and 621 ng/µL. Further evaluation of potential contaminants demonstrated an overall mean A260/280 ratio of 1.98 and an overall mean A260/230 ratio of 2.03, collectively considered to be within the acceptable standards for pure DNA (Arif et al., 2010; Dauphin et al., 2011; Psifidi et al., 2010). All male samples yielded gDNA that met the quantity and quality requirements for WGBS, with no significant differences observed between samples from different treatment groups. Following the successful completion of WGBS and WGS library preparation, pooled bisulfite and whole-genome libraries were subjected to paired-end 100 bp (PE100) sequencing on the MGISEQ-2000. All the samples passed in-house quality control measurements throughout the library preparation and sequencing protocols.

6.3.2 Methylation sequencing and pre-processing

Upon successful completion of WGBS of the 10 libraries on the MGISEQ-2000 platform, an average of approximately 39.21 gigabases (GB) of raw data and 200.54 million paired-end reads were obtained per sample. Consequently, suggesting that the incorporation of the WGBS:WGS pooling strategy allowed for stable base pair calling and the generation of sufficient sequencing read depth. Evaluation of the data with FastQC revealed mean Phred quality scores above 20 throughout the entire read length (100 bp), indicating accurate nucleotide base calling and high-quality sequences. Furthermore, decreased GC content

and uneven per base sequence content distribution across the reads were observed. However, due to the chemical modification employed, bisulfite-treated reads are inherently different from standard sequencing reads regarding of the QC metrices assessed. To this end, forward reads are generally rich in T bases and poor in C bases, whereas the reverse reads are rich in A- and poor in G bases (Nair et al., 2018; Sun et al., 2015). This imbalance in nucleotide base proportions was observed in the present study and further explains the previously mentioned reduction in GC content (Figure 6.2 and Table 6.2).

The pre-processing of FASTQ reads resulted in the removal of roughly 3.42 million paired sequences per sample, with an average of 86.96% of the remaining bases displaying a quality score of at least 30 (Table 6.2). Interestingly, the read duplicate rate remained higher than 10% after pre-processing. While this is still within the acceptable range, it is higher than what has previously been observed with genome sequencing on this platform (Glanzman et al., 2020). This high PCR amplification bias remains one of the main areas of concern with WGBS library preparation protocols that utilise post-bisulfite PCR amplification, such as the one employed in this study, and will be corrected for in the downstream alignment analysis (Olova et al., 2018; Walker et al., 2015). Overall, pre-processing resulted in the loss of a considerably low amount of read pairs and the retainment of exceptionally high-quality bases, with no significant adapter contamination across the read length.

Table 6.2. Read statistics of male sample WGBS datasets following pre-processing.

Group	Sample	Clean reads	%Duplicate reads	%GC content	Q20%	Q30%
	M1	209 304 406	16,5	24,0	96,13	88,21
	M2	211 036 748	16,6	24,0	95,32	86,92
STD	M3	212 577 162	16,6	23,5	95,46	87,28
	M4	200 890 158	18,0	23,5	95,35	86,68
	M5	152 234 820	16,4	24,0	95,55	87,44
	M6	207 592 718	15,0	24,0	93,51	83,80
	M7	221 431 577	14,5	23,5	96,30	88,53
HFHS	M8	202 467 829	16,1	23,0	95,47	87,14
	M9	223 075 662	16,5	23,5	95,55	87,06
	M10	164 795 448	15,8	24,0	94,83	86,51

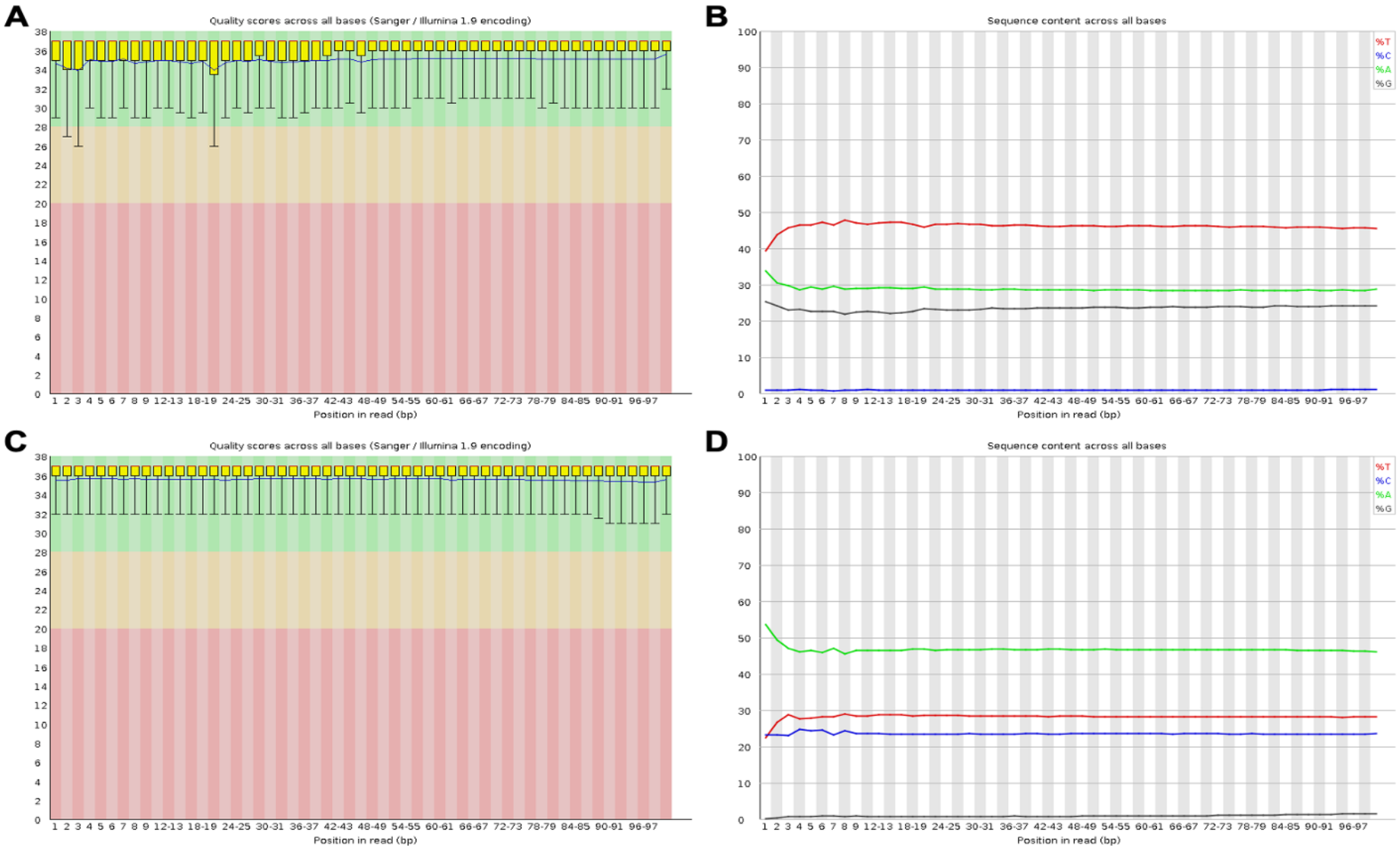

Figure 6.2. Graphical output obtained from FastQC processed WGBS data of a representative sample. Distribution of per base Phred quality scores obtained for **A) forward** and **C) reverse** reads. The nucleotide illustrating composition in terms of the four normal DNA bases across **B) forward** and **D) reverse** reads.

6.3.3 Alignment evaluation

Processed bisulfite treated reads were mapped to the custom reference genome with Bismark and the resulting alignments were assessed using a combination of the built-in scripts provided by the software. An average alignment efficiency of 70.77% was achieved across all samples, which is high for WGBS data considering that the reads are not complementary to the reference (Table 6.3) (Chatterjee et al., 2012). Approximately 9.24 to 10.24% of the reads mapped to multiple locations on the genome, while between 14.75 and 24.53% were not aligned and subsequently removed from downstream analyses (Table 6.3). The bisulfite treatment introduces a reduction in sequence complexity from four bases to three bases (Nair et al., 2018; Sun et al., 2015). Furthermore, while T nucleotides in reads can align to C nucleotides in the reference genome, the reverse is not the case (Rauluseviciute et al., 2019; Xi & Li, 2009). The damaging effects associated with bisulfite treatment thus support the lower rate of alignment when compared to traditional DNA sequencing and increased non-alignments observed in the current study. The assessment of quality metrices of the individual sample alignments revealed no statistically significant differences between or within the STD and HFHS experimental groups, allowing for the incorporation of all sample datasets in downstream analyses (Table 6.3).

Table 6.3. Summary of mapping statistics obtained following the alignment of processed male datasets to the custom reference genome.

Group	Sample	%Unique alignments[a]	%Multiple alignments[b]	%No alignments[c]
	M1	75.00	10.24	14.75
	M2	73.20	9.99	16.83
STD	M3	69.00	9.82	21.15
	M4	73.20	9.24	17.56
	M5	72.80	10.17	17.02
	M6	68.30	9.62	22.04
	M7	72.70	10.13	17.16
HFHS	M8	68.01	10.02	21.97
	M9	69.80	9.92	20.27
	M10	66.30	9.22	24.53

[a] Proportion of reads mapping uniquely to reference genome representative of the alignment efficiency.

[b] Proportion of reads mapping to multiple positions/loci on the reference genome.

[c] Proportion of reads unable to align to any version of the reference genome, including the in silico converted versions.

In addition to efficiently aligning bisulfite treated reads, Bismark also provides CpG-, CHG- and CHH-context specific methylation outputs, where H is representative of any non-G base. To this end, average methylation levels were extracted from the mapping files after performing a mandatory deduplication step. Of the 2318.02 to 5691.13 million Cs covered by uniquely mapped reads across all the samples, a mean of 57.4% was methylated in the CpG context (Table 6.4). However, it should be emphasised that this percentage methylation value is only a rough estimation (Equation 6.1) characterising the methylation profile at the mapping step (Krueger & Andrews, 2011). Definite methylation levels will vary after user-specific filtering and processing steps are completed. DNA methylation at non-CpG sites was historically known to be present more abundantly in plants than differentiated, adult mammalian cells. However, recent scientific studies have described the occurrence of extensive non-CpG methylation in mammalian brain, skeletal muscle, and testis tissue, further stimulating the possibility of considering non-CpG methylation as an alternative form of DNA methylation capable of influencing disease development (Kinde et al., 2015; Pinney et al., 2014; Wang & Kadarmideen, 2019; Yuan et al., 2016; Zhang et al., 2017). The methylation proportions determined in CHG and CHH context in the present study were, however, negligible (below 1%) in all the samples and therefore excluded from downstream differential methylation analysis (Table 6.4) (Deshpande et al., 2020; Tomizawa et al., 2011).

Equation 6.1

$$\%Methylation = 100 \times \left[\frac{Methylated\ Cs}{(Methylated\ Cs + Unmethylated\ Cs)}\right]$$

As a supplementary QC measure, the average percentage methylation for each position across the read length was also visualised using the M-bias plot outputs generated by Bismark to identify possible biases in methylation calls (Krueger & Andrews, 2011). While the theoretical methylation level distribution is expected to be uniform throughout the read, deviation from the horizontal line was observed in the first 10 bases of the reverse reads in the current study (Supplementary Figure 1). This deviation, observed at either end of the read, is in line with previous findings and the bases involved were removed from further analyses (Hansen et al., 2011; Lin et al., 2013).

Table 6.4. Summary of methylation statistics extracted from Bismark alignment files.

Group	Sample	Total Cs analysed (million)	%Methylation			Conversion rate%[d]
			CpG[a]	CHG[b]	CHH[c]	
	M1	5586.75	56.7	0.7	0.8	99.52
	M2	5290.10	54.5	0.6	0.7	99.64
STD	M3	2318.02	60.9	0.6	0.7	99.69
	M4	3989.57	54.6	0.6	0.7	99.67
	M5	3844.49	55.3	0.6	0.6	99.68
	M6	4973.89	56.8	0.6	0.7	99.67
	M7	5691.13	60.6	0.6	0.7	99.65
HFHS	M8	4606.58	56.1	0.8	2.9	99.72
	M9	3807.98	60.9	0.6	0.7	99.69
	M10	2516.32	57.1	0.7	0.8	99.68

[a] Percentage methylation in terms of the symmetrical CpG dinucleotide context.

[b] Percentage methylation in terms of the symmetrical CHG dinucleotide context.

[c] Percentage methylation in terms of the asymmetrical CHH dinucleotide context.

[d] Bisulfite conversion rate calculated using the lambda phage DNA spike-in.

The accuracy of DNA methylation detection depends on the conversion efficiency (C > T) of the bisulfite treatment reaction, and the incomplete transformation of cytosine in sequences may lead to false-positive results. In this study, lambda phage DNA was spiked in prior to sequencing, as an unmethylated control to calculate the conversion rate using the previously described formula. A conversion rate above 99% was obtained for all the samples in this study, suggesting that the bisulfite treatment was performed optimally and that any biases observed in methylation calls are not due to incomplete bisulfite conversion (Table 6.4). The latter was confirmed with M-bias plots illustrating the almost complete conversion of non-CpG cytosines, enabling the use of all biological replicated in the differential methylation comparison of HFHS diet-fed rodents to those on the STD diet (Supplementary Figure 1).

6.3.4 Genome-wide DNA methylation patterns

Given the low levels of CHG and CHH methylation observed in this study, all analyses from this point forward were conducted in CpG dinucleotide context only (Jang et a., 2017; Laurent et al., 2010; Lindroth et al., 2001; Lister et al., 2009). Initial descriptive statistics revealed a mean per base methylation of 72.93 and 71.31% for STD and HFHS samples respectively. No samples were flagged for excessive PCR duplication bias in terms of per base read coverage, suggesting the effectiveness of the deduplication steps performed. Following secondary filtering and normalisation comparative analysis was conducted on 70 183 sites covered in the 10 samples. Overall, a similar trend was observed in the genome-wide methylation status between STD and HFHS samples, with Pearson's correlation scores ranging between 0.58 and 0.98 (Figure 6.3 A). Variable methylation levels were observed across the 22 *Rattus norvegicus* chromosomes.

While majority of the CpG sites analysed were located on chromosome 1 (21.18%) and 2 (11.32%), chromosome 10 (0.75%) and Y (0.45%) contained the least amount of CpG sites. This variation in CpG site per chromosome could possibly be explained by several factors, including stringent coverage filtering methods, genome stability and chromosome size. Regional annotation performed on the methylated CpG sites (70 183) revealed the distribution of 1.71, 1.51, 10.24 and 86.87% within promoter, exon, intron, and intergenic regions, respectively (Figure 6.3 B). The increased methylated CpG dinucleotides located within non-coding regions (intergenic) observed in the current study is in line with previous findings and supports the essential role of DNA methylation in processes required for normal development such as genomic imprinting and transposable element repression (Dahlet et al., 2020; Huff & Zilberman, 2014; Tost, 2010). Moreover, 4.31% of the global sites analysed were annotated within CpG islands (CpGi), 3.30% in CpG island shores (CpGi shore) and 97.79% in other regions (Figure 6.3 B).

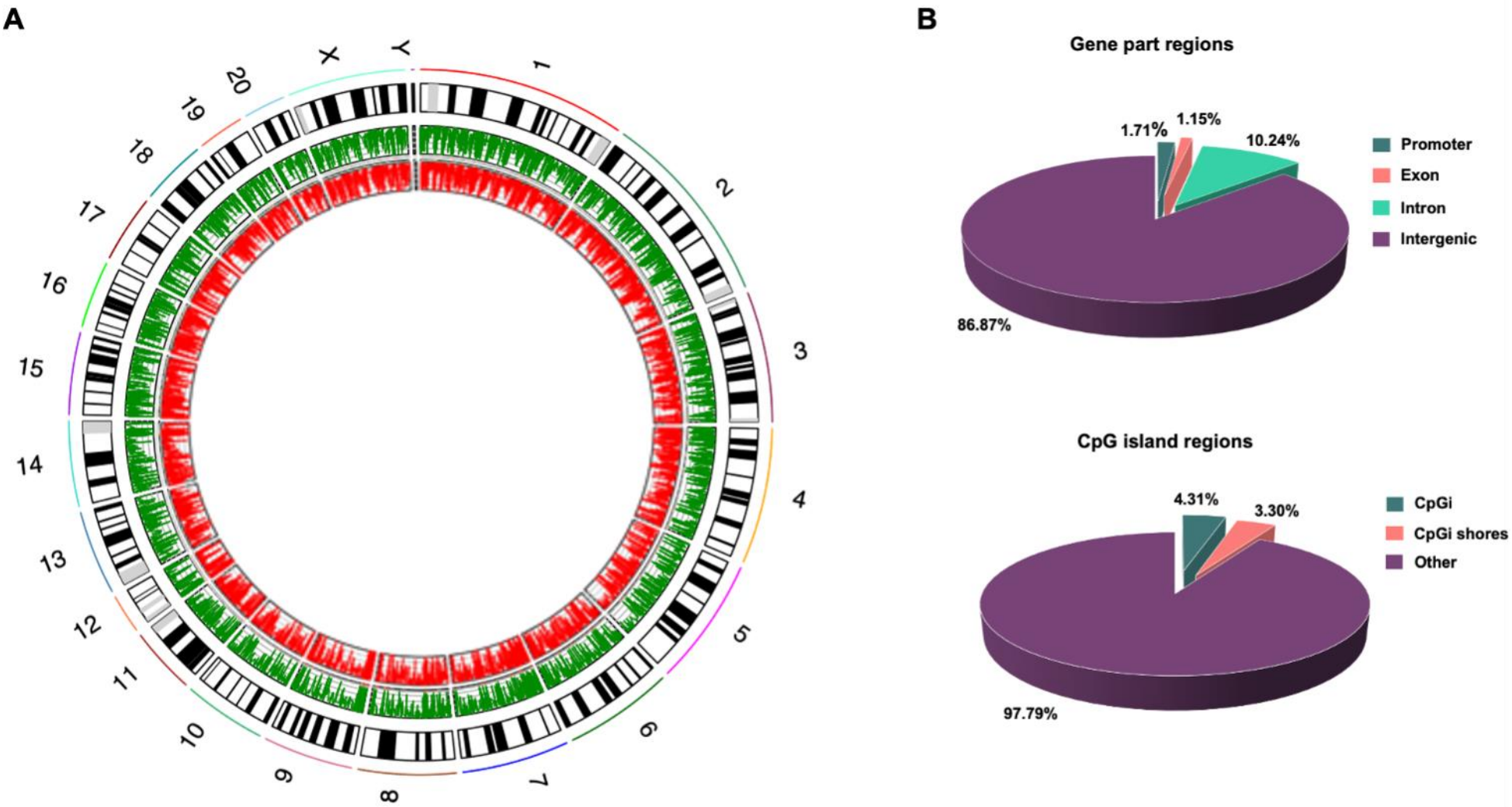

Figure 6.3. A) Genome-wide distribution of methylated sites with average **STD (green)** and average **HFHS (red)** methylation. The methylation levels of n = 5 samples per group are shown by lines in track 1 and 2 from inside to outside. The labels of the outside track represent the chromosomes of the Rattus norvegicus genome. **B)** Regional annotation of global methylated sites in terms of gene parts (promoter, exon, intron and intergenic) and CpG island regions (CpGi = CpG island, CpGi shore = CpG island shores).

6.3.5 Differentially methylated cytosines (DMCs) and annotations

As global methylation status is often insufficient to characterise the biological significance of observed changes in DNA methylation levels, differential methylation analysis was performed on a per base level using the previously identified 70 183 CpG sites annotated in the *Rattus norvegicus* (rnor6.0) assembly. A total of 5 531 DMCs (FDR $\leq$ 0.05) were identified in male Wistar rats maintained on the HFHS diet compared to those in the STD diet-fed control group. DMCs located on the Y chromosome were excluded from downstream analysis in the present study, thus reducing DMCs by 0.43% (5 508). Among these DMCs, 3 047 DMCs were hypomethylated, while the remaining 2 461 were hypermethylated in the HFHS group.

Visualisation of the methylation dynamics between samples was conducted using the methylation levels of the most variable DMCs (FDR $\leq$ 0.01). Accordingly, using Ward's method, hierarchical clustering revealed categorical separation between the HFHS and STD control samples, with all biological replicates correctly classified to the respective groups (Figure 6.4) (Murtagh & Legendre, 2011). While no discernible outliers were detected amongst biological replicates, moderate variation within the methylation profiles of STD samples was observed (Figure 6.4). Although little is known regarding sources of inter-individual differences in methylation among control rats, changes in cellular mechanisms responsible for maintaining of DNA methylation levels and chromatin homeostasis could partially be responsible (Bewick et al., 2016; Niederhuth et al., 2016; Zhang et al., 2020). Alternative plausible influences include circadian rhythms, seasonality, and changes in temperature (Baccarelli et al., 2009; Pinto et al., 2005; Ricceri et al., 2014; Shi et al., 2013; Vollmers et al., 2012; Zhu et al., 2011). While the extent and consequences of these stimuli remain to be fully explored, their influence in the current study can be excluded as the animals were maintained in a temperature-controlled facility. Nonetheless, the variation amongst control samples observed in this study was considered statistically irrelevant and could therefore most likely be ascribed to the small sample size used, the pooling of biological replicates before library construction or the lack of technical replicates.

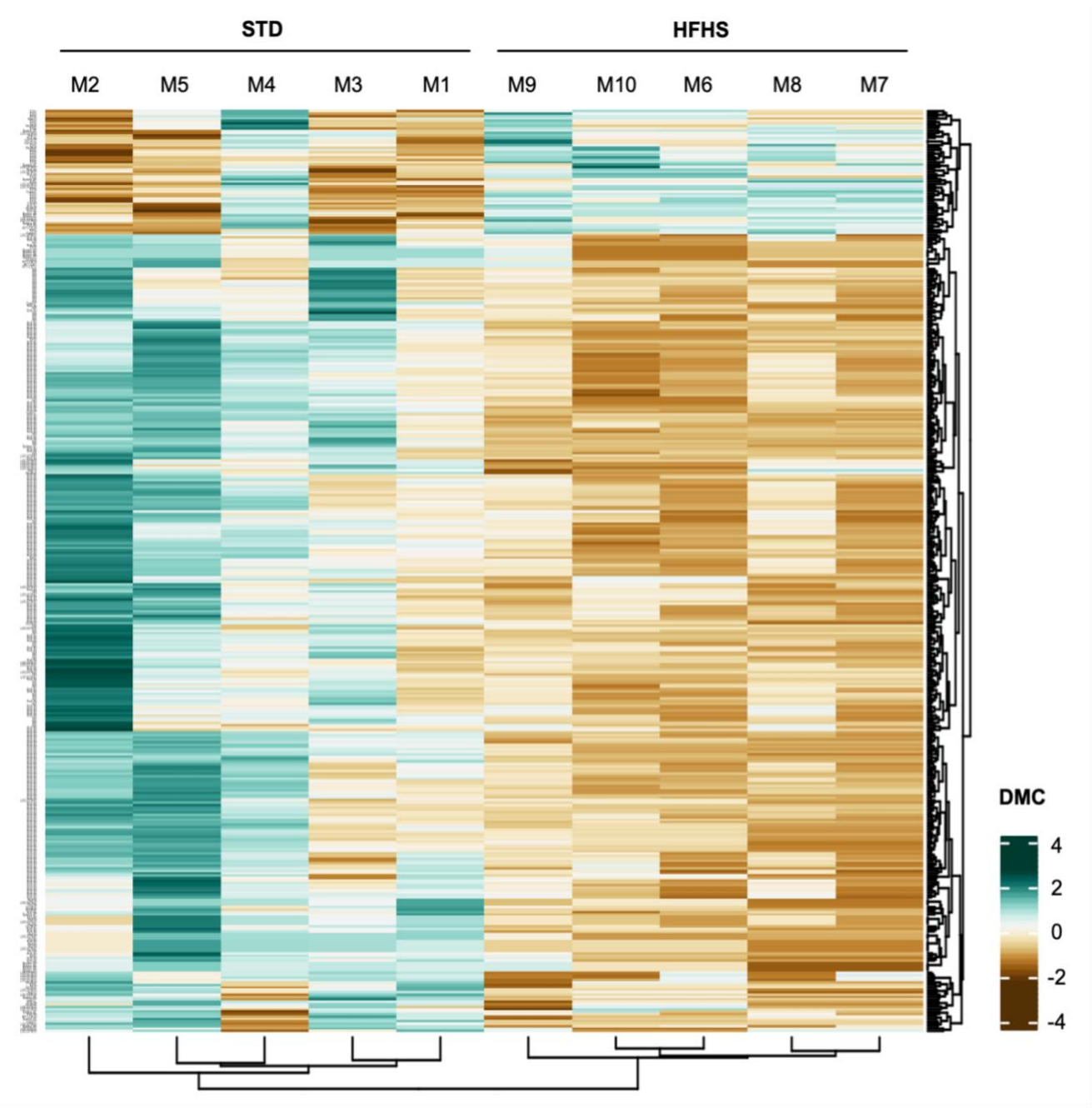

Figure 6.4. Heatmap illustrating methylation levels of differentially methylated cytosines (DMCs). DMCs with a false discovery rate (FDR) ≤ 0.01 in high fat, high sugar (HFHS) diet fed rodents relative to standard (STD) diet fed animals, as determined using whole genome bisulfite sequencing (WGBS) datasets. The heatmap was constructed using the BrBG RColorBrewer palette, where hypermethylation is indicated in varying shades of blue/green and hypomethylation in different degrees of gold/brown. Hierarchical clustering via Euclidean distance of DMCs revealed that the HFHS and STD biological replicates were categorised into two groups based on methylation levels.

Investigation into the genomic location of the above-mentioned 5 306 DMCs (FDR ≤ 0.05) across the *Rattus norvegicus* chromosomes, revealed that majority of the DMCs occupied chromosome 1 (29.40%) and 14 (9.77%), while less than 1% of the DMCs were located on chromosomes 10, 11 and 18. The extent to which DNA methylation can influence transcriptional events is largely dependent on DMC position, relative to established genomic features (Jingo et al., 2012; Pepin et al., 2019). Therefore, regional annotation was also performed to assess the distribution of the DMCs within annotated genic features such as promoter (3.23%), exon (2.52%), intron (17.27%) and intergenic (76.99%) regions (Figure 6.5 A). In this study, majority of the DMCs in the heart of HFHS rodents mapped in the intronic and intergenic regions, both known to contain functionally important elements such as enhancers (Jeschke et al., 2015; Qu et al., 2017; Rearick et al., 2011; Takeshi, 2019).

Though promoter methylation has been the focus of numerous diseases and extensively implicated in the regulation of adjacent gene expression, increasing evidence supports the ability of methylation in the gene body and intergenic regions to also influence gene expression (Aran et al., 2011; Ball et al., 2009; Jeschke et al., 2015; Lu et al., 2012; Rauch et al., 2009; Schlesinger, et al., 2013; Shenker & Flanagan, 2012; Thomas et al., 2012). Altered methylation levels in these regions have further been associated with modified gene expression in cancer and CVD incidence (Fernández-Sanlés et al., 2018; Jeschke et al., 2015; Muka et al., 2016; Shenker & Flanagan, 2012). Similarly, a recent study conducted by Deshpande et al. (2020) demonstrated increased CpG sites located within intronic, and intergenic regions resulted in the enrichment of embryonic development signalling pathways in spermatozoa of high fat diet fed male rats. While the aforementioned study does not compensate for the combinatory effect of high fat and high sugar, as the present study does, the confirmation of increased DMCs in non-promoter regions remains a noteworthy observation and subsequent functional enrichment analysis should therefore be extended to include DMCs beyond the promoter context.

The percentage of DMCs located in CpG island regions were evaluated in terms of CpGi (18.26%), CpGi shores (5.63%) and other (76.11%) regions respectively (Figure 6.5 B). Compared to the globally methylated CpG site annotation, the percentage of DMCs annotation within both CpGi and CpGi shores increased, while DMCs annotation within other CpG islands regions decreased (Figure 6.5 B). As anticipated, further investigation regarding the localisation of CpGi to the respective genic features revealed that majority of the DMCs associated with both CpGi and CpGi shores were located within intronic (55.92%) and intergenic (29.99%) regions.

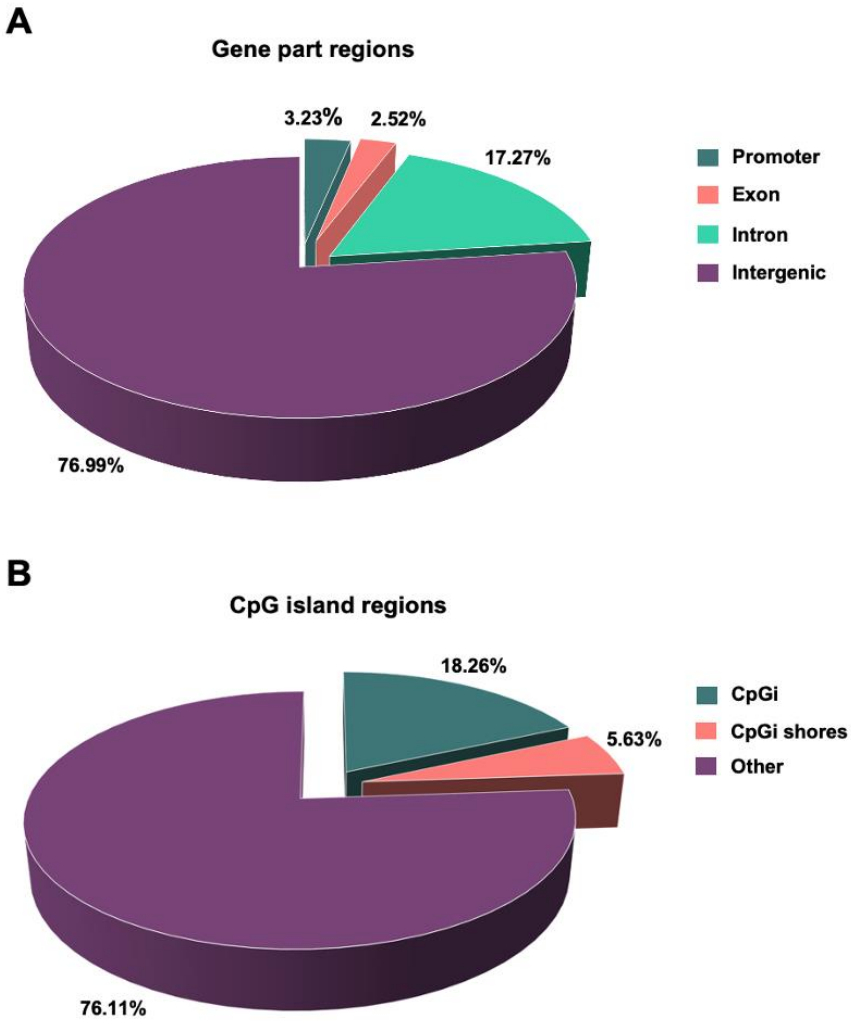

Figure 6.5. Regional annotation of differentially methylated cytosines (DMCs) in terms of **A)** gene parts (promoter, exon, intron and intergenic) and **B)** CpG island regions (CpGi = CpG island, CpGi shore = CpG island shores).

6.3.6 Functional analysis of genes associated with DMCs

Transcripts overlapping with DMCs, regarded as differentially methylated genes (DMGs), were identified by matching the 5 508 DMCs to the rat RefSeq database (rno6.0) retrieved from UCSC. A total of 5 253 DMCs were annotated within gene components of 533 unique genes, of which 339 genes were hypermethylated and 194 genes hypomethylated in the HFHS group (Supplementary Data - Table S7). Upon cross referencing of DMGs with the CTD Gene-Disease Associations dataset, 137 DMGs were found to be significantly associated with CVD including very low-density lipoprotein receptor (*VLDR*), platelet derived growth factor subunit A (*PDGFA*) and collagen type I alpha 1 chain (*COL1A1*) amongst

others (Supplementary Data - Table S8) (Johnson et al., 2020; Rouillard et al., 2016; Zhao et al., 2011).

To gain a holistic view regarding the biological significance of the identified DMGs, hyper- and hypomethylated genes were jointly categorised into the three organised GO vocabularies namely: biological processes (BP), cellular components (CC) and molecular function (MF) using the R/Bioconductor package topGO. Overall, GO terms were assigned to 498 of the 533 DMGs in HFHS diet fed male Wistar rats. Evaluation of significant ($p \leq 0.05$) ontologies demonstrated that major GO categories included gene products involved in intracellular signal transduction and G protein-coupled receptor signalling pathway (biological processes: GO:0035556; GO:0007186), gene products localised in the cytoplasm and intracellular membrane-bounded organelle (cellular component: GO:0005737; GO:0043231), as well as gene products involved in protein- and metal ion binding (molecular function: GO:0005515; GO:0046872) (Figure 6.6). Aside from the processes relating to cellular development, function and signalling, GO analysis also revealed the enrichment of DMGs involved in central nervous system development, immune response, negative regulation of apoptotic process and response to drug, which are of particular interest to this study (Figure 6.6).

Additionally, KEGG pathway analysis was conducted using the KEGGREST package and subsequent rat specific (rno) pathway assignments. The top 15 significant pathways enriched with genes associated with DMCs included phagosome, type I diabetes mellitus, platelet activation, steroid hormone biosynthesis, toll-like receptor signalling pathway, cell adhesion molecules and diabetic cardiomyopathy (Figure 6.7). Upon further investigation, the most significant pathway was the phagosome pathway ($p = 1.04 \times 10^{-7}$) containing 18 genes including *ATP6V1G1, C1R*, *CD209B*, *CTSS*, *DYNC1I1*, *EEA1*, *FCGR2A*, *IMPG1*, *FCGR2B*, *CD36 antigen-like*, *SEC61G* and *TLR4*. Phagocytosis, defined as the process of particle consumption and removal through the formation of phagosomes, is essential for tissue homeostasis and the activation of an immune response (Flannagan et al., 2012; Freeman & Grinstein, 2014; Gordon, 2016; Uribe-Querol & Rosales, 2020). The significant enrichment of DMGs involved in this pathway could possibly indicate the potential presence of pathogenic microorganisms. However, the inclusion DMGs encoding membrane proteins such as cluster of differentiation 36 (CD36 antigen-like) and toll-like receptor 4 (TLR4) indicates that this pathway may rather have been aimed at the elimination of lipids or apoptotic cells instead (de Kleijn & Pasterkamp, 2003; Dirks et al., 2002; Kawai & Akira, 2011; Uribe-Querol & Rosales, 2020).

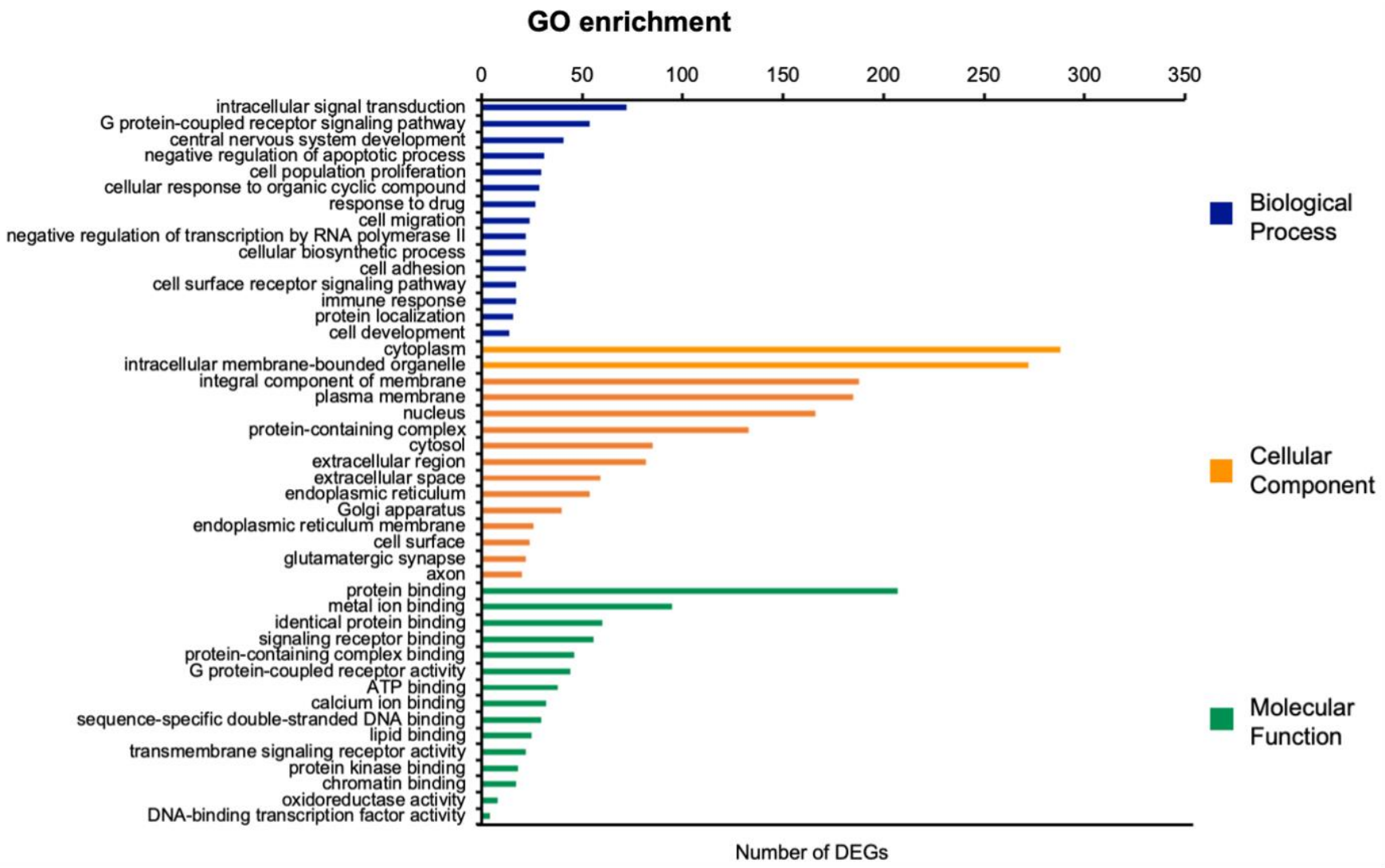

Figure 6.6. Gene ontology (GO) enrichment analysis of genes associated with differentially methylated cytosines (DMCs) in high fat, high sugar (HFHS) diet fed male Wistar rats. The top GO enrichment terms are illustrated separately in terms of the controlled vocabularies for describing Biological Process, Cellular Component and Molecular Function.

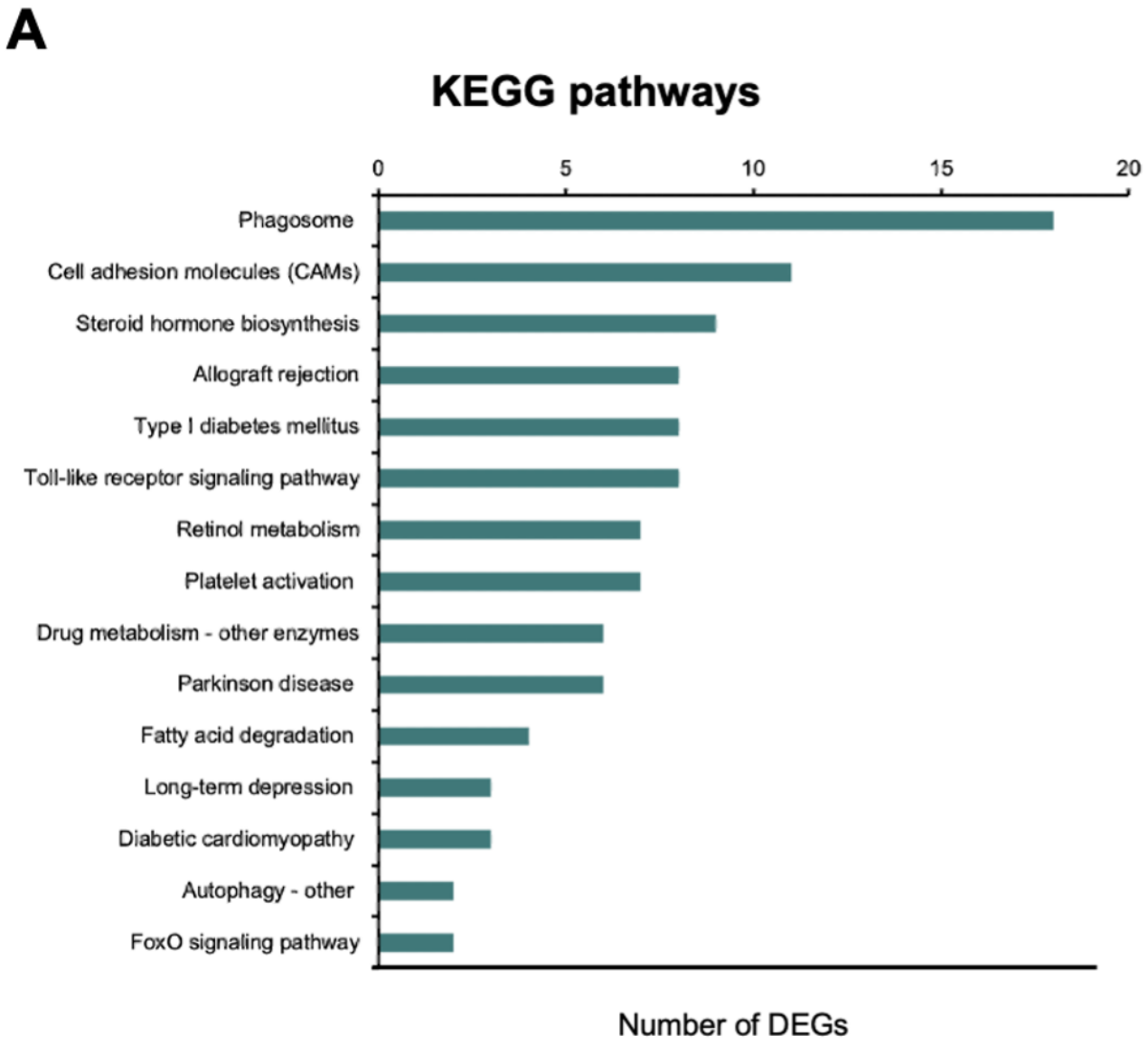

Figure 6.7. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis of genes associated with differentially methylated cytosines (DMCs) in high fat, high sugar (HFHS) diet fed male Wistar rats. The top KEGG enrichment items of differentially methylated genes (DMGs) are listed and sorted by significance ($p \leq 0.05$).

Collectively, the demonstrated pathways and gene ontology enrichments suggest that differential methylation in HFHS diet fed rodents accompanies inflammation (possibly promoted by oxidative stress), altered lipid transport and apoptotic processes, known for their underlying role in CVD (Figure 6.6 and 6.7) (Goldberg et al., 2012; Kim & Kang, 2010; Montecucco et al., 2017; Ridker & Luscher, 2014; Tan & Norhaizan, 2019).

6.3.6.1 DNA methylation changes in HFHS diet fed rats result in the activation of acute inflammatory processes

In depth analysis revealed a significant overlap of DMGs involved in six of the above-mentioned enriched pathways namely: phagosome, cell adhesion molecules (cAMS), allograft rejection, type 1 diabetes mellitus, toll-like receptor signalling pathway and retinol metabolism (Figure 6.7). The involvement of immune-related mechanisms in CVD, particularly atherosclerosis, has been supported by a magnitude of scientific evidence (Chroni et al., 2010; Husain et al., 2015; McMaster et al., 2015; Montecucco et al., 2017; Ridker & Luscher, 2014; Sexena et al., 2015; Stenvinkel et al., 2007; Timmers et al., 2012; Zhou et al., 2001). The innate immune system, often considered as the body's first line defence mechanism, is a dynamic complex involving the interaction of numerous signalling molecules and pathways (de Kleijn & Pasterkamp, 2003). To this end, toll-like receptors (TLRs) have been identified as one of the major receptors capable of recognising exogenous pathogen-associated structures and consequently activating a rapid immune response (Bijani et al., 2012; Frantz et al., 2007; Takeuchi & Akira, 2010). However, increasing evidence suggests that TLRs are also capable of responding to endogenous stimuli produced in response to injury and oxidative stress (Bianchi, 2007; Frantz et al., 2007; McGuire & Arthur, 2015; Yang et al., 2016).

In the current study, male Wistar rats fed a HFHS diet displayed *TLR4* hypomethylation within the gene body when compared to the STD diet fed controls. Amongst all TLRs, TLR4 displays the highest expression levels in the heart and has consequently been reported to play a crucial role in myocardial inflammation associated with atherosclerosis, hypertension, and heart failure (Bagheri et al., 2014; Bijani et al., 2012; de Kleijn & Pasterkamp, 2003; Liu et al., 2015; Satoh et al., 2014; Yang et al., 2016). Additionally, studies involving TLR4 knockout mice models identified TLR4 as a key mediator of high fat diet induced cardiac complications such as vascular inflammation, ROS, contractility, and myocardial dysfunction (Hu & Zhang, 2017; Kim et al., 2007). Evaluation of RNA sequencing analyses conducted on the same animals in Chapter 5, revealed that *TLR4* mRNA was not amongst the significantly differentially expressed genes (DEGs). Possibly suggesting that hypomethylation of the specific DMCs located in the intronic region of *TLR4* did not affect gene expression. Interestingly, TLR4 interactor with leucine-rich repeats (*TRIL*) mRNA was found to be significantly upregulated ($p = 0.007$) in HFHS diet fed male rodents. Furthermore, low density lipoprotein receptor (*LDLR*) mRNA expression was also considerably increased ($p = 0.003$) in the HFHS group (Chapter 5) possibly corresponding

to elevated LDL levels, which in its oxidised form (OxLDL), is a known ligand recognised by TLR4 (de Kleijn & Pasterkamp, 2003; Kruth, 2013; Thirunavukkarasu & Khader, 2019). Mechanistically, the stimulation of TLR4 by either exogenous or endogenous factors (such as HFHS diet as proposed in the current study) initiates several signalling events that ultimately trigger mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) signalling pathways (Arthur & Ley, 2013; Coll & O'Neill, 2010; McGuire & Arthur, 2015; Yang et al., 2016). Briefly, TLR4 activation initiates the recruitment of the myeloid-differentiation factor 88 (MyD88) adaptor protein, which facilitates the formation of a myddosome structure through binding interleukin-1 receptor associated kinases (IRAKs) (Figure 6.8) (Flannery et al., 2010; Gay, 2019; Latty et al., 2018; Ve et al., 2017; Yamamoto et al., 2010). In this study, IRAK4, a key component of the myddosome was also hypomethylated in HFHS diet fed male Wistar rats. The myddosome is required for downstream activation of tumor necrosis factor receptor (TNF-R)-associated factor 6 (TRAF6), ultimately leading to the activation of transcription factors such as NF-κB and interferon regulatory factors (IRFs), as well as MAPK pathways (Balka & De Nardo, 2018; Lopez-Pelaez et al., 2014; McGuire & Arthur, 2015; Takaoka et al., 2005). Pertinent to the present study, genes encoding MAPK kinases responsible for activating MAPKs namely MAP2K and MAP3K7, were hypomethylated in the HFHS group. Induction and binding of the previously mentioned transcription factors to related gene promoters induces the expression of proinflammatory cytokines and chemokines that elicits an inflammatory response (O'Neill & Bowie, 2007; Terrel et al., 2006; Vallejo, 2011; Yang et al., 2016). Alternatively, signalling cascades downstream of TLR4 can also be mediated by the TIR-domain-containing adapter-inducing interferon-β (TRIF) adaptor protein (Figure 6.8) (Balka & De Nardo, 2018; Gay et al., 2006; McGuire & Arthur, 2015; Tatematsu et al., 2010;). The association of TRIF with TLR4 leads to the activation of both NF-κB and IRF via TRAF (De Nardo, 2015; Hoebe, et al., 2003; O'Neill, 2006; O'Neill & Bowie, 2007; Sato et al., 2003).

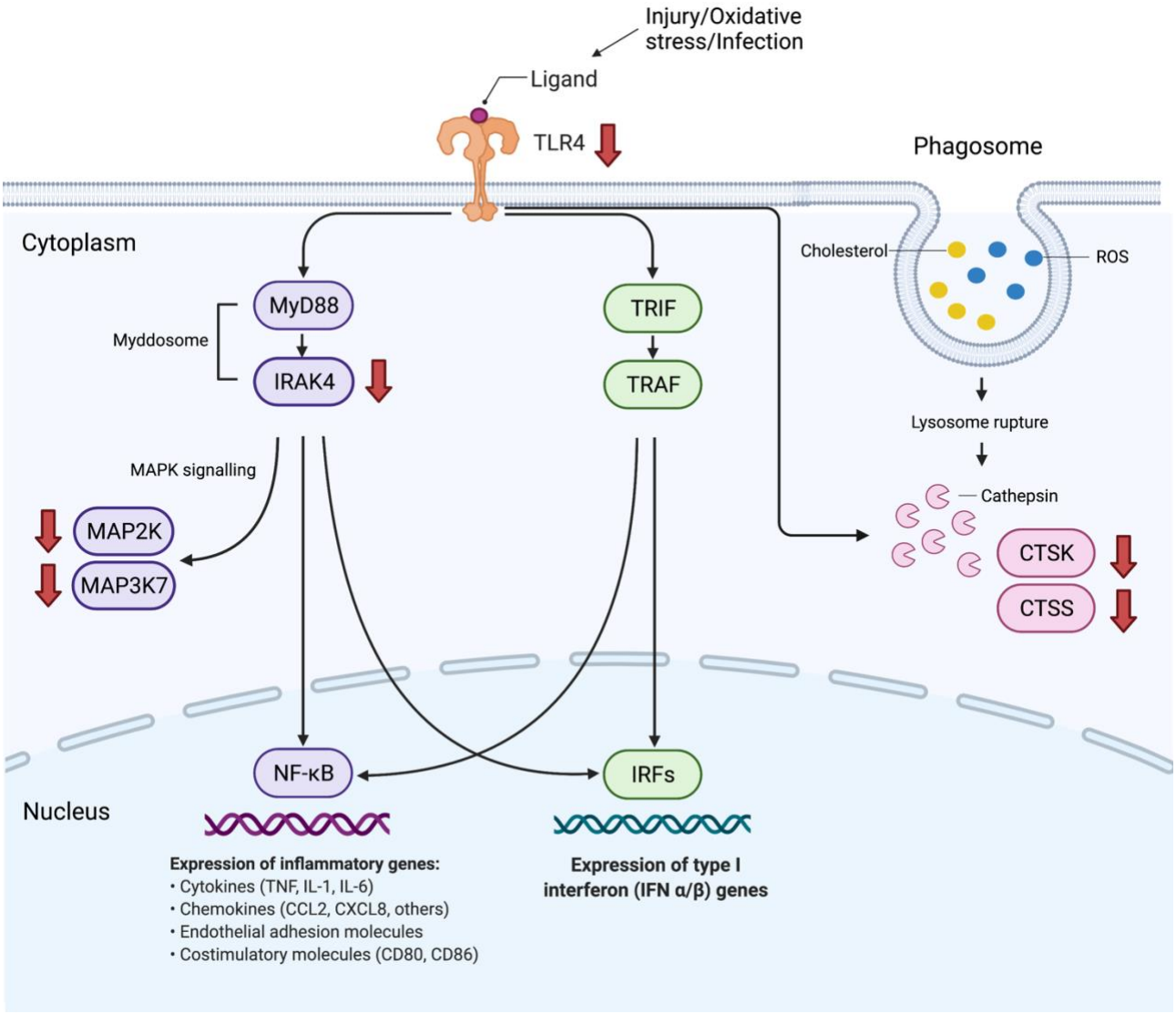

Figure 6.8. Schematic diagram to illustrate the proposed involvement of TLR4 and its ligands in underlying CVD processes. Downstream signalling of TLR4 is either mediated by the MyD88 or TRIF adaptor proteins, ultimately inducing the expression of proinflammatory cytokines and chemokines. The ability of cathepsin to mediate TLR4 signalling with the aid of the phagosome pathway is also displayed. Genes labelled with a red arrow represents differentially methylated genes identified in the present study where an up-arrow indicates hypermethylation and a down-arrow hypomethylation. **TLR4**, toll-like receptor 4; **MyD88**, myeloid-differentiation factor 88; **IRAK4**, interleukin-1 receptor associated kinases; **MAP2K**, mitogen-activated protein kinase kinase; **MAP3K7**: mitogen-activated protein kinase kinase kinase 7; **TRIF**, TIR-domain-containing adapter-inducing interferon-; **TRAF**, tumor necrosis factor receptor-associated factor; **NF-κB**, nuclear factor κB; **IRFs**, interferon regulatory factors; **CTSK**, cathepsin K and **CTSS**, cathepsin S. Adapted from "TLR Signalling Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

Two additional genes encoding cathepsin, *CTSK* and *CTSS*, were also amongst the overlapping DMGs in the pathways associated with immune response. These two cathepsins, known for their role in protein degradation and cellular turnover, have been associated with CVD and were first described in atherosclerotic tissue (Liu et al., 2004; Nomura & Katunuma, 2005; Plat et al., 2007). Since then, numerous studies involving *in vitro*, animal, and human models have confirmed the involvement of increased cathepsin levels in several CVDs, including myocardial infarction, cardiac hypertrophy, cardiomyopathy, hypertension, and atherosclerosis (Chen et al., 2013; Hua et al., 2013; Liu et al., 2006; Rodgers et al., 2006; Sharir et al., 2014; Stypmann et al., 2002; Sun et al., 2010). Of particular interest to the current study, is the regulation of cathepsin expression in the heart through stimuli such as cholesterol, ROS, oxLDL, elevated glucose, as well as oscillatory shear stress (Liu et al., 2018; Plat er al., 2007). Not only did male rodents on the HFHS diet display diminished glucose clearance ability and atherogenic lipid profiles (Chapter 4), but genes involved in oxidative stress were also differentially expressed in these animals compared to the controls (Chapter 5). Additionally, cathepsins share overlapping ligands with TLRs (e.g., oxLDL) and have been shown to indirectly contribute to CVD development by regulating the immune response through TLR activation (Liu et al., 2018).

Taken together the current study supports the concept of a HFHS being able to induce aberrant DNA methylation within the gene body of genes associated with a proinflammatory response in the heart. The hypomethylation of *TLR4* is proposed to have increased TLR activation either through ligands associated with ROS or cardiac injury binding directly to TLR4 or via the hypomethylation and subsequent increase in cathepsins CTSS and CTSK. The activation of TLR4 was accompanied by hypomethylation of genes involved in the myddosome structure (*IRAK4*) and MAPK signalling pathway (*MAP2K* and *MAP3K7*), possibly affecting gene expression in favour of cytokine and chemokine release via NF-κB and IRF.

6.3.7 Correlation analysis

To explore whether changes in the cardiac methylome corresponded with altered gene expression, WGBS analysis was compared to RNA sequencing analysis, described in Chapter 5, performed on the same samples. To this end, overlapping targets were identified by matching genes identified to be differentially expressed in HFHS vs STD male rats to genes associated with DMCs identified in the current study. A total of 10 genes fulfilled the

previously mentioned criteria namely: C1D nuclear receptor corepressor (*C1D*); cathepsin K (CTSK); cluster of differentiation 36 (*CD36*); cyclin G2 (*CCNG2*); early endosome antigen 1 (*EEA1*); eukaryotic translation initiation factor 4A2 (*EIF4A2*); phosducin like (*PDCL*); peroxiredoxin 4 (*PRDX4*); SEC61 translocon subunit gamma (*SEC61G*) and retinitis pigmentosa 1 (*RP1*) (Supplementary Data – Table S9). To enable a more comprehensive understanding on the relationship between HFHS diet induced DNA methylation and CVD pathophysiology, the 10 genes were subjected to an additional filtering step to include only genes with a validated experimental and/or clinical CVD association (Table 6.5). Following completion of the correlation and filtering procedures, five eligible genes were identified to fulfil all selection criteria (Table 6.5).

Table 6.5. DMC-related genes (DMGs) most likely associated with CVD pathogenesis.

Gene	Chromosome	Total DMCs[a]	Genomic feature[b]	Methylation		Expression	
				%Diff.[c]	P-val	Log2FC[d]	P-val
CCNG2	14	5	Intergenic	-18.7	0.004	-0.266	0.007
CD36	4	2	Intron	-5.6	0.015	-0.451	<0.001
SEC61G	14	6	Intergenic	-19.3	0.009	-0.895	0.008
CTSK	2	14	Intergenic	-17.3	0.002	-0.579	0.003
PRDX4	X	8	Intergenic	-19.9	0.004	-0.832	0.002

[a] Differentially methylated cytosines (DMCs) to which target genes have been associated.

[b] Genomic region in which DMCs are located following regional annotation analysis.

[c] Percentage methylation difference in HFHS group compared to STD group. The mean difference in methylation of all corresponding DMCs represents the gene level difference.

[d] Log2 fold changes for genes obtained as contrasts between HFHS group and STD group.

Of the six genes fulfilling all three selection criteria, *CCNG2* responsible for encoding cyclin G2, a protein involved in cell cycle regulation (Bennin et al., 2002; Bernaudo et al., 2016; Liu et al., 2004; Xu et al., 2008; Zhang et al., 2021). This unconventional cyclin is expressed at high levels in cells experiencing cell cycle arrest and terminal differentiation, conferring a potential tumor supressing role in cancer (Adorno et al., 2009; Choi et al., 2009; Gao et al., 2018; Kim et al., 2004; Li et al., 2019; Nakae et al., 2008; Xu et al., 2008; Zimmermann et al., 2012). Cyclin G2 is capable of inhibiting G1/S phase cell cycle progression through binding to protein phosphatase 2A (PP2A) and peroxisome proliferator-activated receptor (PPARγ) (Aguilar et al., 2010; Bennin et al., 2002; Zhao et al., 2018). Data generated in this study revealed significantly decreased methylation levels within the intergenic region of

CCNG2 accompanied by reduced gene expression in HFHS rats compared to STD rats (Table 6.5). Similarly, previous studies have demonstrated altered CCNG2 mRNA and protein expression levels in adipose tissue, indicating an inverse association between CCNG2 expression and obesity and insulin resistance (IR) (Abdalla et al., 2021; Aguilar et al., 2010; Garrido-Sánchez et al., 2014; Pivovarova et al., 2015). Additionally, a study by Zimmermann et al. (2016) showed that insulin and insulin-like growth factor-1 (IGF-1) was able to significantly lower the expression of CCNG2, ultimately promoting cell proliferation. Considering that both obesity and IR are major metabolic risk factors of CVD, the reduced CCNG2 expression associated with these disorders could be linked to the involvement of CCNG2 in underlying CVD pathology (Haywood et al., 2020; Minta et al., 2010; Zhang et al., 2021).

The second gene identified with correlation analysis, *CD36* (multifunctional receptor), is expressed in various tissues including the vascular system (Couturier et al., 2019; Hou et al., 2015; Son et al., 2018; Yang et al., 2018; Yu et al., 2020). In line with its involvement in numerous pathological processes including fatty acid (FA) uptake, immune response, platelet activation and metabolic regulation, CD36 ligands include both lipid and protein molecules such as oxLDL and thrombospondin, respectively (Glatz & Luiken, 2017; Magwenzi et al., 2015; Wang & Li, 2019; Zhao et al., 2018). As FAs are considered the predominant source of energy (ATP) production in cardiac muscle tissue and approximately 70% of FA uptake is arbitrated by CD36, CD36 is essential in myocardial lipid metabolism (Glatz & Luiken, 2018; Kim & Dyck, 2016; Shu et al. 2020). The DMCs identified in this study, located within the intronic region of CD36, were hypomethylated in the HFHS group when compared to animals in the control group (Table 6.5). Similarly, *CD36* mRNA expression levels in HFHS rats were significantly lower than in the STD rats (Table 6.5). Decreased CD36 expression has previously been observed in hypertrophy and ischemia, resulting in a decreased FA oxidation and impaired contractile function (Abumrad & Goldberg, 2016; Dehn & Thorp, 2018; DeLeon-Pennell et al., 2016; He et al., 2019; Lesnefsky et al., 2017). Similarly, pressure overload has been shown to inhibit CD36 synthesis via the downregulation of peroxisome proliferator-activated receptor a (PPARa), which subsequently leads to the accumulation of toxic lipids and heart failure (Dobrzynet al., 2013; Iemitsu et al., 2003; Karam et al., 2017; Madonna et al., 2011; Oka et al., 2015; Sung et al., 2017). The hypomethylation and decreased expression of CD36 observed in this study, accompanied by increased hypertrophic gene expression (Chapter 5), therefore further supports the ability of the HFHS diet to act as a pathological stimulus on a molecular

level. Conversely, in CVDs such as diabetic cardiomyopathy and atherosclerosis, increased CD36 expression in the sarcolemma facilitates excessive myocardial FA uptake and overall disease progression (Ackers et al., 2018; Carley & Severson, 2008; Coort et al., 2004; García-Rúa et al., 2012; Li et al., 2020; Ouwens et al., 2007; Sato et al., 2018; Xu et al., 2019). Interestingly, studies have shown that the inhibition of CD36 can impede lipid accumulation and ROS generation in high fat diet-induced cardiomyopathy by promoting glycolysis (Angin et al., 2012; Glatz et al., 2021; Yang et al., 2007; Zhang et al., 2015). Considering this, the reduction in CD36 expression observed in the current study could also be viewed as a possible defence mechanism against the HFHS diet insult aimed at decreasing FA uptake and normalisation of cardiac substrate metabolism. Collectively, these studies suggest that the role of CD36 in cardiac muscle tissue is mainly dependent on the pathological stimuli and how it influences CD36 synthesis and translocation (Shu et al., 2020). Furthermore, considering the significant role of CD36 in cardiac metabolism, it may present as a potential target for metabolic modulation therapy (Glatz et al., 2021; Revenco & Morgan, 2009).

Additional genes meeting the selection criteria utilised in comparative analysis included SEC61 translocon gamma subunit (*SEC61G*), peroxiredoxin 4 (*PRDX4*) and cathepsin K (*CTSK*) (Table 6.5). While the latter has previously been described for its role in the immune response via the TLR signalling pathway (Figure 6.8), *SEC61G* encodes a subunit of the protein translocation complex of the endoplasmic reticulum (ER) and PRDX4 prevents the accumulation of hydrogen peroxide within the cell (El Eter & Al-Masri, 2015; Greenfield & High, 1999; Liebthal et al., 2018; Linxweller et al., 2017; Nebendahl et al., 2013; Rhee et al., 2018). This study found that intergenic DNA methylation, as well as expression of *SEC61G*, *PRDX4* and *CTSK* was significantly reduced in HFHS diet fed rats compared to those maintained on the STD diet (Table 6.5). While *SEC61G* expression is induced under conditions conducive of ER stress, complete silencing of *SEC61G* results in cellular apoptosis (Li et al., 2020; Liu et al., 2019; Lu et al., 2009; Marslli et al., 2020; Reis-Filho et al., 2006; Tsukamoto et al., 2008). Of particular interest to the current study is the hypomethylation of *SEC61G* in skeletal muscle of males clinically diagnosed with T2DM demonstrated by Mudry et al. (2017). On the other hand, increased levels of PRDX4 have been known to attenuate cardiovascular events through oxidative stress (Forman et al., 2010; Jiang et al., 2020; Jing et al., 2011; Martinez-Pinna et al., 2011).

In the current study, the lowered expression and intronic methylation of CD36 indicates the HFHS diet was able to stimulate a pathological background closely related to that observed

with in ischemic and pressure overload conditions. Additionally, the hypomethylation and downregulation of *SEC61G* and *PRDX4* suggests the cumulative decrease in antioxidant activity and increased apoptosis associated with HFHS feeding. While these hypotheses are supported by the GO enrichment of DEGs involved in cardiac muscle contraction, response to oxidative stress and hypertrophic gene regulation, demonstrated by RNA sequencing analysis conducted on the same animals (Chapter 5), further investigation is required in order to confirm this.

6.4 Conclusions

The exponential increase and availability in high throughput technologies over the past few years has facilitated a deeper understanding into the molecular mechanisms underpinning multiple diseases including CVD. Despite the efforts to advance precision medicine and develop personalised treatments for CVD, the diverse and asymptomatic nature of the disease, hinders progress significantly. Therefore, shifting the provisional scientific focus from single etiology to converging mechanisms driving CVD pathogenesis. Amongst the multiple factors contributing towards CVD development, epigenetics has been suggested to provide a mechanistic link between heritable and environmental components. Moreover, DNA methylation, the most widely studied epigenetic mechanism, has been extensively implicated in CVD development and progression (Baccarelli et al., 2010).

While there are currently several methods available to evaluate DNA methylation, WGBS remains the gold standard and is more comprehensive than targeted approaches. The present study is the first study demonstrating the successful application of WGBS technology in South Africa and allowed for the comprehensive profiling of DNA methylation in male Wistar rats maintained on a HFHS diet and its relation to underlying CVD pathophysiology. In order to explore differentially methylated bases in cardiac tissue, DNA from five biological replicates in the experimental groups previously described were subjected to WGBS using a PE100 strategy. Following QC assessment and trimming, an average of 200.54 million paired-end reads were obtained per sample with approximately 70.77% all remaining reads mapping uniquely to the latest version of the annotated reference genome and no samples displaying excessive PCR duplication bias. Furthermore, a conversion rate above 99% was obtained for all the samples in this study, indicative of efficient C to T conversion during the bisulfite treatment reaction.

Genome wide evaluation of DNA methylation levels revealed a mean CpG methylation of 57.4%, while non-CpG methylation in the CHG and CHH context was negligible. Comparative analysis between groups, conducted in the CpG context, showed a per base methylation of 72.93 and 71.31% for STD and HFHS samples respectively. In line with previous findings, regional annotation performed on the methylated CpG sites revealed increased distribution of methylated bases in intergenic regions compared to promoter regions. Differential methylation analysis, performed on a per base level, identified a total of 5 329 DMCs (FDR $\leq$ 0.05) in male Wistar rats maintained on the HFHS diet compared to those in the STD diet-fed control group. The exclusion of DMCs located on the Y chromosome further reduced the number of DMCs by 0.43% (5 306), of which 2 936 DMCs were hypomethylated and 2 370 hypermethylated in the HFHS group. Hierarchical clustering revealed the distinct classification of the most variable DMCs (FDR < 0.01) by treatment groups, suggesting the possible use of these DMCs to distinguish between the disease and the control groups. In this study, transcripts overlapping with DMCs were regarded as DMGs, with a total of 5 254 DMCs annotated within gene components of 533 unique genes, of which 339 genes were hypermethylated and 194 genes hypomethylated in the HFHS group. These DMCs mainly remained in the intergenic and gene body regions suggesting the possibility of non-promoter methylation correlating with gene expression involved in the pathological processes of HFHS diet feeding and its resulting cardiovascular complications.

Moreover, GO enrichment and KEGG pathway analysis conducted in this study demonstrated a distinct link between DMC-related genes and the enrichment of molecular functions underlying CVD pathology. Some of the major findings associated with the HFHS group included the differential methylation of genes involved in phagosome, type I diabetes mellitus, platelet activation, toll-like receptor signalling pathway and diabetic cardiomyopathy. The enrichment of immune related terms corresponded with intergenic and gene body hypomethylation of several overlapping genes including *TLR4*, *MAP2K*, *MAP3K7*, *IRAK4*, *CTSK* and *CSS*. Collectively these results demonstrate the ability of the HFHS diet to act as a pathological stimulus capable of inducing altered DNA methylation of genes associated with lipid metabolism and proinflammatory responses in the heart. The notable changes in cardiac DNA methylation induced by the HFHS diet were further associated with increased risk of CVD development and the possible induction of a hypertrophic background through comparative analysis of WGBS and RNA sequencing data. The methylation sites identified in the present study could be regarded as new

biomarkers or targets for diet-induced CVD, which may play significant roles in early diagnosis and clinical treatment. However, considering that the epigenome comprises numerous additional modifications and that DNA methylation has been shown to accompany chromatin remodelling, further investigation aimed at characterising these interactions in this particular animal model is required.

6.5 Supplementary Data

Supplementary Data: Table S7 - List of DMCs and corresponding annotation in HFHS males.

Supplementary Data: Table S8 - List of DMGs associated with CVD according to the CTD database.

Supplementary Data: Table S9 - List of DMGs corresponding with DEGs identified in RNA sequencing analysis.

Supplementary Figure 1. M-bias plots of a representative samples generated with Bismark.

6.6 References

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Chapter 7: Conclusions, limitations, and prospects

7.1 Concluding findings

Unhealthy diets, rich in fat and sugar derivatives, remain one of the major contributors to the worldwide increase in cardiovascular disease (CVD) risk factors including dyslipidaemia insulin resistance (IR) and type 2 diabetes mellitus (T2DM). In an era where these non-communicable diseases continue to place a colossal burden on the health system and economy of developing countries such as South Africa, research exploring the contributing mechanisms is crucial. Recent breakthroughs in omics-based technologies have transformed genomic research. Next generation sequencing (NGS) is being utilised to decode various diseases including CVD pathophysiology, to develop improved diagnostic, prognostic, and therapeutic markers. Furthermore, the limited cardioprotective capacity of drugs aimed at treating CVD risk factors such as hyperglycaemia and hyperlipidaemia, strongly supports the need to explore alternative therapies. In recent years, studies focused on the use of plant derived nutraceuticals as an alternative therapy to alleviate diet induced metabolic disarrangements and decrease cardiovascular risk. In this study, a Wistar rat in combination with NGS technologies was utilised to explore the influence of nutrition in CVD development and the role of AfriplexGRT^{TM} to ameliorate observed alterations.

The results obtained showed that treatment of cardiomyocytes with high glucose plus palmitate (HG + Pal) induced a cardiac insult capable of reducing ATP production and the host antioxidant defence system through modulating a key redox regulator, NRF2. Additionally, HG + Pal treatment was significantly associated with membrane depolarization and impaired mitochondrial bioenergetics, resulting in mitochondrial dysfunction and a subsequent increase in apoptosis. Interestingly, this study also showed that post-treatment with either Asp or Afriplex GRT^{TM}, had no significant beneficial effect on the parameters assessed and further exacerbated HG + Pal-induced mitochondrial dysfunction and apoptosis. Gene expression and functional enrichment analyses revealed that HG + Pal treatment induced transcriptional changes associated with altered lipid metabolism, inflammatory response, and increased contractile dysfunction, promoting the development of atherosclerotic and hypertrophic phenotypes, unable of being ameliorated by Asp or Afriplex GRT^{TM} treatment. These findings contradict previous findings reporting on the ability of Asp to reverse HG-induced stress on cardiomyocytes and possibly suggests that the HG + Pal treatment resulted in a severe insult incapable of being remediated by the polyphenols.

To explore whether the previously described findings translated to an obesogenic diet-induced in vivo model, Wistar rats maintained either on a high fat, high sugar (HFHS) or standard (STD) rodent diet. This study demonstrated that HFHS diet feeding resulted in increased caloric intake and body weight, corresponding to the augmented retroperitoneal fat (RF) weight in both male and female animals. While a definite IR phenotype was not induced, rodents maintained on the obesogenic diet displayed diminished glucose clearance abilities. Taken together with the elevated serum triglyceride levels and TG/HDL ratio, these results confirm that the HFHS diet was successful in establishing a pre-clinical, CVD risk prediction model that was used to assess the molecular mechanisms underlying disease pathophysiology. In this study, HFHS diet-induced effects on the biometric parameters assessed were more prominent in male rats and Afriplex GRT^{TM} supplementation, had no significant adverse effect on these parameters.

Moreover, while this study provided the first large scale, cardiac transcriptomic profiling of Wistar rats maintained on this particular diet and treated with the Afriplex GRT™ extract, using RNA sequencing (RNA-Seq). In depth bioinformatics analysis allowed for the identification of 571 and 414 differentially expressed genes (DEGs) in HFHS diet fed males and females, respectively. Furthermore, animals receiving Afriplex GRT™ supplementation clustered closely with those in HFHS group, with a total of 120 and 337 DEGs identified in males and females in the Afriplex GRT™ group, respectively. The results demonstrated the ability of the HFHS diet to act as a pathological stimulus capable of increasing host inflammatory response whilst decreasing antioxidant activity, which could increase oxidative stress. The decrease in host defence mechanisms is further accompanied by increased hypertrophic gene expression, possibly affecting cardiac muscle functionality. As expected, the described mRNA enrichments were more prominent in males than females, correlating with the limited changes in the metabolic risk parameters of female animals. Functional analyses of DEGs associated with Afriplex GRT™ supplementation yielded no significant enrichment of disease or pathology related processes and no high confidence results for the amelioration of the transcriptomic signatures resulting from HFHS diet feeding.

The known influence of epigenetic modifications on gene expression and the obesogenic diet induced disruption of cardiac gene regulatory network observed in males in the present study, supported the complementation of cardiac transcriptomic signatures with comprehensive DNA methylation analysis. Whole genome bisulfite sequencing (WGBS), successfully employed for the first time in South Africa, identified 5 329 differentially methylated cytosines (DMCs) (FDR $\leq$ 0.05) in male Wistar rats maintained on the HFHS diet

compared to those in the STD diet-fed control group. A total of 533 unique transcripts overlapped with DMCs located mainly in the intergenic and gene body regions. Interestingly, approximately 77% of the DMCs identified in this study were located within intergenic regions known to house regulatory elements and non-coding RNA. Considering that the degree to which DNA methylation effects the transcriptome significantly depends on the genomic location of methylated sites, exploring the functional relevance of intergenic DNA methylation in the context of diet induced CVD is an avenue worth exploring. Moreover, this study demonstrated a distinct link between DMC-related genes and the enrichment of molecular functions underlying CVD pathology. Some of the major findings associated with the HFHS group included intergenic and gene body hypomethylation of genes related to lipid metabolism and immune related terms in the heart. The notable changes in cardiac DNA methylation induced by the HFHS diet were further associated with increased risk of CVD development and the possible induction of a hypertrophic background through comparative analysis of WGBS and RNA sequencing data.

Taken together, this study demonstrated that HFHS feeding induced traditional CVD risk factors of dyslipidaemia and decrease glucose clearance that may have contributed to the observed sex disparities. This study further provides new insight into the deleterious effect HFHS feeding has on altered gene expression and overlapping DNA methylation profiles, with the underlying molecular mechanism of low-grade inflammation (e.g., TLR4, MAP2K, IRAK, CTSK and CSS), oxidative stress (e.g., NRF2, PDX4 and SEC61), lipid metabolisms and hypertrophy governing early onset of CVD.

7.2 Study limitations

As the case with multiple studies, particularly those involving the use of NGS technologies, this study was considerably impacted by financial constraints. Additional limitations include:

- The HG + Pal treatment conditions used in the vitro model resulted in cell viability levels lower than 50%, which could have limited the recovering ability of the cells and the action of the antioxidant.
- The inability of the HFHS diet to induce a significant metabolic disorder and CVD phenotype.
- The lack of cardiac functional parameter assessments due to limited access to echocardiogram infrastructure.
- The low sample number and technical replicates in both RNA-Seq and WGBS.

7.3 Future prospects

This study provided insight into the cardiac transcriptomic and epigenomic alterations in an obesogenic diet induced Wistar rat model displaying moderate CVD risk. Furthermore, it generated scientific knowledge regarding the molecular mechanisms through which Afriplex GRT™ confers its potential cardioprotective effects. The transcriptomic signatures and methylation sites identified in this study could potentially serve as novel targets for diet-induced CVD, ultimately playing a significant role in early diagnosis and clinical treatment.

Future studies should:

- Further explore the Afriplex GRT™ datasets explicitly, to provide a better understanding of the associated transcriptional signatures, pathway enrichments and possible mode of action.
- Include gender specific evaluation of the mRNA sequencing data, to elaborate on potential gender differences.
- Aim to characterise alternative epigenetic alterations such as histone modifications in this particular animal model.
- Utilise WGBS to perform DNA methylation analysis on female, as well as Afriplex GRT™ samples.
- Include functional studies involving high priority candidates, to explore signalling cascades and downstream targets.
- Explore the regulatory elements associated with intergenic DMCs and their transcriptional relevance in terms of disease pathophysiology.
- Utilise an alternative diet capable of inducing a severe cardiac disease phenotype, to validate the current study findings.

Supplementary data

Chapter 3

Supplementary Table 1. Complete list of differentially expressed genes (DEGs) identified from RT^2 Profiler PCR Arrays. DEGs categorised in terms of their role in major underlying CVD mechanisms.

		Fold-regulation		
Gene name	**Gene symbol**	**HG + Pal**	**Asp**	**Afriplex GRT™**
Lipid transport and metabolism				
ATP-binding cassette transporter A1	*ABCA1*	-3.44	-3.34	-3.75
Low-density lipoprotein receptor	*LDLR*	2.40	2.16	2.67
Nuclear receptor 1H3	*NR1H3*	-1.53	-1.22	-1.17
Prostaglandin-endoperoxide synthase 1	*PTGS1*	1.53	1.62	1.44
Perilipin 2	*PLIN2*	2.23	2.59	2.46
Retinoid X receptor alpha	*RXRA*	-6.01	-6.66	-6.48
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	*PPARGC1A*	2.20	2.15	2.87
Insulin resistance				
Serpin peptidase inhibitor, clade E, 1	*SERPINE1*	1.37	1.48	1.77
Vascular endothelial growth factor A	*VEGFA*	1.19	1.61	1.77
Protein kinase, AMP-activated, gamma 3	*PRKAG3*	7.06	7.53	7.18
Insulin-like growth factor binding protein 3	*IGFBP3*	2.91	2.87	2.45
Neuropeptide Y	*NPY*	6.42	6.61	6.06
Insulin-like growth factor binding protein 5	*IGFBP5*	1.84	1.36	1.94
Inflammation				
Interleukin 6	*IL6*	1.64	1.73	1.47
Chemokine (C-C motif) ligand 5	*CCL5*	-2.55	-2.20	-2.76
Tumor necrosis factor	*TNF*	4.72	4.21	4.78
Colony stimulating factor 1	*CSF1*	2.83	2.56	2.10
Colony stimulating factor 2	*CSF2*	3.94	3.30	3.64
Early growth response 1	*EGR1*	3.10	3.52	3.60
Fibroblast growth factor 2	*FGF2*	2.51	2.23	2.67
Interferon gamma	*IFNG*	2.30	2.42	2.06
Interleukin 1 beta	*IL1B*	2.09	2.22	2.17
Vascular cell adhesion molecule 1	*VCAM1*	-3.24	-3.32	-3.01
Nitric oxide synthase 2	*NOS2*	4.87	5.01	4.93
Nitric oxide synthase 3	*NOS3*	2.53	2.00	2.10
Leukemia inhibitory factor	*LIF*	3.63	3.05	3.58
Titin	*TTN*	1.57	1.44	1.73
Troponin I type 2	*TNNI2*	1.58	1.26	1.84
Troponin T type 3	*TNNT3*	1.61	1.95	1.89
Nebulin	*NEB*	1.65	1.33	1.82
Platelet derived growth factor receptor beta	*PDGFRB*	-1.94	-1.44	-1.22
C-X-C motif chemokine ligand	*CXCL1*	2.29	2.53	2.62

Fibrosis				
Collagen type 3 alpha 1	*COL3A1*	-1.44	-1.27	-1.25
Connective tissue growth factor	*CTGF*	-1.39	-1.84	-1.40
Transforming growth factor beta 2	*TGFB2*	-2.10	-2.26	-2.19
Matrix metallopeptidase 3	*MMP9*	1.80	1.14	1.38
Endoglin	*ENG*	-1.56	-1.09	-1.65
Integrin alpha 2	*ITGA2*	2.97	2.24	2.36
Apoptosis				
B-cell leukemia/lymphoma 2 related protein A1d	*BCL2A1*	2.04	2.59	2.88
Baculoviral IAP repeat-containing 3	*BIRC3*	2.87	2.91	2.18
Secreted phosphoprotein 1	*SPP1*	3.80	3.32	3.27
CASP8 and FADD-like apoptosis regulator	*CFLAR*	1.61	1.83	1.94
Paired box 3	*PAX3*	1.45	1.68	1.36
Myogenic differentiation 1	*MYOD1*	-1.21	-1.80	-1.06
Tenascin C	*TNC*	-2.53	-2.70	-2.01
Calpain 2	*CAPN2*	-1.29	-1.70	-1.47
Mitogen activated protein kinase 3	*MAPK3*	-1.27	-1.63	-1.86
Hypertrophy				
Actin alpha 1	*ACTA1*	-1.34	-1.66	-1.73
Activin A receptor type IIB	*ACVR2B*	1.51	1.22	1.19
Adrenergic beta 2	*ADRB2*	3.24	3.08	3.61
Agrin	*AGRN*	-1.54	-1.82	-1.39
Emerin	*EMD*	-1.49	-1.93	-1.84
Myosin heavy polypeptide 1	*MYH1*	1.62	1.78	1.99
Myosin heavy chain 2	*MYH2*	-5.39	-5.02	-5.85
Tripartite motif-containing 63	*TRIM63*	1.40	1.59	1.97
Desmin	*DES*	1.50	1.30	1.94
Elastin	*ELN*	-4.25	-4.56	-4.30
Catenin beta 1	*CTNNB1*	-1.27	-1.29	-1.47

All DEGs associated with HG + Pal were either statistically significant ($p \leq 0.05$) or displayed a fold change ≤ 2 in either positive or negative direction. In all instances post-treatment with neither Afriplex GRT™ nor Asp was able to significantly impact gene expression derangements resulting from glucolipotoxicity.

Chapter 5

Supplementary Table 2. GO enrichment analysis of **overexpressed** DEGs in **HFHS males.**

GO.ID	Term	Annotated	Adjusted P-value
GO:2001222	regulation of neuron migration	4	0.018
GO:0045471	response to ethanol	5	0.025
GO:0042493	response to drug	13	0.033
GO:0006357	regulation of transcription by RNA polymerase II	43	0.035
GO:0045830	positive regulation of isotype switching	2	0.036
GO:0055003	cardiac myofibril assembly	4	0.037
GO:0002244	hematopoietic progenitor cell differentiation	3	0.049
GO:0043627	response to estrogen	3	0.049
GO:0060048	cardiac muscle contraction	6	0.052
GO:0090263	positive regulation of canonical Wnt signaling pathway	4	0.054
GO:0051091	positive regulation of DNA-binding transcription factor activity	8	0.056
GO:0043410	positive regulation of MAPK cascade	7	0.089
GO:0001666	response to hypoxia	9	0.094
GO:0010628	positive regulation of gene expression	41	0.099
GO:0031175	neuron projection development	28	0.100

Supplementary Table 3. GO enrichment analysis of **under expressed** DEGs in **HFHS males**.

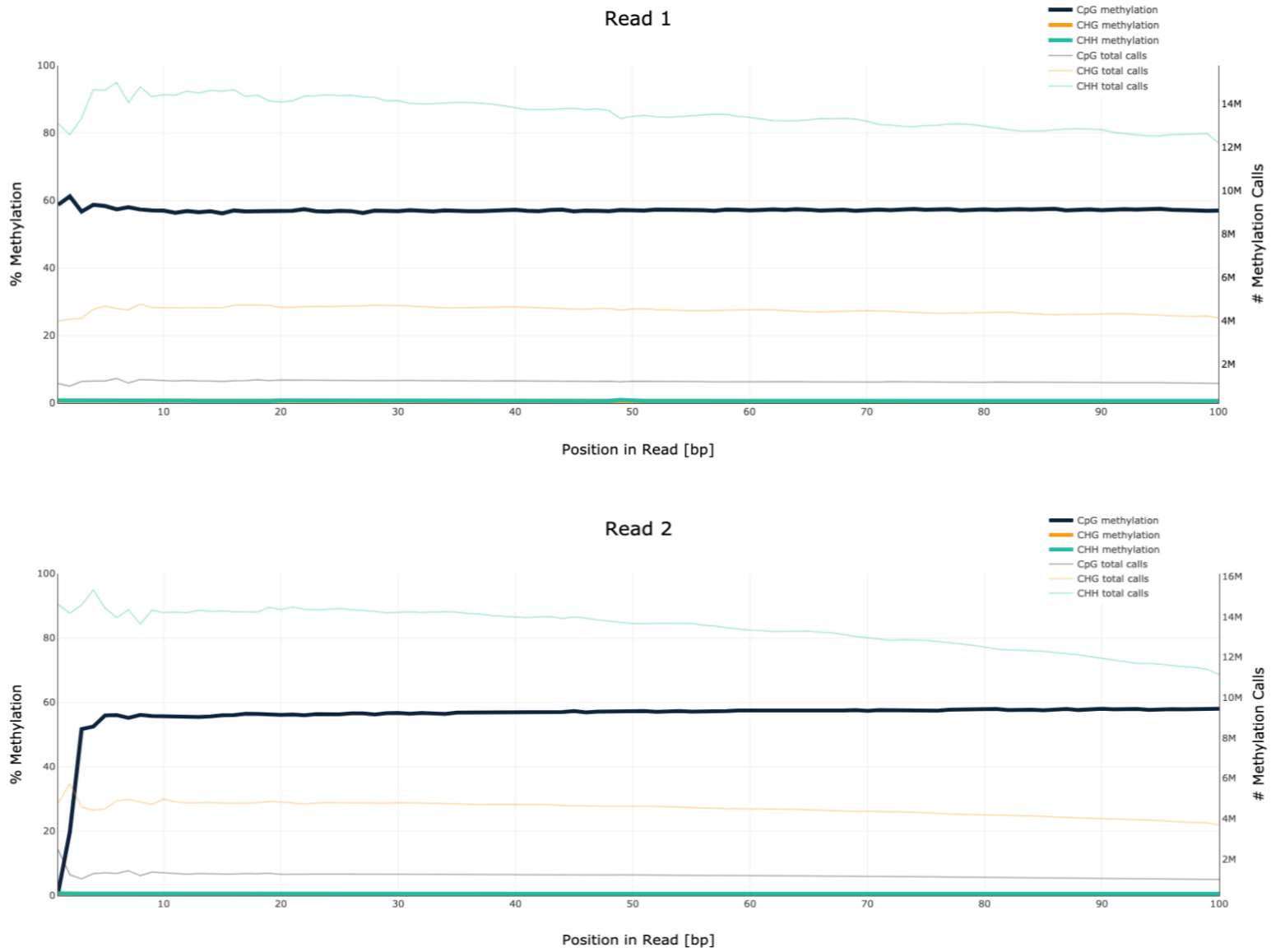
GO.ID	Term	Annotated	Adjusted P-value
GO:0030182	neuron differentiation	12	0.037
GO:0051602	response to electrical stimulus	2	0.052
GO:0014823	response to activity	3	0.054
GO:0000422	autophagy of mitochondrion	3	0.075
GO:0046326	positive regulation of glucose import	2	0.077
GO:0016192	vesicle-mediated transport	27	0.086
GO:0045184	establishment of protein localization	36	0.088
GO:0007568	aging	11	0.089
GO:0006979	response to oxidative stress	15	0.019
GO:0030855	epithelial cell differentiation	8	0.098
GO:0042493	response to drug	9	0.100
GO:0035335	peptidyl-tyrosine dephosphorylation	3	0.104
GO:0006954	inflammatory response	18	0.028
GO:0006886	intracellular protein transport	22	0.113
GO:0016567	protein ubiquitination	14	0.123

Supplementary Table 4. GO enrichment analysis of **overexpressed** DEGs in **HFHS females**

GO.ID	Term	Annotated	Adjusted P-value
GO:0061077	chaperone-mediated protein folding	6	0.074
GO:0035264	multicellular organism growth	4	0.093
GO:0030198	extracellular matrix organization	5	0.097
GO:0046330	positive regulation of JNK cascade	3	0.1514
GO:0007229	integrin-mediated signaling pathway	5	0.1111
GO:1900026	positive regulation of substrate adhesion-dependent cell spreading	2	0.1174
GO:0033209	tumor necrosis factor-mediated signaling pathway	4	0.1282
GO:0032755	positive regulation of interleukin-6 production	3	0.1314
GO:1990090	cellular response to nerve growth factor stimulus	2	0.1012
GO:0032760	positive regulation of tumor necrosis factor production	2	0.088
GO:0048013	ephrin receptor signaling pathway	2	0.077
GO:0032331	negative regulation of chondrocyte differentiation	2	0.1031
GO:0051209	release of sequestered calcium ion into cytosol	4	0.073
GO:0042733	embryonic digit morphogenesis	2	0.076
GO:0042127	regulation of cell population proliferation	23	0.076

Supplementary Table 5. GO enrichment analysis of **under expressed** DEGs in **HFHS females**

GO.ID	Term	Annotated	Adjusted P-value
GO:0032868	response to insulin	6	0.061
GO:0032922	circadian regulation of gene expression	3	0.071
GO:0007565	female pregnancy	4	0.074
GO:0042127	regulation of cell population proliferation	12	0.089
GO:0001649	osteoblast differentiation	4	0.103
GO:0045893	positive regulation of transcription, DNA-templated	14	0.116
GO:0042438	melanin biosynthetic process	2	0.120
GO:0001570	vasculogenesis	2	0.121
GO:0030318	melanocyte differentiation	2	0.137
GO:0009617	response to bacterium	8	0.080
GO:0044342	type B pancreatic cell proliferation	2	0.090
GO:0045944	positive regulation of transcription by RNA polymerase II	12	0.104
GO:0009749	response to glucose	3	0.161
GO:0043433	negative regulation of DNA-binding transcription factor activity	2	0.084
GO:0007219	Notch signaling pathway	3	0.085



Supplementary Figure 1. M-bias plots of a representative samples generated with Bismark. The percentage methylation is illustrated throughout the read length of the forward and the reverse reads.