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Paul C. Guest *Editor*

Reviews on Biomarker Studies of Metabolic and Metabolism-Related Disorders

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Preface

Obesity has now reached epidemic proportions since an estimated 20% of the population is affected worldwide and this proportion appears to be rising each year. It has struck all socioeconomic backgrounds and ethnicities throughout the world, although some populations appear to be more affected than others. Being obese or overweight is now a well-known risk factor for other diseases including metabolic syndrome, which is characterized by varying degrees of insulin resistance, dyslipidaemia and hypertension. Critically, individuals suffering from metabolic syndrome are at a high risk of developing life-threatening illnesses such as cardiovascular diseases or type 2 diabetes or both. Furthermore, recent evidence has suggested that some aspects of metabolic syndrome like insulin resistance can also occur in psychiatric conditions like schizophrenia. For example, weight gain and development of insulin resistance are common side effects of currently available antipsychotic medications. However, reports of metabolic dysfunction in schizophrenia patients have also been observed in first-onset patients prior to their receiving antipsychotic treatment, indicating that this may also be involved in the aetiology.

Insulin resistance is thought to be the underlying pathophysiology in metabolic syndrome since it is precursor of type 2 diabetes and is associated with dyslipidaemia and a pro-inflammatory state. However, an association of insulin resistance with hypertension and cardiovascular disease has yet to be confirmed in large-scale clinical studies. Central fat accumulation is also considered to be an important factor as this can lead to hyperglycaemia, hypertension, insulin resistance and cardiovascular conditions. Considering the health risks and enormous costs of this syndrome to the healthcare services, it is of critical importance that individuals who are at high risk of developing metabolic syndrome are diagnosed and treated as early as possible.

It is now recognized throughout the scientific, medical and economic communities that improved tests incorporating biomarkers are needed to improve the diagnosis of patients suffering from metabolic disorders. The early identification of those at risk of developing this disease will help to place these individuals on the best treatment course as early as possible for improved treatment outcomes. This will also help to

cut costs incurred by the healthcare services. For all of this to occur, new research efforts are needed to identify novel biomarkers that can be used to predict the disease in the presymptomatic stage, for disease monitoring and for prediction of treatment response. It is also possible that new drug targets can be identified using these approaches which, in turn, can lead to the development of new treatment approaches.

This book includes a series of reviews on biomarker discovery and usage in the study of diseases marked by perturbations in metabolism. The various subjects include psychiatric disorders, diabetes, gestational diabetes, insulin biosynthesis, endothelial dysfunction, fatty liver, mitochondrial targeting, the role of microRNAs, circular RNAs, cancer cell metabolism, targeting the mitochondria in cancer, aging, overnutrition and nutritional programming. The authors carry out cutting edge research on the six habitable continents in countries such as Brazil, France, Germany, Japan, New Zealand, South Africa, Spain, Thailand, Turkey, the United Kingdom and the United States, in line with the importance of this subject throughout the world. The subject matter is of interest to scientific and medical research scientists, as well as pharmaceutical companies, government agencies and the healthcare services.

Campinas, Brazil Paul C. Guest

Contents

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Chapter 1
Insulin Resistance in Schizophrenia

Paul C. Guest

Abstract Schizophrenia and diabetes have been known to be linked disorders for decades. One reason is due to the fact that a major side effect of antipsychotic medication treatment is metabolic syndrome, which increases the risk of the patients developing type 2 diabetes and cardiovascular disorders. However, signs of metabolic syndrome in schizophrenia patients were identified more than 100 years ago, even before the development of antipsychotic drugs. This suggests that schizophrenia itself predisposes towards diabetes and, in turn, insulin resistance may be a risk factor for the development of schizophrenia. This review summarizes the findings surrounding this issue and places them into context with regards to increasing our understanding of the aetiology of schizophrenia and in support of biomarker and drug discovery efforts.

Keywords Schizophrenia · Psychosis · Insulin resistance · Antipsychotic · Antidiabetic · Biomarker

1.1 Introduction

Schizophrenia is a debilitating psychiatric disorder ranked as number 15 on the list of years lived with disability (YLD) [1]. It is characterised by disturbances in perception, cognition and behaviour, resulting in impaired functioning in social settings such as interpersonal relationships, parenting and self-care [2]. It is considered to be a polygenic disorder triggered by one or more environmental risk factors, although these have not been fully elucidated due to the complex nature and heterogeneity of the disorder [3]. Individuals suffering from schizophrenia are more likely to develop co-morbidities such as obesity, type 2 diabetes and metabolic syndrome [4]. These can have a negative impact on life-expectancy as patients with chronic

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schziophrenia have a greater than two-fold higher risk of death, mostly from cardiovascular and metabolic diseases, compared to the general population [5]. This translates to a shortened life expectancy of approximately 14.5 years [6].

The weight gain associated with antipsychotic drug treatment has long been recognized as a health concern [7]. There have been numerous reports on patients who received antipsychotic drugs as far back as their inception in the 1950s and decades later it became evident that the second generation antipsychotic drugs, such as clozapine and olanzapine, were linked with disrupted glucoregulation and the onset of type 2 diabetes. As weight gain and metabolic syndrome are common responses to antipsychotic drug treatment, patients with schizophrenia have a high risk of adverse cardiovascular and cerebrovascular responses, which accounts for part of the high mortality rate [5]. For these reasons, all antipsychotic drugs now have a warning on their labels that their use may be associated with metabolic risks [8].

Although the prevalence of metabolic syndrome is higher in schizophrenia patients treated with antipsychotics, a high rate of this syndrome has been reported to occur in first episode patients who had not yet received these medications [9, 10]. A link between schizophrenia and abnormal glucose metabolism was reported as far back as the late nineteenth century through a study which revealed an increased prevalence of diabetes in families with a history of insanity [11]. Furthermore, some patients with schizophrenia require relatively high doses of insulin, suggesting a degree of insulin resistance [12]. More recently, investigations beginning in the 2000s showed that there was an increased frequency of impaired glucose tolerance, insulin resistance and hyperinsulinaemia in antipsychotic naive first episode patients [13–17].

This review discusses the findings surrounding metabolic syndrome in both antipsychotic-naive first episode and antipsychotic-treated schizophrenia patients with a view to increasing our understanding of the aetiology and progression of schizophrenia. It will also describe the implications on identification of potential novel biomarkers for improved stratification of patients, identification of novel drug targets and treatment strategies. This will enable a personalised medicine approach so that individuals suffering from schizophrenia may be offered earlier and more targeted treatment options for the best possible treatment outcomes.

1.2 Insulin Resistance and Metabolic Syndrome Associated with Antipsychotic Treatment

Since the discovery of clozapine in the 1970s and the subsequent development of other second generation antipsychotics, there has been a noticed improvement and significant recovery of some schizophrenia patients in terms of cognition and psychotic symptoms, along with their increased integration into society [18]. However, these benefits have been overshadowed by potential side effects, such as the risk of agranulocytosis, weight gain and related complications. Schizophrenia patients

have a decreased life expectancy due to increased incidence of co-morbidities such as metabolic syndrome [5, 6] and many of these effects are known to be induced by antipsychotic treatment [19, 20]. These drugs are used to treat individuals with first onset or refractory schizophrenia [21] and two of these, olanzapine and clozapine, are thought to be the most efficacious [22, 23]. However, these two drugs are also the most likely to lead to weight gain and disrupted glucose and lipid metabolism [21, 24]. A meta-analysis published in 2013 on the prevalence of metabolic syndrome in schizophrenia patients found that the overall rate of this syndrome was 32.5% with the highest rates observed for those receiving clozapine at 51.9%, compared to 20.2% for patients who were not receiving medication [25]. A 10-year naturalistic study of schizophrenia and schizoaffective disorder patients who had received clozapine found that 43% developed diabetes with an average weight gain of 13.5 kg [20]. Furthermore, there was a cardiovascular disease-related mortality rate of 9% and this was significantly correlated with body mass index (BMI). However, a more recent meta analysis found a mortality rate from any cause of only 0.6% in schizophrenia patients who had received clozapine for more than 1 year [26]. The reasons for this discrepancy are not clear.

Clozapine binds to the 5-hydroxytryptamine $(HT)_{2A/2C}$ receptor subtypes and has high affinity for several dopaminergic receptors. Unlike most first generation antipsychotics, clozapine only shows weak antagonism at the dopamine D_2 receptor, which was widely assumed to be a key modulator of neuroleptic activity [27]. Most second generation antipsychotics have multiple molecular targets, including adrenergic, histaminergic, serotonergic and muscarinic receptors, with the noted lower antagonistic effects on dopamine D_2 receptors compared to the first generation antipsychotic drugs [28]. The weight gain associated with these drugs has been linked to antagonism of the histaminergic H_1 receptor as meta-analyses have shown a significant correlation between risk of weight gain and histamine H_1 receptor affinity [29, 30]. In turn, this can increase the risk of insulin resistance which can lead to increased risk of type 2 diabetes and cardiovascular disease [31, 32]. Other studies have reported elevations in circulating leptin levels in patients receiving antipsychotics [33]. The secretion of this hormone is a normal response to increased fat deposition. However, the normal associated suppression of food intake by this hormone does not occur in patients receiving antipsychotic drugs. This suggests that the signalling mechanisms that regulate food intake in the hypothalamus may be disrupted by antipsychotic drug action.

There are several factors associated with antipsychotic treatment that appear to have an influence on weight gain, including dosage and duration of treatment, past and current medications, age and gender, stress, smoking, presence of other diseases, genetic predisposition, diet and physical activity [34]. A randomized double-blind prospective study of olanzapine, risperidone and haloperidol treatment found significant weight gain induced by by the treatment in drug-naive patients with first-episode schizophrenia and identified risk factors for this side effect including a lower BMI and use of olanzapine [35]. A recent study found that the greatest influential factor was the choice of antipsychotic drug [36]. A study from 2015 supports the hierarchical ranking of second generation antipsychotics found in previous studies [37]. This

Table 1.1 Molecules identified by Schwarz et al. [39] linked with the increased BMI following treatment of schizophrenia patients for 6 weeks with second generation antipsychotics

Protein	Function
Apolipoprotein C III	Lipid transport
Apolipoprotein H	Lipid transport
Epidermal growth factor	Growth factor
Follicle stimulating hormone	Hormone
Interleukin-18	Inflammation
Interleukin-25	Inflammation
Interleukin-6 receptor	Inflammation
Matrix metalloproteinase I	Inflammation
Placenta growth factor	Growth factor
Thyroid stimulating hormone	Hormone

study found that clozapine and olanzapine had the highest risk, followed by amisulpride, asenapine, iloperidone, paliperidone, quetiapine, risperidone and sertindole as having a medium risk, and aripiprazole, lurasidone and ziprasidone with the lowest risk. This was consistent with an earlier retrospective study which analyzed the weight gain propensity of different antipsychotics. Treatment with clozapine and olanzapine resulted in the most weight gain, while risperidone was intermediate and sertindole was the lowest [38]. In line with the above studies, the respective affinities of these antipsychotics for the histamine H_1 receptor appeared to be the most robust correlated factor with the weight gain.

Along with the above attributes, a number of molecular predictors of weight gain have been identified. Schwarz et al. used a multiplex immunoassay approach to test whether or not serum molecules measured before initiation of treatment could be associated with the subsequent weight gain following a 6-week treatment with antipsychotics [39]. They found that the baseline levels of 10 serum molecules associated with lipid transport, inflammation, growth factor and hormone signalling were significantly correlated with the change in BMI (Table 1.1). In addition to weight gain, they found a significant increase in triglyceride levels and insulin:glucose ratios, consistent with known metabolic side effects of second generation antipsychotic treatments [21, 24, 40, 41]. Another study found that lower baseline levels of leptin predicted the greatest increase in BMI in female schizophrenia patients treated with olanzapine for up to 1 year [42]. This effect was not observed in the case of male schizophrenia patients.

1.3 Insulin Resistance in First Onset Antipsychotic Naive Schizophrenia Patients

Although insulin resistance and other metabolic symptoms can be associated with treatment with second generation antipsychotics, there is substantial evidence which has demonstrated that these effects were seen in schizophrenia patients even before

the original development and availability of antipsychotics [12] and recent studies conducted within the last two decades have confirmed that first onset schizophrenia patients can exhibit metabolic abnormalities including impaired glucose tolerance and insulin resistance even before these individuals were administered antipsychotics [13, 14]. In a study published in 2003, Ryan and Colleagues found that 4 out of 26 hospitalized first onset schizophrenia patients had impaired fasting glucose tolerance before the administration of antipsychotics compared to none of the healthy volunteers [13]. They also found that the schizophrenia patients had significantly higher fasting plasma levels of glucose, insulin and cortisol and they also had an elevated homeostatic model assessment of insulin resistance (HOMA-IR) reading, compared with the controls. In a cross sectional study carried out in 2007, Spelman et al. found that 4 out of 38 non-obese individuals who fulfilled the criteria for first episode drug-naive schizophrenia had impaired fasting glucose tolerance compared with 8 out of 44 of the unaffected relatives of these patients and none out of the 38 healthy controls [14]. Hyperinsulinaemia or insulin resistance have also been found in antipsychotic-free chronic schizophrenia subjects [33, 43], consistent with a study of schizophrenic patrients from the 1966 Northern Finland Birth Cohort which identified insulin resistance in 45% and 33% of the total and non-medicated schizophrenia patients, respectively [44]. Another investigation found impaired glucose tolerance in drug-naïve schizophrenia patients compared to matched controls and separate study found that a similar group of patients had hepatic insulin resistance through the use of a hyperinsulinaemic clamp method [45].

The finding of a high prevalence of impaired glucose tolerance in the non-affected relatives supports the likelihood of a shared environmental or genetic background in these effects, as described in recent reviews [9, 46–49]. The above findings were not supported by a study published in 2008, which found that first episode patients with schizophrenia did not differ from healthy controls in their baseline measurements of glucose, lipids or prevalence of diabetes [50]. In contrast, the results of a study of 160 individuals with schizophrenia who presented to the Early Psychosis Intervention Programme in Singapore showed significantly lower BMI, low density lipoprotein (LDL) and cholesterol, with higher prevalence of diabetes compared to controls [51]. More recent studies found a cardiometabolic risk factor signature in drug-naive adolescents and young adults with first onset psychosis [52].

A study published in 2010 in Molecular Psychiatry showed for the first time that first onset schizophrenia patients who had not received antipsychotics at the time of their admission had high circulating levels of insulin-related molecules [16]. To obtain sufficient numbers of antipsychotic naive patients, subjects were recruited from four independent clinical centres over 2006–2008 along with matched controls. Although glucose levels were not significantly altered, the first onset patients (n = 66) showed increased levels of insulin, proinsulin, des 31,32 proinsulin and C-peptide compared to the levels seen in controls (n = 78). All of these molecules are derived from the pathway involved in proinsulin to insulin conversion and are therefore packaged into the same secretory granules for storage and release along with the mature insulin molecule [53]. For this reason, this study and a follow up investigation showed that first onset antipysychotic naive schizophrenia patients

also had high circulating levels of the secretory granule protein chromogranin A, compared to controls [16, 17]. This indicated that the insulin-producing cells (β cells) in pancreatic islets may be under increased secretory demand in at least some schizophrenia patients. The increased levels of insulin and elevated HOMO-IR have now been confirmed in recent studies from different research groups [11, 54, 55]. In one of these studies, Steiner et al. tested whether or not insulin resistance in first onset schizophrenia patients can be distinguished from stress and medication effects [54]. They found that insulin resistance, serum cortisol, and urinary stress hormones metanephrine and normetanephrine were increased in patients compared to controls but no significant correlations were found between HOMO-IR and the levels of the stress hormones, smoking or clinical symptoms. However, HOMA-IR showed a strong correlation with BMI. Taken together, these findings supported the case that the effects on signalling are related to schizophrenia as opposed to being a side-effect of antipsychotic treatment, hormonal stress axis activation or lifestyle factors.

1.4 Effects of Insulin Resistance in Schizophrenia on Other Neuroendocrine Systems

In addition to disturbances in insulin-related pathways, there have been a number of reports related to altered hypothalamic pituitary adrenal (HPA) and hypothalamic pituitary gonadal (HPG) axes in first onset and chronic schizophrenia subjects [56–58].

1.4.1 Growth Hormone

Insulin and growth hormone share many elements of the same signalling pathways such as the growth hormone receptor/Janus kinase 2/signal transducer and activator of transcription (GHR/JAK2/STAT), GHR/JAK2/SHC-transforming protein 1 (SHC)/ mitogen-activated protein kinase (MAPK) and growth hormone/insulin receptor substrate (IRS)/phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathways [59]. This suggests that there may be some elements of potential cross-talk. For example, one study found that insulin has a direct, inhibitory effect on basal and stimulated growth hormone secretion by GH3 cells [60]. Consistent with this finding, a number of studies have found changes in growth hormone levels in schizophrenia patients. A multiplex immunoassay profiling study found decreased levels of growth hormone in serum from first and recent onset schizophrenia patients compared to controls [17]. Likewise, a combined two-dimensional difference gel electrophoresis (2D-DIGE) and combination of liquid chromatography tandem mass spectrometry analysis found decreased levels of growth hormone A-chain in

postmortem pituitary glands from schizophrenia patients, compared with the levels of growth hormone A-chain in postmortem pituitaries from controls [61]. A case report found that 2 males and 1 female diagnosed with schizophrenia developed acromegaly due to a growth hormone-secreting pituitary macroadenoma [62]. This may be consistent with the above findings considering a possible desensitization of growth hormone signalling pathways related to neuronal functions, due to consistently elevated levels of this hormone. The association of acromegaly with schizophrenia has been well documented [63–68]. In addition, a sleep study of schizophrenia patients found prolonged sleep onset latency, increased time awake and decreased stage 2 sleep, with elevated growth hormone release following growth hormone releasing hormone stimulation, compared to controls [69].

1.4.2 Cortisol

In 2003, Ryan et al. showed that some first-episode, antipsychotic-naive schizophrenia patients have higher circulating levels of insulin and cortisol compared to healthy control subjects [13]. This was confirmed in a multiplex immunoassay study carried out in 2011 which found elevated serum insulin and cortisol levels (along with other hormones) in first onset schizophrenia patients [17]. Other studies have described that psychiatric disorders such as depression can be associated with insulin resistance and changes in the diurnal cortisol curve [70]. In addition, a comparison of ultra high-risk (UHR) and antipsychotic-naïve first-episode schizophrenia patients with matched controls using the Perceived Stress Scale and the Recent Life Events Questionnaire and measurements of day-time saliva cortisol levels found that symptom severity was correlated with altered cortisol levels in the UHR patients [71]. This finding suggested that altered cortisol levels play a role only in the early phases of the disease. The exact mechanism regulating the relationship between insulin and cortisol secretion remains to be determined. A meta-analysis carried out by Pillinger et al. assessed insulin resistance and found an elevated homeostatic model assessment of insulin resistance (HOMA-IR) in drug-naïve first-episode schizophrenia patients compared to controls but they highlighted activation of the stress axis and lifestyle factors as potential confounding factors [11]. However, we found recently that insulin resistance and altered glucose metabolism in first-episode schizophrenia patients were related to schizophrenia, as opposed to being a consequence of antipsychotic treatment, body composition or stress axis activation [54].

1.4.3 Gonadal Steroids

A number of studies have shown that several steroid hormones of the HPG axis can affect the onset and progression of schizophrenia. For example, estrogen has been found to be neuroprotective [72] and this may explain the typically later onset of

schizophrenia with less extreme symptoms in females compared to males [73, 74]. A number of studies have now shown that estrogen supplementation can lead to an abatement of symptoms in both males and females with schizophrenia although further research is required to develop newer and safer drugs which target this pathway [75]. A study of first onset schizophrenia males revealed significantly lower serum levels of estradiol, estrone, total testosterone and free testosterone, compared to controls [76]. These findings were consistent with those of a later study which showed that testosterone levels in male schizophrenia patients were negatively associated with Positive and Negative Syndrome Scale (PANSS ratings) scores with no correlation found for female patients [77].

1.4.4 Other Hormones

In addition to the changes described in circulating insulin, growth hormone and cortisol levels described above, we found that the serum concentrations of chromogranin A, pancreatic polypeptide, progesterone and prolactin were increased in first onset schizophrenia patients (Fig. 1.1) [17]. As many hormones are influenced by ultradian or circadian rhythms, the secretion of these molecules is likely to be coordinated through an oscillatory feedforward–feedback relationship between the islets of Langerhans and other components of the HPA and HPG systems [78]. For example, higher serum prolactin levels have been shown to be associated with insulin resistance in men [79] and hypoglycaemia has been linked to a decrease in the amplitude of growth hormone pulsatility [80]. In addition, studies of high fat diet-induced obese rats showed both hyperinsulinemia and increased progesterone levels [81]. The changes found in chromogranin A may also be of interest since this molecule is found in many neuroendocrine cell types, where it undergoes limited proteolytic processing to produce smaller functional peptides including vasostatin, which has been shown to inhibit vasoconstriction in blood vessels [82], catestatin, which inhibits secretion from catecholaminergic adrenal chromaffin cells [83], and pancreastatin, which inhibits insulin secretion [84, 85]. The other hormone found to be altered in the schizophrenia patients in the multiplex immunoassay study was pancreatic polypeptide [17]. Previous studies have shown that this islet hormone is involved in regulation of energy balance [86]. These findings all demonstrate potential links to the metabolic disturbances found in both first onset and chronic schizophrenia patients.

1.5 Conclusions

It is clear that schizophrenia patients suffer from metabolic abnormalities along with the psychiatric symptoms. This in true in the case of both first onset patients prior to treatment and in more chronic patients who have received medications over

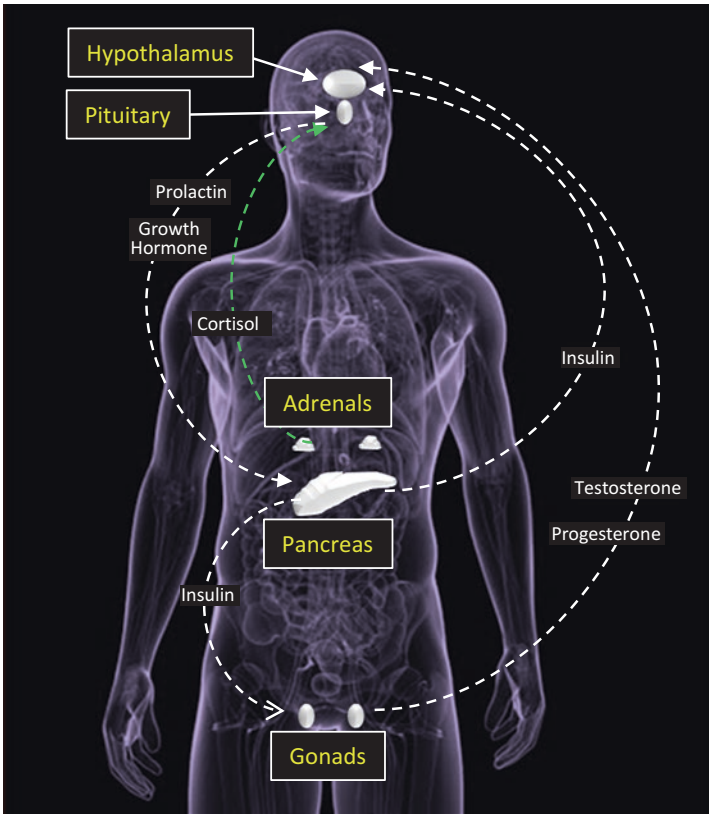

Fig. 1.1 Diagram showing the hormonal pathways known to be affected in schizophrenia

both short- and long-term periods. There is some evidence for the effectiveness of physical activity interventions for people with schizophrenia although the greatest barrier appears to be the poor rates of compliance [87]. One possible way of increasing compliance is through the incorporation of an achievable goal into the exercise program such as shown in a study which used the incentive of training for a 5 kilometer race [88]. In this study, 11 out of the 17 patients participated in all of the training sessions and 14 participated in the actual event. A study carried out in 2016 found a compliance rate of greater than 80% with other health benefits through the use of active-play video games to improve aerobic fitness [89]. One study of 24 schizophrenia patients who participated in an individually tailored 90 min outdoor cycling session per week for 3 months found a high level of adherence with short-term benefits on self-esteem, positive relationship, global function and quality of life [90]. However, long-term benefits were maintained only in the case of positive relationship change. These findings suggest that physical exercise can be effective to improve physical and mental health of individuals with schizophrenia. However, further strategies at increasing compliance are essential.

Given the likelihood of improved compliance, the use of oral medications for control of the metabolic symptoms has received increasing attention. With antipsychotic use, there have been a number of studies which have used a combination therapy with metformin for the management of weight gain [91–93]. Metformin is a biguanide compound which has been widely used in the treatment and management of type 2 diabetes mellitus [94]. Metformin treatment results in mild weight loss and reduces fasting glucose, insulin and triglyceride levels via suppression of hepatic gluconeogenesis and increased peripheral insulin sensitivity while increasing high-density lipoprotein [94–96]. Although there is substantial evidence of weight loss associated with metformin of obese and overweight people who are already taking antipsychotics such as clozapine, there have been no published studies which have investigated the effect of metformin in attenuating weight gain if given at the time of antipsychotic initiation. With this in mind, a 24-week double-blind placebo-controlled trial is planned to study the effects of metformin given at the time of clozapine treatment commencement in 86 people with primary outcome measurements of endpoint compared to initial body weight between the metformin and placebo groups [97]. The researchers also plan to examine potential biomarkers associated with weight change among the trial subjects.

One investigation reviewed the effects of hormones or drugs that target the HPA and gonadal axes as potential candidates for improving cognition in psychiatric disorders [58]. This identified 12 studies that considered the effects of HPA axis drugs and 14 that targeted the HPG axis. Trials reporting positive results were found for treatments of bipolar disorder, major depressive disorder and schizophrenia. In the case of schizophrenia this included the drug dehydroepiandrosterone, a metabolic intermediate in the biosynthesis of the androgen and estrogen sex steroids [98, 99], raloxifene, a drug used in the treatment of osteoporosis in post-menopausal women [100], and pregnenolone, which is a precursor in the biosynthesis of most steroind hormone [101]. The authors also identified at least two positive replication studies for the effects of raloxifene and pregnenolone.

It is concluded that psychiatric disorders such as schizophrenia are linked with metabolic disorders such as insulin resistance and perturbations of the HPA and HPG axes. These disturbances can occur at first onset in drug-naive patients as well as in chronic patients who have been treated with psychiatric medications for varying periods. However, these problems have lent themselves to further studies in search of biomarkers and novel drug targets. This search may eventually lead to the development of tools for improved patient stratification and targeted treatment of patients suffering from schizophrenia in line with personalized medicine approaches [102–105]. It is anticipated that this will lead to improvements in the lives of individuals with schizophrenia as well as their families, along with reduced costs for societies and healthcare services throughout the world.

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Chapter 2
Biogenesis of the Insulin Secretory Granule in Health and Disease

Paul C. Guest

Abstract The secretory granules of pancreatic beta cells are specialized organelles responsible for the packaging, storage and secretion of the vital hormone insulin. The insulin secretory granules also contain more than 100 other proteins including the proteases involved in proinsulin-to insulin conversion, other precursor proteins, minor co-secreted peptides, membrane proteins involved in cell trafficking and ion translocation proteins essential for regulation of the intragranular environment. The synthesis, transport and packaging of these proteins into nascent granules must be carried out in a co-ordinated manner to ensure correct functioning of the granule. The process is regulated by many circulating nutrients such as glucose and can change under different physiological states. This chapter discusses the various processes involved in insulin granule biogenesis with a focus on the granule composition in health and disease.

Keywords Pancreatic islets · Insulin secretory granules · Insulin · Biogenesis · Chromogranin A · Diabetes

2.1 Introduction

Insulin is a vital hormone involved in the regulation of glucose homeostasis and energy balance in most cells of the body. It is synthesized in the beta cells of the islets of Langerhans in the pancreas. After synthesis, it undergoes packaging and storage into secretory granules, characterized by a dense core, when viewed through an electron microscope [1]. There are approximately 10,000 of these granules in each beta cell, each with diameter of around 250 nm and containing somewhere in the region of 250,000 molecules of insulin [2, 3]. These insulin molecules are arranged as hexamers with a central Zn^{2+} ion which gives rise to the characteristic

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dense core (Fig. 2.1). This core is surrounded by a halo of lower density and a phospholipid bilayer, which contain the various other constituents of the granule [4].

Part of the process of secretory granule maturation involves the proteolytic conversion of the precursor protein proinsulin to produce the mature insulin molecule [4]. This begins by acidification of the granule lumen to around pH 5.5 along with accumulation of high concentrations of free Ca^{2+} ions on the order of 10 mM [5]. This intragranule environment leads to activation of prohormone convertases which are co-packaged with proinsulin into nascent granules and these enzymes cleave this precursor polypeptide to generate mature insulin (Fig. 2.2) [6]. The insulin-related peptides account for around 65% of the secretory granule protein content and the remaining 35% consists of approximately 100 minor proteins, including cellular trafficking proteins, ion translocation enzymes, the prohormone convertases, other precursor molecules and bioactive peptides [7–10]. Many of these are known to have functions in the body which can be related to, or distinct from, those of insulin in maintenance of physiological homeostasis.

Once the secretory granules are mature, glucose-stimulated insulin secretion can occur in two distinct phases. The first phase initiates within 2 min after stimulation and lasts 5–10 min. This is followed by a second phase that increases slowly and lasts 1–2 h until circulating glucose levels return to normal (Fig. 2.3) [11–13]. In type 2 diabetes, the first phase may be completely absent and the second phase reduced [14]. Furthermore, the composition of the accessory proteins may be altered leading to either altered production or changes in the composition of the released product [15–17].

This review will describe the process of secretory granule biogenesis in health and disease with a focus on some of the main constituent proteins which are being increasingly recognized as having important physiological roles.

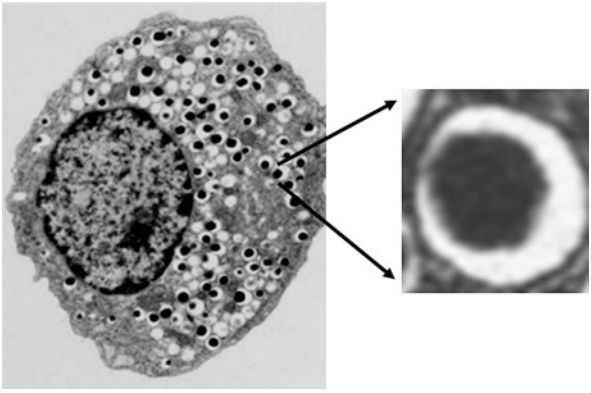

Fig. 2.1 Electron micrograph showing pancreatic beta cell (diameter = 10 μm) on the left and a magnified insulin secretory granule (diameter = 0.2–0.25 μm) on the right. Under normal conditions, each cell contains approximately 10,000 secretory granules, each containing 200,000–250,000 molecules of insulin. The crystalline insulin core appears as an electron dense structure in the centre of each granule

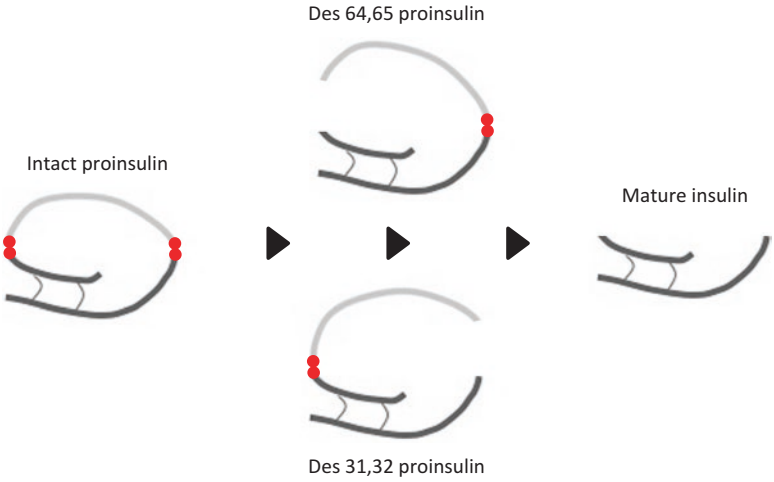

Fig. 2.2 Diagram showing proteolytic conversion of proinsulin by proteolytic cleavage on the C-terminal side of two pairs of dibasic amino acid sequences. This gives rise to the proinsulin conversion intermediates and ultimately mature insulin and C-peptide

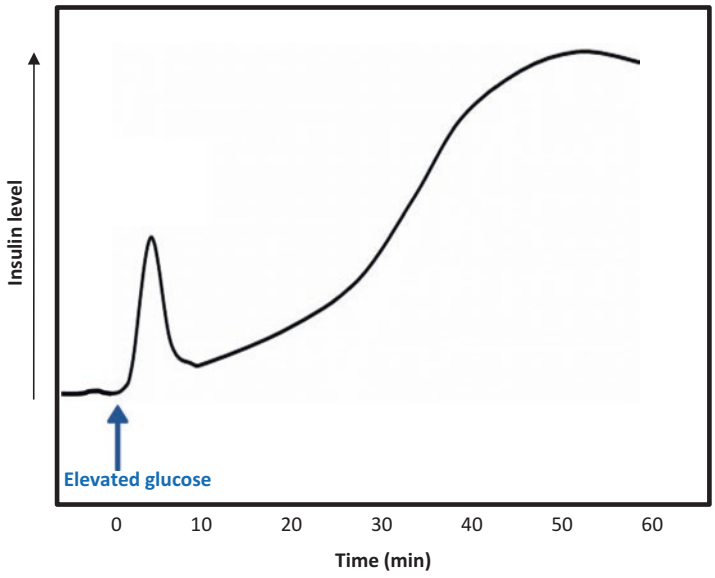

Fig. 2.3 Representative graph depicting the biphasic secretion pattern of insulin in response to elevated glucose levels

2.2 Biosynthesis of Insulin

Insulin biosynthesis is regulated by many factors although the circulating glucose level is the most important of these in stimulating transcription and translation of the insulin gene [18]. A rise in glucose levels can lead to elevations in insulin mRNA levels of 2 to 4 fold within 24 h [19–21] and increased translation from 10- to 30-fold within 1 h of stimulation [22, 23]. The insulin gene encodes a precursor known as preproinsulin, which is comprised of 110 amino acids. Like most secreted proteins, preproinsulin contains an amino-terminal signal peptide, which allows its interaction with ribonucleoprotein signal recognition particles [24]. This leads to translocation of nascent preproinsulin molecules into the lumen of the rough endoplasmic reticulum, where the signal peptide is cleaved by signal peptidase to generate the proinsulin molecule [25, 26]. Within the endoplasmic reticulum, proinsulin is then folded and disulfide bonds are formed through the concerted action of chaperone proteins and protein-thiol reductase [27]. This molecule is then transported via repeated cycles of vesicle budding and fusion from the endoplasmic reticulum to the Golgi apparatus. Within the trans Golgi network (TGN), proinsulin is packaged along with multiple other components into nascent secretory granules (Fig. 2.4). As the immature granules bud from the TGN, the process of condensation and acidification of the granule content begins along with the proteolytic conversion of proinsulin and other intragranule precursors to generate their mature forms [4, 5, 28, 29].

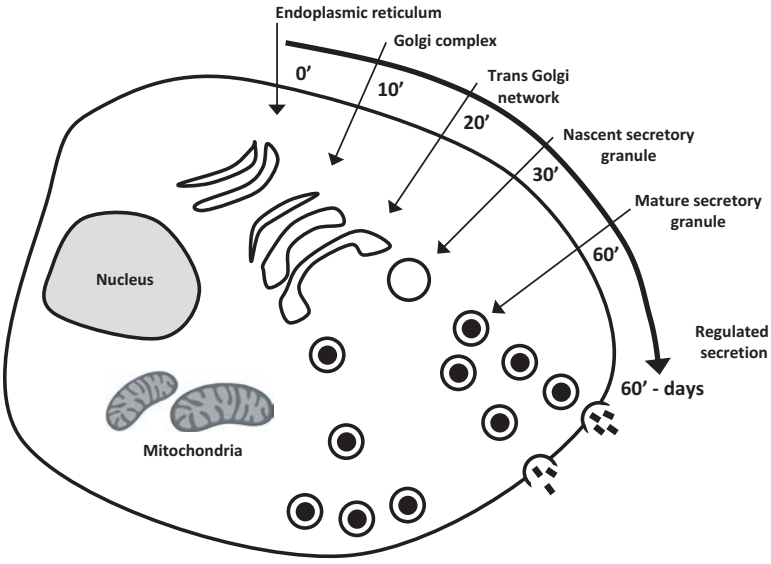

Fig. 2.4 Diagram of a pancreatic beta cell showing the time course of insulin secretory granule biogenesis and secretion in response to the circulating glucose concentration

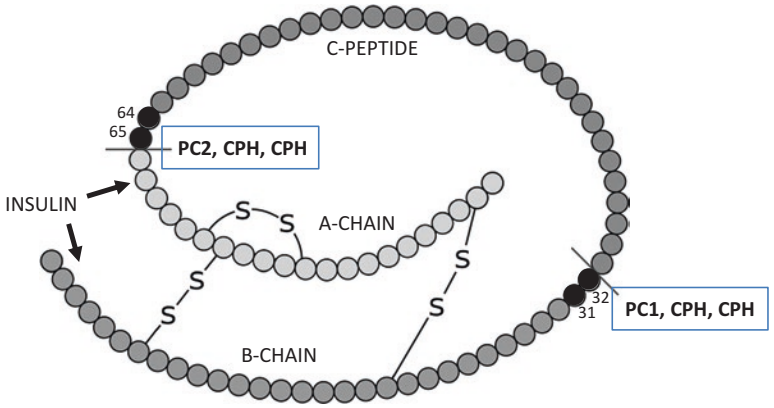

Fig. 2.5 Structure of proinsulin showing the dibasic amino sequences that form the boundaries between the C-peptide and the A- and B-chains of the insulin molecule. Cleavage occurs on the C-terminal side of the dibasic residues through the action of the prohormone convertases (PC1 and PC2) and the exposed residues are removed rapidly by carboxypeptidase H (CPH)

The production of mature insulin requires cleavage of proinsulin by the endoproteases prohormone convertase (PC)1 and PC2 on the carboxy-terminal side of Arginine^{31}-Arginine^{32} and Lysine^{64}-Arginine^{65}, respectively. These dibasic residues mark the boundary of the A- and B-chains of insulin with the connecting (C)-peptide c (Fig. 2.5). The conversion begins approximately 20 min after synthesis in the trans Golgi network and is completed in the nascent secretory granules within 1–2 h post-synthesis. After the endoprotease cleavages, the exposed basic residues are removed rapidly by the Zn^{2+}-dependent exopeptidase carboxypeptidase H (CPH; also known as CPE). This gives transient rise to the conversion intermediates des 31,32- and des 64,65-proinsulins, followed by production of the freed C-peptide and mature insulin. This entire process is optimal in the low pH and high Ca^{2+} environment in the trans Golgi network and secretory granule compartments [4]. Changes in the composition of the proinsulin-related peptides have been reported to occur in type 2 diabetes, indicating that there may be perturbations in proinsulin processing [30, 31].

2.3 Secretion of the Insulin Secretory Granule Contents

In the pancreatic beta cells, insulin secretion is regulated by nutrient availability via calcium mobilization and activation of cAMP-associated signalling cascades. Elevations in circulating glucose levels that normally follow consumption of a meal lead to increased influx of glucose into the cytoplasm of beta cells through the low affinity glucose transporter 2 (GLUT2) [32]. The transported glucose is then rapidly converted to glucose-6-phophate by the low affinity glucokinase, which is the rate-limiting step of glycolysis [33]. Glycolysis results in carbohydrate oxidation and energy production in the form of an increase in the cytosolic ATP/ADP ratio.

In turn, this leads to closure of the ATP-sensitive K+ channels in the β cell membrane resulting in depolarization and opening of voltage-dependent Ca^{2+} channels. The opening of these latter channels leads to Ca^{2+} influx, which triggers insulin secretory granule docking and fusion with the plasma membrane via soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)] complexes and release of the contents into the circulation.

Secretion follows a biphasic pattern as shown in Fig. 2.3, which appears to result from two different populations of insulin secretory granules. One stored pool is responsible for the initial transient phase and then a reserve pool appears to account for the second phase of secretion [11–13]. The fist pool of granules appear to be already docked and primed for secretion at the plasma membrane in an organelle/SNARE complex, comprised of the granules, syntaxin 1 (Stx1), synaptosomal-associated 25 kDa protein (SNAP25), vesicle associated membrane protein 2 (VAMP2) and synaptotagmin [34]. This primed pool of granules accounts for 1–5% of the total cellular content [35, 36]. Following the first wave of secretion, the second pool of granules undergoes recruitment to the same release sites and secretion occurs via the same mechanism. However, this second phase is slower as this requires a sequence of ATP- and Ca^{2+}-dependent reactions to occur, along with formation of the secretion-competent granule SNARE complex [37, 38].

2.4 Insulin Secretory Granule Contents

Apart from the insulin-related peptides, insulin secretory granules contain more than 100 proteins, comprised of other precursor proteins, processing enzymes, and various membrane proteins involved in modulation of the intragranule environment, granule movement and the secretory process itself (Table 2.1). This section describes the major components which have been identified as being localized to insulin secretory granules by immunohistochemistry, electron microscopy or co-secretion studies. As with the proinsulin related peptides, the composition, levels and processing of many of these has been reported to be altered in type 2 diabetes and other diseases. Other insulin granule candidates identified in insulin secretory granule rich subcellular fractions will also be discussed but these require confirmation due to the presence of contaminating organelles in preparations of these fractions [9, 39].

2.4.1 Chromogranin A

The gene for human chromogranin A (*CHGA*) encodes a 439-amino-acid protein that shows high evolutionarily conservation and it is found in the secretory vesicles of neuroendocrine cells such as the pancreatic beta cell (Fig. 2.6) [40, 41]. Markedly

Table 2.1 Major proteins and peptides identified in association with rat insulinoma secretory granules by proteomic analyses [9, 81, 82]

UniProtKB	Gene name	Protein name
Insulin-related peptides		
P01322	INS1	Insulin 1
P01323	INS2	Insulin 2
Other precursor proteins		
P10354	CHGA	Chromogranin A
O35314	SCG1	Secretogranin 1
P10362	SCG2	Secretogranin 2
P47868	SCG3	Secretogranin 3
P20156	VGF	Neurosecretory protein VGF
P12969	IAPP	Islet amyloid polypeptide
P01346	IGF2	Insulin-like growth factor 2
Prohormone convertases		
P28840	PCSK1	Neuroendocrine convertase 1 (PC1)
P28841	PCSK2	Neuroendocrine convertase 2 (PC2)
P15087	CPE	Carboxypeptidase E (CPH)
P14925	PAM	Peptidyl-glycine alpha-amidating monooxygenase
Chaperones		
P27682	SCG5	Neurosecretory protein 7B2
Q9QXU9	PCSK1N	ProSAAS
Other proteins		
Q63475	PTPRN2	Receptor-type tyrosine-protein phosphatase N2

The codes (UniProtKB) for Rattus norvegicus are given. Proteins which have not been confirmed as being present in insulin secretory granules by immunohistochemistry, electron microscopy or co-secretion studies have not been included

Fig. 2.6 Amino acid sequence of the human chromogranin A proecursor. The dibasic and other cleavage sites are indicated below using the single letter amino acid codes (K = lysine, R = arginine, G = glycine). The major cleavage products which have been observed are also indicated (vasostatin I and II, pancreastatin, WE14, catestatin and serpinin)

elevated levels of chromogranin A have been identified in the circulation of individuals with various neuroendocrine tumors [42, 43], and alterations have also been detected in patients with type 2 diabetes [44, 45] and cardiovascular disorders [46]. The human chromogranin A sequence has 9 known dibasic, single basic or peptide-amidating cleavage sites and is proteolyzed by prohormone convertases, in a

manner similar to that for proinsulin cleavage. From N-terminus to C-terminus, this leads to the generation of peptides which have been termed vasostatin I [47], pancreastatin [48], WE14 [49], catestatin [50] and serpinin [51]. All of these peptides have been demonstrated to have physiological functions. Vasostatin and catastain have antiadrenergic properties and serpinin is proadrenergic, meaning that these two peptides have opposing effects on cardiac contractility [52]. In addition, vasostatin inhibits angiogenesis although catastatin has proangiogenic properties. WE14 may be an autoantigen in type 1 diabetes [49]. Finally, catastatin and pancreastinin are important modulators of insulin sensitivity and glucose metabolism [53].

The chromogranin A precursor has been reported to undergo differential processing to produce distinct peptides in different tissues of the diffuse neuroendocrine system [54, 55]. Biosynthetic radiolabelling of isolated rat islets followed by immunopreciptation using sequence specific antibodies have demonstrated that chromogranin A undergoes proteolytic processing over a similar time course to that seen for insulin, generating multiple fragments corresponding to the N-terminal, central and C-terminal regions of the molecule [56]. The possibility that chromogranin A processing may be altered in disease has been supported by Western blot analyses of isolated islets from diabetic Goto-Kakizaki (GK) versus normal rats [16]. This revealed an accumulation of the intermediate forms in the GK rats, although this could be due to the fact that chromogranin A is produced in multiple islet cell types and the distribution of both beta and alpha cells is altered in the GK rat pancreas [56].

The biosynthesis of chromogranin A is co-regulated with that of proinsulin in isolated islets. Studies in isolated rat islets found an approximate 20-fold increase in the incorporation of ^{35}S methionine into the nascent chromogranin A precursor after changing the glucose in the media from 2.8 to 16.7 mM [23, 57]. The fact that these peptides are co-packaged into the granules with insulin suggests that they will be co-released with insulin in response to high glucose levels. The secretion of a number of these peptides has been demonstrated in pulse-chase radiolabelling studies under stimulatory glucose concentrations. One of these is the N-terminal peptide identified originally as betagranin, which corresponds to the vasostatin region of the human chromogranin A molecule [57]. Taken together, these findings indicate that the biosysnthesis and secretion of chromogranin A and insulin are co-ordinately regulated by the circulating glucose concentrations.

2.4.2 PC1

The mRNAs of PC1 and PC2 have been detected in various neuroendocrine cell types, where the encoded proteins are involved in the proteolytic maturation of proneuropeptides and prohormones [58–60]. It is known that proinsulin is first converted to des 31,32-proinsulin by the concerted action of PC1 and CPH and subsequently to insulin and C-peptide by PC2 and CPH [61]. A number of biosynthetic radiolabelling studies have confirmed that PC1 itself undergoes proteolytic

conversion from a larger precursor to produce the active form of the enzyme in various neuroendocrine cell types [62–64] and this occurs with similar kinetics as proinsulin to insulin conversion in pancreatic beta cells [16].

The increase to higher proportions of proinsulin and des 31,32-proinsulin in type 2 diabetes has been linked to increased demand on the beta cells and thus proinsulin not being retained within the secretory granules long enough for complete processing to occur [31, 65, 66]. Another study using mice lacking active PC1 found a severe block in proinsulin conversion with the precursor accounting for approximately 90% of the total immunoreactive insulin like molecules [67]. This would be expected to lead to severe metabolic defects as proinsulin has a low binding capacity at the insulin receptor and low metabolic activity compared to insulin [68]. Consistent with this, the lack of PC1 in human patients with mutations that lead to inactivation of the enzyme leads to metabolic alterations such as obesity [69–71].

2.4.3 PC2

PC2 was first detected as the type 2 endopeptidase in insulin secretory granules in the late 1980s by the laboratory of John C. Hutton at the University of Cambridge, in the United Kingdom [29]. This activity cleaves the proinsulin molecule on the C-terminal side of Lysine^{64}-Arginine^{65} and the Ca^{2+} and pH requirements of this activity suggested that it would be active in the Golgi apparatus and the secretory granule compartments. The identification of enzyme responsible for this activity came from another study from Hutton and co-workers, which demonstrated that PC2 immunoreactivity co-eluted with the type 2 proinsulin endopeptidase in gel filtration and ion-exchange chromatography experiments and antiserum raised against the PC2 catalytic domain immunoprecipitated the type 2 activity from insulin secretory granule extracts [72]. Interestingly, pulse chase radiolabelling studies showed that the maturation of proPC2 to the mature form occurs significantly slower than the conversion of proinsulin, chromogranin A and PC1 to their mature forms, and high glucose concentrations only led to a moderate 2 to 3-fold increase in the biosynthetic rate [73]. These findings suggested that the proportions of PC2 and proinsulin, as well as other granule components, may vary under different physiological states.

A study conducted using islets isolated from the GK diabetes rat model showed a relatively low proportion of PC2 immunoreactivity compared with that found in islets isolated from control Wistar rats [16]. However, both in vivo and in vitro analyses carried out in the same study found that the reduction in PC2 levels did not have a significant effect on the rate of proinsulin processing. In contrast, a study using mice lacking active PC2 found a significant accumulation in the circulating levels of both proinsulin and des 31,32 proinsulin although approximately 40% of the immunoreactivity was accounted for by mature insulin [74]. Thus, this effect does not appear to be as extreme as in cases involving loss of PC1 activity, as described above.

2.4.4 CPH

CPH was also identified originally in the Hutton lab in the early 1980s as an exopeptidase activity involved in proinsulin conversion [75]. This was confirmed in the same lab in 1987 by purification and characterization of the enzyme from insulinoma secretory granules [76]. The activation properties of this enzyme are similar to those of the endopeptidases, indicating the highest degree of activity in the intragranular environment. Pulse-chase radiolabelling of rat islets followed by immunoprecipitation using sequence-specific antisera showed that CPH was synthesized initially as a larger precursor and processed to the mature form by proteolytic processing over the same approximate time course as proinsulin [77]. Furthermore it was secreted in parallel with the insulin-related peptides in response to glucose-stimulation. As with PC2, the biosysnthetic rate of CPH was stimulated only 2 to 3-fold following an increase in the media glucose concentration, suggesting that this enzyme may also be depleted under extended periods of hyperglycemia [23]. An alternative explanation could arise from the fact that this enzyme is present in other islet cell types and the true effect of glucose on its synthesis in beta cells could be obscured by this.

In two types of mutant mice, the loss of normal CPH activity leads to a number of disorders, including diabetes, hyperproinsulinemia and deficits in learning and memory [78–80]. In addition, single nucleotide polymorphisms in the CPH gene have been associated with obesity in a European population along with several other genes [81]. However, no differences could be detected in CPH biosynthesis, secretion or effect on proinsulin processing in the GK rat model, suggesting that more profound effects on this enzyme are required to manifest as physiological disturbances [16].

2.4.5 Other Insulin Secretory Granule Proteins

Two dimensional gel electrophoresis studies of purified insulinoma granules in the 1980s and 1990s revealed the presence of approximately 150 protein spots in addition to the insulin-related peptides [7, 8]. Although many of these are likely to be post-translationally modified forms of the same proteins, and contaminants from other cellular compartments may also be present, some of these proteins are known to be co-packaged with insulin into nascent granules and co-released in response to high circulating glucose levels. Three different nano liquid chromatography tandem mass spectrometry (LC/MS/MS) analyses of a secretory granule-enriched fraction from the rat INS-1E cell line together identified more than 150 different proteins [9, 39, 82]. Apart from insulin 1 and insulin 2, this included several known insulin granule proteins such as various members of the granin

family and the proinsulin conversion enzymes (Table 2.1). Rats and mice are the only mammalian species to contain two copies of the insulin gene, which vary only slightly in sequence [83]. The biological significance of this is not known. The granins all contain multiple basic and dibasic amino sites, consistent with their roles as precursors of multiple bioactive peptides [84]. In addition to PC1, PC2 and CPH, another conversion enzyme was identified known as peptidylglycine alpha-amidating monooxygenase, which catalyzes C-terminal amidation of many peptides, as in some of those derived from chromoganin A processing [85, 86]. Other secretory granule proteins indentified in insulin secretory granules from these and other studies included islet amyloid polypeptide [87], receptor-type tyrosine-protein phosphatase N2 [88] and insulin-like growth factor 2 [89].

In addition to these proteins, several others were found to be enriched in the secretory granule fraction of the INS-1E cells, although this could be due to contamination from other cellular compartments and require further confirmation. This included proteins involved in post-translational modifications, granule movement and exocytosis, and various endoplasmic reticulum, Golgi apparatus, mitochondrial and membrane-associated proteins [9, 81, 82]. Also, multiple components of the SNARE complex involved in granule docking, fusion and exocytosis were identified. A reduction in the islet levels of these SNARE proteins has been proposed as the mechanism of impaired glucose-stimulated first phase of insulin secretion in type-2 diabetes [90–93].

2.5 Conclusions

Taken together, these findings highlight the critical role of the secretory granule contents and the so-called accessory proteins in maintenance of metabolic health. In the case of pancreatic beta cells, this translates to finely tuned insulin production, insulin sensitivity and metabolic homeostasis. Besides the insulin-related peptides, the insulin secretory granule appears to contain more than 100 other proteins, which play essential roles in insulin biosynthesis, trafficking and secretion, as well as in other functions within the beta cell. Some of the co-secreted peptides, such as those derived from chromogranin A, also appear to have functions outside the beta cell with biological activities detected on multiple target tissues, including effects on angiogenesis, the adrenergic system, autoimmunity, insulin sensitivity and glucose handing. Further studies of these proteins could lead to identification of novel biomarkers and drug targets in type 2 diabetes and other metabolic disorders. In turn, this could lead to new drug discovery efforts aimed at producing newer and better treatments for these disorders.

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Chapter 3
Current Models of Fatty Liver Disease; New Insights, Therapeutic Targets and Interventions

Karen R. Jonscher and Kimberley D. Bruce

Abstract Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of disorders ranging from simple steatosis to steatosis with inflammation and fibrosis. NAFLD is currently the most prevalent chronic liver disease worldwide, with a global prevalence of 25%, and is soon projected to be the leading cause for liver transplantation in the US. Alarmingly, few effective pharmacotherapeutic approaches are currently available to block or attenuate development and progression of NAFLD. Preclinical models are critical for unraveling the complex and multi-factorial etiology of NAFLD and for testing potential therapeutics. Here we review preclinical models that have been instrumental in highlighting molecular and cellular mechanisms underlying the pathogenesis of NAFLD and in facilitating early proof-of-concept investigations into novel intervention strategies.

Keywords Non-alcoholic fatty liver disease · NAFLD · Steatosis · Liver · Preclinical models · Intervention strategies

3.1 Introduction

The liver carries out key metabolic functions including the synthesis, storage and metabolism of lipoproteins, triglycerides, cholesterol, bile acids and glycogen. It is responsible for maintaining metabolic homeostasis and for detoxification of xenobiotics in the bloodstream [1, 2]. A plethora of mechanisms regulate hepatic metabolism, and as we study these in more detail it becomes increasingly evident that genetic, developmental and environmental factors can perturb these regulatory

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mechanisms, leading to impaired liver function and severe liver disease. Non-alcoholic fatty liver disease (NAFLD) is currently the most prevalent chronic liver disease, estimated to affect approximately 1 billion individuals worldwide and 30% of American adults [3]. This condition presents with few initial outward symptoms and has an increasing incidence concomitant with those of type 2 diabetes (T2DM), cardiovascular disease (CVD), obesity and the metabolic syndrome [4]. NAFLD encompasses a spectrum of disorders characterized by the accumulation of ectopic fat in the liver (hepatic steatosis) without significant alcohol use [5]. In many patients, NAFLD is characterized by simple steatosis which can be reversed with diet and exercise. However, 25% of NAFLD patients develop more severe steatosis coupled with inflammation and hepatocyte ballooning, termed non-alcoholic steatohepatitis (NASH) [6]. NASH can progress further still to fibrosis (26–37%), leading to cirrhosis, portal hypertension liver failure and hepatocellular carcinoma (HCC) [7–15]. Importantly, it is projected that NASH will be the leading cause of liver transplantation in the US by 2020, overtaking hepatitis C [16, 17]. Although NAFLD is already a major health issue, FDA-approved pharmacotherapeutics are limited [1]. Physiologically relevant preclinical models of NAFLD and NASH are therefore important platforms for investigating mechanisms of disease pathogenesis and performing proof-of-concept studies for emerging therapeutic targets and interventions. The prevalence of NAFLD is tightly correlated with that of obesity, particularly in adults [18]. Many preclinical models therefore incorporate an obesogenic diet to induce NAFLD in a way that more closely mimics pathogenesis in humans. However, it is imperative to emphasize that NAFLD may originate from multiple contributing factors and therefore some models are more relevant to specific disease states than others. Thus, the aim of this review is to critically appraise current models of diet-induced fatty liver disease across multiple disease contexts.

3.2 NASH Pathogenesis

Steatosis is a major factor leading to NAFLD and NASH. Excess fat accumulates in hepatocytes in the form of lipid droplets due to an imbalance between anabolic and catabolic programs in multiple cell types [19–23]. Accumulation of excess triglycerides in hepatocytes induces apoptosis and release of signals portending cellular damage. These damage signals act in concert with markers of oxidative stress, lipotoxicity or gut-derived bacterial endotoxins to activate hepatic macrophages, resulting in secretion of inflammatory cytokines. In turn, the release of inflammatory cytokines activates neighboring endothelial cells, resident and infiltrating macrophages, and hepatic stellate cells (HSCs) [24]. A consequence of HSC activation is remodeling of the extracellular matrix (ECM) and deposition of collagen into the Space of Disse, establishing a positive feedback loop that continues to diminish hepatocyte function [24]. Obesity, insulin resistance, inflammation, oxidative stress,

mitochondrial dysfunction, microbiome dysfunction, endoplasmic reticulum stress, and genetic predisposition are all risk factors and mechanisms by which excessive triglycerides are accumulated in hepatocytes [25–28], leading to liver injury. Moreover, reactive oxygen species (ROS) critically mediate hepatic fibrosis in each cell type. Indeed, elevated ROS is a general feature of both initiation and progression of liver disease [29].

Insulin resistance, resulting from deficits in lipid metabolism that occur systemically due to increased adipose tissue lipolysis, is the primary pathophysiological defect leading to hepatic steatosis [30]. Most patients with NAFLD exhibit adipose tissue insulin resistance, whether or not they are obese [30], and ~60% of the free fatty acids (FFA) taken up by the liver and used for synthesis of hepatic triglycerides are generated by adipose tissue lipolysis [31]. Increased secretion of very low-density lipoprotein (VLDL) is characteristic in NAFLD and results in serum lipid defects such as hypertriglyceridemia, decreased high-density lipoprotein (HDL), and higher low-density lipoprotein (LDL) [32]. Elevated FFA uptake, in concert with reduced FFA oxidation and de novo lipogenesis, results in accumulation of intra-hepatic triglycerides that cannot be compensated for by VLDL-triglyceride export [33, 34]. Insulin resistance is associated with impaired glycogenesis, increased gluconeogenesis and glycogenolysis, accompanied by activation of carbohydrate response element-binding protein (ChREBP) and sterol regulatory-element binding protein 1c (SREBP-1c), the "master regulator" of hepatic de novo lipogenesis [35–37]. Accumulation of bioactive lipids such as diacylglycerol and ceramides further inhibits insulin signaling in the liver [38], additionally impairing liver lipid metabolism. The complex etiology of NAFLD and NASH may therefore require a multi-factorial therapeutic approach, including control of insulin resistance and oxidative stress, which can be tested using preclinical models, described below.

3.3 Diet-Induced Models

Since metabolic diseases such as obesity and T2DM are associated with NAFLD onset and NASH progression, many existing preclinical platforms for investigating fatty liver disease utilize established models of diet-induced obesity. In rodent models, C57BL/6 mice are often used due to their capacity for genetic manipulation and intrinsic susceptibility to develop obesity, T2DM and NAFLD. Dietary models are generally preferred over genetic manipulations since the focus on a single gene/pathway may be overly reductionist and lead to compensation and confounding results. Although the endocrine system in guinea pigs and other higher mammals may more closely resemble human hepatic metabolism, the increased resources and time required to develop abnormal liver function makes them less desirable as preclinical models of NASH. The established dietary models are summarized in Fig. 3.1 and are discussed in more detail below.

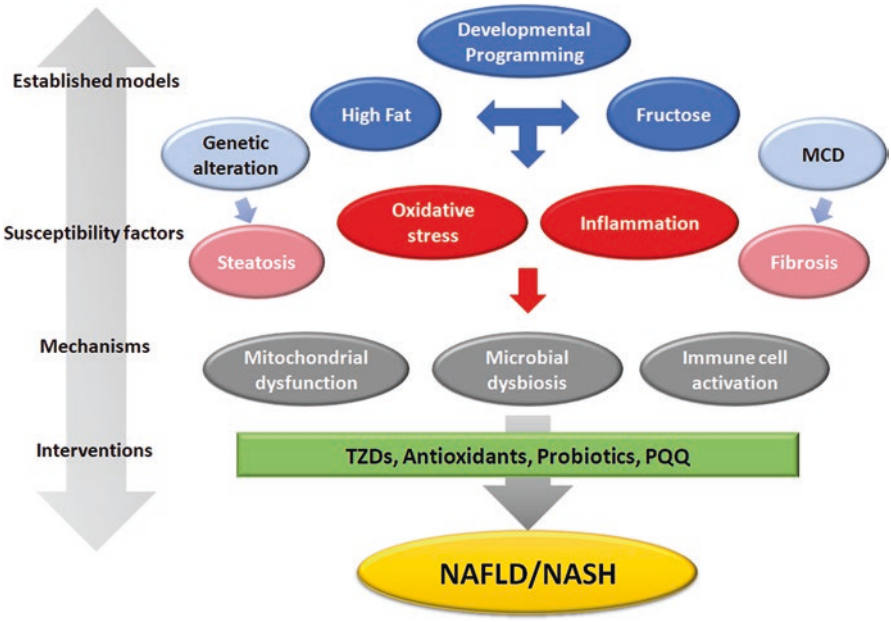

Fig. 3.1 Schematic representation of preclinical models used to establish metabolic perturbations and mechanisms leading to NAFLD and NASH. In general, dietary regimens such as high-fat and fructose feeding, alone or in combination, lead to steatosis, oxidative stress, inflammation and fibrosis, which all may be exacerbated when given during early life due to developmental programming of metabolic function. In contrast, genetic models tend to be primarily associated with steatosis, whereas MCD is an excellent model for fibrosis. Development of these metabolic susceptibility factors are tightly associated with synergistic mechanisms key to NAFLD/NASH pathogenesis (e.g., mitochondrial dysfunction, microbial dysbiosis and immune cell activation), which are targets for novel intervention strategies (i.e., TZDs, antioxidants, probiotics and PQQ)

3.3.1 High Fat Diet (HFD)

High fat feeding establishes a phenotype that mimics the pathogenesis of NAFLD, leading to NASH. High fat feeding produces excess systemic FFA, via both direct intake and lipolysis, leading to increased hepatic triglyceride accumulation [39]. Therefore, HFDs are commonly used to model NASH. In a longitudinal analysis of C57BL/6J mice fed a 60% kcal fat diet for up to 50 weeks, features of the metabolic syndrome including obesity, glucose intolerance, insulin resistance, hyperlipidemia, and hepatic steatosiswere observed by 10–12 weeks [40]. This preceded a NASH-like inflammatory phenotype that developed by 19 weeks. By 39 weeks, markers of liver dysfunction (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) increased [40]. It is important to note that disease onset and severity are largely influenced by both quantity and quality of dietary fat. HFDs can range from 45–85% kcal fat. The first HFD-induced model of NASH was a 71% kcal fat liquid diet given to male Sprague-Dawley rats. Rats developed panlobular steatosis after

only 3 weeks [41]. Although an abnormal liver phenotype develops rapidly in rodents fed very high fat diets, the pathophysiology does not mimic the human condition as well as diets that are somewhat lower in fat (e.g., 45% kcal fat). In addition, higher fat content is less physiologically relevant and may be associated with compensation (i.e., reduction) in overall food intake resulting in mineral and vitamin deficiencies. Furthermore, diets greater than 45% kcal fat have been associated with reproductive issues, making transgenerational studies difficult.

Fats in commercially available HFDs are often a mixture of saturated (lard) and unsaturated (corn oil). These diets, perhaps due to palatability and composition, are good obesogenic diets. In contrast, diets rich in trans-fats are comparatively less obesogenic, yet are associated with greater levels of steatosis and ALT [42]. Notably, features of NAFLD/NASH may be mitigated by substituting the fat in standard HFDs with n-3 polyunsaturated fatty acids (PUFAs) [43, 44], medium chain FA s [45], and long-chain monounsaturated FAs (e.g., oleic acid) [46], demonstrating the importance of finely orchestrated lipid metabolism in NASH pathogenesis.

3.3.2 Modified High Fat Diets

To better represent dietary exposures experienced in human populations, NASH-inducing dietary regimens have been optimized to combine high fat with factors such as fructose or cholesterol. Fructose is consumed in high quantities in the US in the form of high-fructose corn syrup. It requires fewer metabolic steps for conversion to lipids than either sucrose or glucose, and its consumption is associated with increased NAFLD severity [47]. Fructose may be administered in the diet or in the drinking water. In a detailed study, Sellmann et al. evaluated deleterious metabolic effects of fructose mixed in HFD versus in drinking water [48]. Greater weight gain and more pronounced steatosis resulted from combining fructose with HFD, suggesting the interaction between sugar and fat metabolizing pathways is a target of NAFLD. Diets have also been modified with the addition of cholesterol. Increased VLDL and LDL cholesterol is associated with increased risk of developing CVD. Thus, a high fat and high cholesterol diet (HFC) has been used to study interactions between NASH and cardiac or vascular dysfunction in the stroke-prone (SP) hypertensive rat [49].

Many groups now recapitulate a Western-style diet (WD), containing high fat, high sugar, and high cholesterol, with milk fat used in some formulations [50–52], mimicking the fast food-like dietary composition associated with the increasing incidence of NASH in humans [53]. Studies have shown that WD feeding induces obesity and insulin resistance with high fidelity to human conditions [53]. However, even in established murine WD-based models, it can take up to 24–52 weeks for severe NASH and advanced fibrosis to develop, which makes testing interventions on later disease stages difficult and costly. In response, recent models have adopted a combination of WD and carbon tetrachloride (CCL_4) to more rapidly induce key metabolic and histological features of human NASH by 12 weeks, and consistent

development of HCC by 24 weeks [54]. Finally, as discussed below, progression toward NASH in HFD/WD-fed mice may also be accelerated in offspring by maternal consumption of HFD/WD during gestation and lactation.

3.3.3 Methionine- and Choline-Deficient Diet (MCD)

The MCD diet is a well-described model that induces a NASH-like phenotype with hepatocyte ballooning and lobular inflammation by 2–8 weeks, much more rapidly than the standard HFD. Mechanistically, choline deficiency causes impaired hepatic VLDL secretion, increased hepatic fat accumulation, hepatocyte necrosis and oxidative stress [39, 55]. When choline deficiency is combined with methionine deficiency, pronounced inflammation characterized by infiltration of Kupffer cells, increased AST and ALT levels and early fibrosis are also observed [56]. Although the onset of severe NASH is very rapid in the MCD model, the other features of metabolic syndrome such as obesity, changes in glucose tolerance, insulin resistance and hyperlipidemia, associated with the onset of human NAFLD, are not observed. The physiological relevance and utility of the model is further confounded by the fact that animals on the MCD often display significant weight loss [39, 55, 56].

3.4 Mechanistic Insights, Susceptibility Factorsand Interventions

Mitochondrial stress, inflammation and oxidative stress are major pathways perturbed in NAFLD and NASH that can be activated or mitigated using dietary models of NAFLD/NASH (HFD, WD, MCD). Below, we will discuss detailed molecular mechanisms within these pathways important for driving liver pathophysiology, dietary models that have been used to highlight these mechanisms, and ways in which these models have been used to investigate novel interventions that may have translational relevance to human NAFLD.

3.4.1 Mitochondrial Stress

In mitochondria, metabolic homeostasis is maintained by the coordinated action of β-oxidation, the hepatic tricarboxylic acid (TCA) cycle, ketogenesis, electron transport chain (ETC) activity and adenosine triphosphate (ATP) synthesis. Impaired mitochondrial metabolism is a central feature of human NAFLD, characterized by elevated intermediates of the tricarboxylic acid (TCA) and accumulation of

lipotoxic intermediates of incomplete lipid oxidation (e.g., long-chainacyl carnitines, diacylglycerols and ceramides) [57]. Impaired mitochondrial metabolism also is tightly associated with disease stage and severity, as well as the flexibility of the mitochondria to "adapt" and facilitate catabolism of excess lipid intermediates [58–61]. Recent studies suggest that mitochondrial inflexibility plays a key role in disease progression from steatosis to the more severe NASH [59, 60]. For example, in earlier stages of disease (i.e., obesity), maximal respiration is increased in obese vs. lean individuals [58]. However, as the disease progresses toward NASH, patients show significantly reduced maximal respiration, increased hepatic insulin resistance and mitochondrial uncoupling [58]. It is likely that early increases in mitochondrial function represent compensatory mechanisms needed to cope with nutritional overload and to dispose of excess acetyl-CoA via oxidation. When nutritional overload becomes long-term, these compensatory mechanisms fail, leading to impaired ETC activity [58], diminished ATP synthesis [62] and more severe disease pathology. Interestingly, despite attenuation of ETC activity and ATP synthesis, in humans, TCA cycle activity remains elevated [63]. However, this may be an insufficient adaptation to counter the effects of chronic nutritional overload and metabolic challenge.

Importantly, mitochondrial dysfunction has been recapitulated in rodent models of NAFLD/NASH. In a short-term study, after only 8 weeks on HFD, mitochondrial function does not seem to be dysregulated. However, mice exhibit hepatic insulin resistance, steatosis and accumulation of lipotoxic by-products such diacylglycerols and ceramides [59]. These results suggest that although mitochondrial energetics are not "impaired", mitochondria are not able to adapt in response tothe increased metabolic strain of HFD feeding. In longer-term studies, mice were fed HFD for 32 weeks, developing insulin resistance and fatty liver [60]. By 32 weeks, ETC efficiency was reduced but TCA cycle activity remained elevated. Indeed, the induction of TCA cycle activity was associated with ETC dysfunction, oxidative stress and inflammation. Results from these studies suggest that during insulin resistance, the TCA cycle facilitates mitochondrial impairment by providing electrons to a defective ETC that is vulnerable to oxidative stress [60].

One of the key mechanisms driving the pathogenesis of NAFLD is increased fatty acid oxidation (FAO) due to nutritional overload [64, 65]. In the presence of excess FFAs, mitochondria adapt by increasing FAO in the attempt to catabolize excess substrate. Subsequently, in HFD-fed mice, surplus acetyl-CoA generated from FAO is shuttled toward ketone production [66]. In severe forms of NAFLD, acetyl-CoA is also shuttled towards the TCA cycle [63], providing carbon for gluconeogenesis and contributing to the development of glucose intolerance and type 2 diabetes. Mechanistically, activation of peroxisome proliferator-activated receptor (PPAR)-α in the liver in response to elevated FFA flux in NAFLD and NASH can upregulate transcription of carnitine palmitoyltransferase (CPT)-1 [67], potentially promoting FFA delivery to the mitochondria for increased FAO.

Regardless of etiology, increased FAO contributes to lipid peroxidation, ROS and oxidative stress, leading to a feed-forward cycle of mitochondrial dysfunction

and disease progression. In addition, elevated malonyl-CoA production resulting from increased TCA cycle activity inhibits CPT-1-mediated uptake of FFAs into the mitochondria, eventually limiting FAO [68]. Thus, in later stages of the disease, it is likely that mitochondrial function is impaired and FAO is reduced, resulting in accumulation of excess acetyl-CoA which is subsequently shuttled to lipid storage rather than catabolism.

Sirtuins, a family of seven deacetylases (three of which are mitochondrial), critically mediate mitochondrial function and the development of NAFLD. Under conditions of nutrient restriction, SIRT3 has been shown to deacetylate a broad range of mitochondrial proteins to regulate mitochondrial activity, including enzymes involved in FAO, TCA cycle, and oxidative defense [69]. HFD-fed mice exhibit decreased SIRT3 and dysfunctional ETCactivity associated with elevated protein acetylation [70]. This phenotype is recapitulated in SIRT3-deficient HFD-fed mice. These animals develop obesity, insulin resistance and a NASH-like phenotype [71], as well as increased acetylation of mitochondrial proteins and reduced FAO [72]. One of the hyper-acetylated mitochondrial proteins found in the acetylome of SIRT3-deficient mice is mitochondrial trifunctional protein (MTP), a rate-limiting multi-enzyme regulating oxidation of long-chain fatty acids. Interestingly, while MTP−/− mice develop profound hepatic steatosis and neonatal death [73], chow-fed mice that are heterozygous for MTP deficiency develop insulin resistance and NAFLD with aging (>9 months old), concomitant with reduced FAO [74]. Recently, Nassir et al. used an adenovirus to overexpress SIRT3 in MTP+/− mice. Results from these studies showed decreased MTP acetylation and increased FAO. Mitochondrial function was improved and NAFLD onset attenuated [75], suggesting a key mechanistic role for SIRT3 and MTP in driving the pathogenesis of NAFLD.

In addition to genetic strategies to improve fatty liver disease, rodent models have also been used to demonstrate in vivo efficacy of a number of pharmacological interventions targeting mitochondrial dysfunction in NAFLD and NASH. In one example, administration of the mitochondrial protonophore 2,4-dinitrophenol (DNP) prevented weight gain and insulin resistance. Due to fatalities, its toxicity prevented further research. However, Perry and colleagues generated a controlled release form of DNP which was able to reduce insulin resistance and hepatic steatosis in an MCD rat model of NASH via mild mitochondrial uncoupling [76]. Importantly, controlled release DNP also prevented hepatic steatosis in a mouse model of lipodystrophy [77]. It remains to be determined if this agent can rescue NAFLD onset in a typical HFD rodent model of metabolic disturbance and fatty liver. In another recent study, treatment with the thiazolidinedione (TZD) pioglitazone improved mitochondrial function and NASH outcomes in a mouse model of high-fructose and trans-fat induced NASH. Specifically, pioglitazone administration resulted in reduced toxic lipid intermediates (e.g., diacylglycerol and ceramides) and attenuated TCA cycle flux, suggesting the mechanism of action was to diminish mitochondrial stress, decelerating progression of liver disease [57].

3.4.2 Oxidative Stress

Multiple lines of evidence from human and animal studies suggest an association between NAFLD/NASH and biomarkers of oxidative stress or lipid oxidation [78–84]. Excessive iron accumulation can be one contributing factor. Free radicals generated via the Fenton or Haber-Weiss reactions appear sufficient to damage hepatic reticuloendothelial cells [85, 86]. Elevation in hepatic iron is often found in adult patients with NAFLD and may be associated with disease severity [87]. Similar trends are also observed in patients with hepatitis C [88]. In humans, iron-induced oxidative stress can occur when the hepatic iron concentration is greater than three times the upper limit of normal (i.e., ~90 micromoles/g dry liver) [89]. Transferrin saturation, an early indicator of increasing iron stores, is abnormally high in obese children with NAFLD [90].

In rodent and murine models, iron overload accelerates the development of NASH, demonstrated in Lepr-deficient genetically obese (db/db) mice [86]. Remarkably, fibrosis persists in rats fed a choline-deficient, iron-supplemented diet, even when choline is replaced in diets [91]. The composition and function of gut microbiota and iron have also been linked to the development and progression of NAFLD. Moreover, the availability of iron stores critically influences both glucose homeostasis and microbial function [92]. Consistent with these observations, Kumar and co-workers reported that when iron regulation was normalized in rats fed an iron-deficient diet (by chronic administration of a genetically engineered pyrroloquinoline quinone (PQQ)-secreting probiotic), hepatic function was restored [93]. Treatment with probiotic-secreting PQQ also restored liver function and activity of oxidative defense enzymes (superoxide dismutase and catalase) when cadmium or mercury were used to induce liver damage [94]. Other dietary antioxidants, including resveratrol, quercetin, epigallocatechin and curcumin, have demonstrated positive effects on reversion of hepatic steatosis in animal models of NAFLD [95]. Although their effect on iron stores has not been widely tested, emerging evidence suggests that dietary antioxidants have potential as NAFLD or NASH therapeutics.

Recently, we found that hepatic steatosis, inflammation and fibrosis induced in mice by chronic feeding of a Western-style diet, both during gestation and postnatally, can be ameliorated by chronic, low dose (~10 ug/mouse/d) treatment with the novel dietary antioxidant, PQQ, provided in drinking water [50, 51]. Lipid peroxidation is a common marker of oxidative stress and liver damage and in models of T4-induced hyperthyroidism and streptozotocin-induced diabetes mellitus, Kumar and coworkers demonstrated that PQQ directly administered (by intraperitoneal injection) for as few as 6 days at doses between ~1–20 mg PQQ per animal per day reversed histological evidence of liver injury and diminished levels of lipid peroxidation products [96–99]. Similar results were also observed using chicks and commercial laying hens as models [100, 101]. Taken together, these studies illustrate the important role of oxidative stress in the pathogenesis of NAFLD and the potential for intervention using a dietary antioxidant. Mechanistically, PQQ activates antioxidant networks, preventing oxidative stress-induced lipid peroxidation. In multiple

rodent models, PQQ administration resulted in increased activity of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase [51, 93, 94, 96, 98, 99, 102–108]. In vitro, PQQ rescued antioxidant activity in hepatic stellate cells, hepatocytes and macrophages isolated from livers of mice with fibrosisinduced by thioacetamide [109]. Undoubtedly, PQQ's antioxidant action in the various cell types driving hepatic pathophysiology is a major contributor to its protective effects in liver.

3.4.3 Inflammation

Macrophages are one of the most prevalent cell types in liver inflammation associated with NASH and other chronic inflammatory and fibrosis-inducing liver injuries [110–113]. Macrophages and macrophage-derived mediators (cytokines) contribute to the pathogenesis of NAFLD and NASH by mechanisms that remain poorly understood [111–115]. Liver resident macrophages (Kupffer cells) maintain tissue homeostasis under physiologic conditions [110, 116]. During pathological conditions, such as in response to danger signals from injured hepatocytes, macrophages are recruited to the liver from peripheral blood monocytes which arise from hematopoietic stem cells in the bone marrow [117, 118]. Once differentiated, recruited macrophages in the liver either promote resolution of inflammation and prevent progression to fibrosis [119] or remodel the tissue, provoking fibrosis [110, 112, 120–123]. Mounting clinical and experimental evidence indicates that macrophages critically contribute to the initiation and progression of chronic inflammatory conditions and fibrotic tissue remodeling [110–113]. On the other hand, tissue repair and resolution of inflammation, and even reversal of established fibrosis, depends on macrophages [110–113, 119]. This apparent discrepancy in macrophage function may be directly related to the high functional plasticity of macrophages which allows them to adapt to differing microenvironments and equips them with a variety of metabolic functions through which they, in turn, affect the microenvironment [110, 111]. Macrophage polarization to a pro-inflammatory phenotype is also causally linked to reprogramming of macrophage metabolism [110, 124, 125]. Hallmark features of the pro-inflammatory macrophage phenotype are metabolic adaptation to Warburg metabolism, characterized by increased aerobic glycolysis, a dysfunctional TCA cycle (with an accumulation of intermediates such as lactate and succinate), and reduced oxidative phosphorylation (OXPHOS), resulting in increased mitochondrial ROS [124, 126–131]. In contrast, anti-inflammatory macrophages, or those promoting homeostatic or reparative function, are characterized by OXPHOS, normal TCA cycle function and minimal glycolysis [110, 130]. Restoration of ETC and physiologic TCA cycle function may be prerequisite for adopting an anti-inflammatory and homeostatic macrophage phenotype to effect liver regeneration or repair [124, 125], serving as a potential interventional target.

3.4.4 Gut-Liver Axis and Microbiome

Because the liver and intestines are connected *via* the hepatic portal system, the liver is exposed to metabolic products of gut microbiota (such as ethanol, short-chain fatty acids [SCFA], lipopolysaccharide and even bacteria themselves). Therefore, the gut microbiota and their metabolic products may influence hepatic pathophysiology [132]. A causative role for microbiota in NAFLD has been established in both human and animal studies. In the early 1980's, treatment with metronidazole was shown to improve hepatic steatosis in patients with small intestinal bacterial overgrowth [133]. Subsequently, when germ-free mice were inoculated with cecal contents from normal, control mice, the germ-free mice gained 60% body fat and more than doubled their level of hepatic triglycerides [134]. Aron-Wisnewsky et al. [135] summarized five ways in which the gut microbiota contributes to promoting NASH:

1. Microbiota from obese animals extracts and store more energy from food as compared with lean counterparts [136].
2. Levels of SCFAs produced by microbiota were elevated in feces of obese individuals. Since SCFAs are fatty acid and cholesterol precursors, as well as substrates for gluconeogenesis [137], SCFA elevation results in upregulation of fat storage pathways [134], promoting obesity.
3. Increased intestinal permeability is associated with both NAFLD and NASH in humans [138, 139] and animals [140]. This suggests that microbial dysbiosis impairs the homeostasis between bacteria and host at the level of intestinal epithelial cells. Such dysbiosis may promote translocation of bacteria and bacterial products from the gut into the portal circulation, inducing inflammation in the liver [141, 142] and other metabolically active tissues such as adipose [143, 144].
4. Underlying mechanisms by which adaptive and innate immune responses are modulated include signaling through Toll-like receptors (TLR), which recognize conserved microbial products (pathogen-associated molecular patterns; PAMPS) and initiate signaling cascades that lead to transcriptional activation of pro-inflammatory genes [141]. Animal studies have confirmed that TLR4 is required for steatosis and NASH development [145–147] as well as for fibrosis via activation of hepatic stellate cells [148, 149]. In addition to altering choline [150] and bile acid [151] metabolism to promote NAFLD and NASH.
5. Gut microbiota products such as ethanol may travel to the liver and activate resident macrophages, providing substrates for fatty acid synthesis (acetate) and stimulating the production of ROS (acetaldehyde) [152]. Studies in children with NASH have shown an increase in alcohol-producing bacteria in feces [152] and NAFLD and NASH are associated with elevated blood alcohol levels in the absence of alcohol consumption in humans [152, 153] and animals [154].

3.5 Animal Models to Study Developmental Origins of NAFLD

Results from multiple epidemiological studies showed an association between impaired fetal nutrition and increased risk of metabolic disease, diabetes and hypertension, leading to the theory of Developmental Origins of Health and Disease (DOHaD) [155, 156]. Data from large human cohort studies such as the Helsinki Birth Cohort Study [157, 158], the Generation R study [159–161] and the Viva Cohort [162] showed high maternal BMI and excessive weight gain were associated with greater adiposity and increased cardio-metabolic risk in infants. Animal models of developmental programming have been used to explore mechanisms underlying DOHaD and clarify critical windows of development (preconception/implantation through lactation) through diet-switching approaches. Furthermore, animal models are used to study effects of various in utero exposures (i.e., under- and over-nutrition), maternal metabolic status (i.e., obesity, diabetes) and interventions for correcting programming and its adverse cardiometabolic effects (see recent reviews from Williams et al. [163] and Chavatte-Palmer et al. [164]). The use of animal models affords elucidation of molecular mechanisms driving long-term offspring phenotypes through multidisciplinary approaches and interventions that may ultimately be translated to human studies (Fig. 3.1).

3.5.1 Rodents

Rodent models are most commonly utilized. They are low cost, easy to maintain, and numerous transgenic lines are available. Rodent hemotrichorial placentation is also somewhat similar to placentation in humans, with the trophoblast in direct contact with maternal blood, allowing for detailed studies on effects of maternal diet on placental function [165–167]. Compelling evidence from rodent models demonstrates the critical effects of perinatal exposure to maternal HFD on offspring risk of liver disease. Offspring weaned onto an HFD exhibited accelerated progression of NAFLD to NASH when also exposed to HFD during gestation and lactation [168–170]. In late gestation (embryonic day 18.5), hepatic triglycerides were significantly elevated and accumulation of lipid droplets (as determined by coherent anti-Stokes Raman Spectroscopy [CARS]) increased in fetuses from dams fed an HFD as compared with those from dams fed a standard chow diet [51]. Furthermore, programming of the adaptive and innate immune systems may also occur during gestation, as suggested by Kamimae-Lanning et al., and may prime offspring for future development of NASH [171]. They observed restriction of hematopoietic stem and progenitor cell expansion in livers from fetuses (embryonic day 14.5) exposed to an HFD. Furthermore, lineage commitment was skewed toward lymphopoiesis and myelopoiesis at the expense of stem cell self-renewal [171], potentially biasing bone marrow populations to allow for promotion of monocyte and macrophage

recruitment in response to future inflammatory stimuli. Lactation may also independently contribute to programming NAFLD. Oben et al. found elevated levels of hepatic triglycerides, as well as increased mRNA expression of inflammatory genes (*Tnf* and *Il6*), in offspring born to dams fed a control diet and subsequently fostered by obese dams fed an HFD [172]. The peri-conception period is of particular interest for identifying effects of the earliest exposures on offspring metabolic outcomes. In mice, Dahlhoff et al. showed that peri-conceptional exposure to an HFD resulted in insulin resistance and hepatic steatosis in male offspring fostered onto dams fed a control diet whereas female offspring exhibited impaired fasting glucose and reduced fat mass [173]. Sasson et al. used reciprocal two-cell embryo transfers in mice to show that pre-gestational exposure to maternal HFD impaired both fetal and placental growth but did not affect obesity and glucose tolerance in adulthood. In contrast, gestational exposure to maternal HFD resulted in adverse metabolic outcomes in adult offspring [174] suggesting a key role of gestational exposure to an obesogenic diet in programming metabolic disease. Studies such as these demonstrate the utility of rodent models for elucidating mechanistic origins of NAFLD. However, the small size, particularly of mice, is a significant limitation of this model as is the short length of gestation which makes longitudinal sampling and the study of chronic perturbations somewhat problematic.

3.5.2 Non-human Primates

Non-human primates (NHP) and rabbits also possess a hemochorial placenta [175], similar to those of rodents and humans. Non-human primates are the "gold standard" model due to their anatomical and biochemical similarity with humans. However, elevated cost of housing and maintenance, as well as ethical considerations based on their social organization and human-like behaviors, limit wide-scale adoption of this model for biomedical research. Nonetheless, important insights into developmental programming of NAFLD have been obtained. Using Japanese macaques, McCurdy et al. found increased hepatic triglycerides and signs of oxidative stress in liver from fetuses exposed to maternal HFD, detectable in the early third trimester [176]. Even when dams were switched to a control diet during a subsequent pregnancy, fetal liver triglyceride levels were only attenuated and not rescued to control levels [176]. Although dams were not obese or diabetic, their chronic consumption of anHFD increased the risk of fetal NAFLD that persisted postnatally. At 1 year of age, Thorn et al. determined that liver triglycerides were increased in juveniles exposed to maternal HFD then weaned onto a control diet. Moreover, activation of inflammatory gene expression in response to lipopolysaccharide (LPS) in hepatic macrophages was elevated in control diet-fed offspring of obese and insulin resistant dams [177], potentially priming the offspring toward development of NASH. Aberrant lipid management was also observed by Puppala et al. in livers from baboon fetuses [178]. RNASeq analysis revealed downregulation of the TCA cycle, the proteasome, oxidative phosphorylation and

glycolysis/gluconeogenesis in liver from fetuses exposed to a high fat-high fructose maternal diet, with concomitant up-regulation of Wnt/β-catenin signaling. Differential expression of miRNAs inversely correlated with numerous target genes within these pathways, suggesting miRNA-gene interactions might be epigenetic mechanisms regulating lipid metabolism and NAFLD progression. Furthermore, Ma et al. showed that offspring of dams fed an HFD were subject to microbial dysbiosis that was only partially corrected by post-weaning switch to a control diet [179]. Exposure to maternal HFD resulted in reduced abundance of non-pathogenic Campylobacter, suggesting early shaping of the microbiome may drive persistent metabolic processes within the offspring.

3.5.3 Other Models

Although rodents and NHP are most commonly utilized, rabbits, pigs and sheep also serve as models for studies in developmental programming. Here we will focus on sheep. Research carried out using large animals such as ruminants affords unique analysis of organ development and fetal physiology, evaluation and development of new pharmacologic treatments, gestational imaging, and elucidation of the mechanisms and consequences of fetal programming [180]. Sheep are human-sized animals and resemble humans in that they have a long gestational period (5 months) and only one to two lambs per pregnancy. Compelling epidemiological evidence suggests that the metabolic syndrome originates, in part, from a suboptimal intrauterine environment [181, 182] which can be directly and mechanistically studied in sheep. Pregnant ewes tolerate invasive procedures well and elegant functional studies have recently been performed on fetuses in utero using fetal catheters to infuse compounds and monitor metabolic response [183–185]. These types of studies are not possible to carry out in smaller animals. However, epitheliochorial placentation, ruminant digestion, high cost of housing and costly maintenance deter from use of this model. Also, use of sheep is highly regulated by government agencies due to Q fever.

3.6 Brain-Liver Axis

The regulation of hepatic lipid and lipoprotein metabolism plays a key role in the development of dyslipidemia and NAFLD. Although historically overlooked, the central nervous system (CNS) has recently emerged as an important regulator of systemic energy balance and hepatic metabolism. Specifically, neurons of energy-sensing nuclei of the hypothalamus are thought to sense nutritional status, as well as hormones and neuropeptides, to modulate the sympathetic regulation of hepatic glucose and lipid metabolism. The brain-liver axis involves bidirectional sympathetic and parasympathetic signaling pathways via the spinal cord and vagus nerve. Rodent models manipulating neuronal control of peripheral and hepatic lipid

metabolism have been used to begin to understand mechanisms underpinning regulation of the brain-liver axis. Anand and Brobeck were the first to show that specific regions of the hypothalamus played an important role in the regulation of food intake and metabolism. They demonstrated that lesions to the ventromedial nucleus (VMH) caused hyperphagia, while lesions to the lateral hypothalamus resulted in anorexia and weight loss [186, 187]. It is now well established that the arcuate nucleus (ARC), located within the medio-basal hypothalamus (MBH), contains distinct populations of neurons that regulate systemic energy balance and metabolism (see review by Waterson and Horvath [188]). For example, agouti-related peptide (AgRP)- and neuropeptide Y (NPY)-expressing neurons stimulate hyperphagia, whereas pro-opiomelanocortin (POMC)- and cocaine amphetamine-related transcript (CART)-expressing neurons inhibit appetite, stimulating anorexia and weight loss. POMC-expressing neurons project to a sub-population of second order melanocortin-3 (MC3) and MC4 receptor-expressing neurons, which have also been implicated in the regulation of food intake and body weight. Interestingly, activation of MC3/4R signaling in streptozotocin-induced diabetic mice can reduce hepatic expression of genes, such as Srebp1c and Scd1, involved in de novo lipogenesis [189]. In contrast, inhibition of this circuit, either via pharmacological interventions or genetic deletion in mice, resulted in increased SNS activity and elevated hepatic triglyceride synthesis [190]. Considering these findings, recent studies have suggested that ablation of MC4R (MC4R-KO) may constitute a novel mouse model of NASH that recapitulates the natural history of diet-induced NAFLD/NASH [191]. Specifically, HFD-fed MC4R-KO mice develop obesity, insulin resistance, dyslipidemia and histologic features of steatohepatitis such as inflammatory cell infiltration, hepatocyte ballooning, and pericellular fibrosis [191]. Similar to the end stages of NAFLD progression, MC4R-KO mice developed HCC following 1 year of exposure to HFD. Although more studies are required to elucidate mechanistic details, it is plausible that MC4R signaling to the liver involves the neuronal sensing of fatty acids, since eicosapentaenoic acid supplementation can ameliorate NASH-like features in MC4R-KO mice [192]. In support of this hypothesis, fatty acid sensing in the MBH can directly modulate hepatic lipid metabolism [193] as shown in studies performed by Yue et al. where direct infusion of oleic acid into the MBH reduced hepatic VLDL-secretion and Scd1 gene expression, mediated by the MBH-vagal axis [193]. Although this suggests that central administration of fatty acids negatively regulates hepatic lipoprotein production and lipid mobilization, more work is required to resolve the effects of physiologically relevant modes of lipid delivery and discreet neuronal sub-populations involved in brain-liver cross talk.

3.7 Conclusions

The identification and validation of effective therapies to treat NAFLD onset and progression warrants the use of physiologically relevant preclinical models. This is complicated by the fact that NAFLD has a highly complex pathogenesis and

multi-factorial etiology. Nonetheless, a plethora of recent studies have shown that NAFLD may arise from dysfunction in a number of susceptibility factors including development, systemic lipid metabolism, mitochondrial function, diet, lifestyle, inflammation, gut microbiota, and integration of signals from the central nervous system. Identification of these mechanisms have helped to develop promising new therapeutic strategies to ameliorate features of NAFLD, including (although not limited to) dietary supplementation, improving mitochondrial function and reducing oxidative stress. Diet-induced models of obesity are well-established and extensively used. They are arguably the most physiologically relevant models for study of NAFLD associated with insulin resistance and metabolic disorders. Emerging areas of research, such as the interaction between the brain-liver-axis and the regulation of hepatic lipid metabolism, may require new models, or modifications to established approaches. Careful consideration of target disease pathology, in concert with use of available tools and development of new tools by the scientific community, will allow for generation of clinically relevant in vivo models in which to perform essential proof-of-concept studies to vertically advance the therapeutic space in NAFLD and NASH.

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Chapter 4
Preclinical Models of Altered Early Life Nutrition and Development of Reproductive Disorders in Female Offspring

Pania E. Bridge-Comer, Mark H. Vickers, and Clare M. Reynolds

Abstract Early epidemiology studies in humans have and continue to offer valuable insight into the Developmental Origins of Health and Disease (DOHaD) hypothesis, which emphasises the importance of early-life nutritional and environmental changes on the increased risk of metabolic and reproductive disease in later life. Human studies are limited and constrained by a range of factors which do not apply to preclinical research. Animal models therefore offer a unique opportunity to fully investigate the mechanisms associated with developmental programming, helping to elucidate the developmental processes which influence reproductive diseases, and highlight potential biomarkers which can be translated back to the human condition. This review covers the use and limitations of a number of animal models frequently utilised in developmental programming investigations, with an emphasis on dietary manipulations which can lead to reproductive dysfunction in offspring.

Keywords Reproductive health · Animal model · Early-life nutrition · Biomarkers · Fertility · Diet

4.1 Introduction

Growing global awareness around obesity and related morbidities such as cardiovascular disease, hypertension, infertility, and type 2 diabetes (T2DM), has resulted in numerous studies investigating the influence of nutrition on metabolic and reproductive health. Furthermore, it is now clear that *in utero* stressors play a significant role in the origins of infertility. Human epidemiological studies and animal models have illustrated how compromised maternal nutrition and environment, and postnatal nutrition can influence fetal growth and development, and the later health of

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offspring through what has come to be termed developmental programming. This overview addresses these preclinical models of altered early-life nutrition, and the potential biomarkers which offer measureable insight into the reproductive and metabolic changes in offspring. Attention has been drawn to important considerations surrounding the design of preclinical studies, with advantages and disadvantages of specific animal models detailed. Furthermore, explanation of many of the common early life nutritional insults used will help expand on the role early-life nutrition has on reproductive health in later life.

4.1.1 Developmental Origins of Health and Disease

The developmental origins of health and disease (DOHaD) model explores the relationship between the maternal environment, fetal and neonatal development and later disease risk. The hypothesis emphasises the ability of the fetus to develop in reaction to various prenatal cues taken from the intrauterine environment. These alterations of fetal phenotypes, known as predictive adaptive responses (PARs), may offer selective advantages in later life by preparing for potentially adverse environments [1]. However, when there is mismatch between the perceived and actual postnatal environment, for example maternal undernutrition followed by a contradicting high-fat diet (HFD) postnatally, offspring may be at risk of later life diseases. Various studies have linked such environmental differences with increased risk of T2DM, obesity, insulin resistance, and hypertension [2–4]. Early evidence from human studies include David Barker's work on the Hertfordshire Cohort, which links reduced fetal growth to higher incidence of cardiovascular disease in later life [5, 6]. Further human cohort studies, such as the Dutch Hunger Winter [7, 8], have solidified and expanded on Barker's initial research findings.

4.1.2 The Importance of Preclinical Models

Human studies have a range of limitations that hinder collection of data, such as the quality and availability of data from retrospective studies, ethical restrictions, the extended length of experimental and gestational periods necessary for adequate investigation, the unavailability of measurement methods for nutritional intakes, inability to investigate mechanisms at the level of individual tissues, and various confounding environmental factors which are unavoidable in human cohorts. Animal models therefore help to elucidate and extend our knowledge of how altered early life nutrition influences offspring metabolic and reproductive health, as well as offering the opportunity to identify and validate biomarkers which may then be used to detect conditions in humans. The most valuable animal models, with respect to DOHaD and early-life nutrition, are those which mimic adverse maternal diets,

complications of human pregnancy and altered neonatal nutritional environments. The ability to study short- and long-term effects of altered diets in preclinical models is essential before the effective translation of any possible interventions to human populations.

Many preclinical models have already illustrated the effects of an altered early life diet on fetal and neonatal development during key windows of developmental plasticity. Small animal models have offered evidence of metabolic dysfunction caused by modification of nutrients in pre- and postnatal life, as well as indicated the risk of intergenerational 'inheritance' of these issues. Moreover, while comparatively fewer studies have looked at how nutritional variance might impact later life reproductive health, work in small animals has demonstrated effects on reproductive function both independent of and associated with metabolic dysfunction (Table 4.1).

4.1.3 Metabolic Effects on Reproductive Health

Hypertension, hyperinsulinemia, dyslipidaemia, and high fasting glucose are characteristic of metabolic dysfunction [9]. These metabolic conditions may also be associated with reproductive disorders through altered energy metabolism and nutrient signalling. Insulin, for example, influences many features of ovarian development and function, with effects on steroidogenesis, regulation of luteinising hormone (LH) receptors, insulin receptors, ovarian growth and cyst development [10]. These may be direct influences, through insulin receptors found on theca and granulosa cells [11], or indirect, through enhancement of gonadotropin-releasing hormone (GnRH) sensitivity in the pituitary [10] and over-stimulation in the hypothalamus [12]. GnRH is one of the main hormones involved in the initiation of puberty, stimulating the pituitary's production of LH and follicle stimulating hormone (FSH), which in turn act on the gonads to produce 17-β-estradiol (E2). E2 assists follicular development and acts as a negative inhibitor of GnRH [13]. Given GnRH's pulsatile action, these primary and secondary products of GnRH act as better markers of reproductive health [14].

Obesity and increased adiposity, particularly in the abdominal region, is also associated with metabolic dysfunction and a number of reproductive issues such as anovulation, infertility, and pregnancy complications [15]. In obese female rats, increased adiposity and higher leptin concentrations have been linked to early puberty onset [16]. Leptin, a satiety hormone produced from adipocytes, acts permissively in the activation of the hypothalamic-pituitary-gonadal (HPG) axis via the initiation of GnRH production [17, 18]. This metabolic signal may also act as a 'gate', inhibiting the neuroendocrine reproductive axis when leptin concentrations are low, and communicating the body's nutritional state to the reproductive system [19, 20]. Furthermore, leptin receptors are expressed on human oocytes, indicative of another role in the regulation of follicular development and oocyte maturation

Table 4.1 Summary of animal models used to understand the influence of early-life nutrition on the reproductive health of female offspring

		Timing				
Animal model	Diet	Early gestation	Mid-gestation	Late gestation	Lactation	Outcomes in female offspring
Rat	Global UN	■	■	■	■	Reduced ovarian follicle numbers [89, 95] Reduced ovarian mRNA ER-B [89] Reduced mRNA follicular developmental genes [89] Disruption of HPG axis [87], Abnormal estrus cycles [87]
	Protein restriction	■	■	■	■	Delayed puberty onset [37, 105, 106] Disruption of HPG axis [106] Reduced in sex steroid receptor expression [37] Abnormal estrus cycles [105]
					■	Reduced LH and FSH concentrations [37] Reduced ovarian follicle numbers [108]
	High-fat diet	■	■	■	■	Early puberty onset [118] Abnormal estrus cycles [118] Impaired folliculogenesis [36]
	Sugar	■	■	■	■	Heightened leptin [125] Reduced steroidogenic enzymes [127] Upregulates ovarian genes for ovulation, folliculogenesis [78]
	Cafeteria diet	■	■	■	■	Heightened leptin [133] Upregulation of Igf-1 [132]
Mice	Global UN	■	■	■		Delayed puberty onset [94]
				■		Impaired folliculogenesis [85] Reduced mRNA follicular developmental genes [85]

(continued)

Table 4.1 (continued)

		Timing				
Animal model	Diet	Early gestation	Mid-gestation	Late gestation	Lactation	Outcomes in female offspring
	High-fat diet					Reduced ovarian follicle numbers [116] Reduced expression follicular developmental genes [116] Variations in Clock genes [116] Abnormal estrus cycles [117] Reduced ovarian weight [117]
Sheep	Global UN					Reduced ovarian development [91, 92] Reduced ovulation rate [90], Reduced lambing rates [79]
	High-fat diet					Reduced ovarian follicle number [119]

[21], with findings indicating elevated leptin concentrations play an inhibitory role on ovarian steroidogenesis [18] and ovulation rates [22]. As a key factor associated with the initiation of puberty, measurement of circulating leptin concentration is therefore an important biomarker that can link metabolic and reproductive dysfunction.

4.2 Study Design

4.2.1 Animal Husbandry Guidelines

The use of animals as preclinical models allows for the manipulation of complex biological systems, the control and standardisation of genetic and environmental cues, and the use of potentially invasive interventions which may not be ethical or practical in human trials. However, ensuring the validity of preclinical early-nutrition models, through adherence to experimental guidelines (such as those outlined by Animal research: Reporting in vivo experiments (ARRIVE) [23]) and control of confounding factors, is essential to allow comparison between studies and models alike. The ARRIVE guidelines detail the many considerations necessary for designing an animal model. Sample size, number of biological replicates, species, strain, sex, developmental stage, and genetic history of the experimental

animal should be detailed and selected with the desired outcomes in mind. Standardisation of the postnatal litter size is another factor to be considered, particularly in studies using multiparous animals. In rats and mice, for example, culling typically occurs at day 4–6 postnatally, where randomised reductions standardise litters to 8 pups – 4 females and 4 males [24]. Normalising the size of each litter may help control for differences in offspring food intake, maternal care, and pup growth and body weight [25], as well as more specific effects such as the reduction in antral follicles seen in female mice reared in smaller litters [26], and an increase in ovulation rate in female mice following selection for increased litter sizes [27]. Environmental factors such as singleton or group housing, bedding, temperature, enrichment, circadian rhythm are further essential factors to be considered, given their contribution to animal well-being and the potential quality of research [23]. Sheep, for example, are flock animals and grow stressed in isolation so acclimation to single-housing is necessary to minimise these effects. Lack of or limited bedding and nesting material following birth can induce maternal stress and impair cognitive development in offspring in both rats [28] and mice [29], while extreme variation in temperature and housing humidity can put the animal's well-being at risk and impact research, as physiological, metabolic and behavioural changes may be made by the animal to adjust to varying temperatures [30].

4.2.2 Diets

Early-life nutrition studies generally utilise specific diets that can induce human-like diseases and phenotypes, such as obesity, malnutrition, and anovulation. These dietary exposures in early life, explored in-depth in the later sections of this chapter, make use of deficient to excessive levels of a particular nutrient—global undernutrition, macro- and micronutrient restriction, and overnutrition primarily via use of energy dense diets, during the pre- and/or postnatal period. Consistency between studies using a similar diet has long been an issue, given the potential variance in the amount of treatment nutrient and other secondary macronutrients. Furthermore, secondary effects caused by excess or deficiency of other nutritional components may unexpectedly skew results. For instance, single nutrient diets high in one macronutrient such as fat, may inadvertently decrease carbohydrates. It is therefore recommended to use previously validated diets, and ensure adequate knowledge of the selected diet and the treatment sought. Furthermore, purified versus natural diet components can lead to alterations in hepatic gene expression [31], and upregulation in fatty acid synthesis and lipogenesis in mouse models, due in part to the significant increase in sucrose in standard purified diets compared to non-purified diets [32].

4.2.3 Preventing Bias

Typically, in early-life nutritional studies, diets are administered randomly to the mothers, with outcomes measured in the offspring at a later point. This random assignment is essential for reducing potential bias within the study and is deemed an essential element in any dietary study. However, careful regard needs to be taken to ensure possible neonatal mortality caused by the dietary treatments do not compromise the study. For example, particularly in multiparous animals, treatments which may reduce the fertility of the dams, or survival of subsequent litters, may result in only the healthiest offspring surviving in the treatment cohort, while the control cohort has a range of offspring. The possibility that this may skew the results of a study needs to be considered in the design, potentially through the inclusion of further measurement points in both generations of animal [33].

4.2.4 Timing

As seen in human cohorts, such as the Dutch Hunger Winter (1944–45) where pregnant women were exposed to famine at different periods of gestation, the timing of any early life challenge can greatly influence the effects seen in fetal development and later life health. Studies of the Dutch Hunger Winter cohort have illustrated this, with undernutrition during the first trimester of gestation shown to increase the later life risk of cardiometabolic diseases in offspring, while those in utero mid- to late-gestation during the famine had reduced glucose tolerance in later life [34]. Early-life nutrition can extend beyond gestation and early neonatal life, with important windows of development extending through to adolescence and influencing later life health [35]. These windows may differ between species, where developmental milestones occur at different times, such as the postnatal folliculogenesis of rodents compared to prenatal of humans. Experiments in Wistar rats demonstrated the contributions of early developmental periods to later offspring health, where under- and overnutrition during gestation and/or lactation induced earlier puberty onset in the female offspring, with the suggestion that nutritional insults may influence changes at multiple points during fetal and postnatal development [36]. Further studies illustrate how altered nutrition during lactation can negatively impact rodent reproductive capability given the continued development of key gonadal systems [37]. A nutrient restrictive diet in pregnant ewes during the period of maximal fetal growth only (gestational day 28–80), followed by return to standard diets resulted in increased leptin concentration and adipose tissue mass in offspring compared to controls, emphasising again how the specific timings of nutritional insults can differentially influence development [38].

4.3 Animal Models

4.3.1 Rodents

Animal models vary in gestational length, cost, and comparability to human physiology and fetal development. Commonly, rodents – rats (Rattus norvegicus) and mice (Mus musculus) – are the animal models of choice for preclinical assessments of multi-generational effects. The advantage of decreased gestation time, higher offspring yield compared to large animal models, cost, and relative ease of genetic manipulation make rodent models, of which rats are primarily used, an attractive animal model (Table 4.2). The average gestation period of rodents is relatively short compared to larger animals (~19.5 days in mice, 22 days in rats) [39], with weaning occurring within 21–28 days of birth. Puberty occurs at age 40–45 days for males and 32–36 days for females [40], marked in females by vaginal canalisation and the beginning of short estrus cycles of 4–5 days [41]. Rodent life-span is comparatively short (2–4 years), which allows the assessment of early life challenges over the rodents' life course and intergenerational effects in future offspring. Given that many developmental effects can be propagated through multiple generations, the ability of preclinical models to assess how later generations of offspring are influenced by a previous insult is also essential. In females, this means three generations post-insult, and two generations for males (Fig. 4.1). Though the difference in litter size may seem a disadvantage when compared to singleton or twin births common in humans and other large animal models, the increased number of offspring allows for the adequate investigation of both sexes exposed to the same maternal challenge, as well as the ability to collect tissues within the same litter at different, significant time-points. However, the ratio of a rat's weight to that of her pups does not correlate well with a human mother to her baby, resulting in several physiological differences, such as dissimilar fetoplacental transport [42], relative to humans.

The increased size of rats in comparison to mice allows for greater collection of blood and tissue samples. However, the translation of physiological results from the rodent model to humans is not always straightforward. Humans are precocial, with many neurological pathways developing during gestation. Similarly, sheep and guinea pigs are precocial, while altricial species, such as rats and mice, develop postnatally. Studies of nutrient restriction in rodents are often more informative postnatally due to their later brain development [43, 44]. Equally, the differences in timing of gonadal development between species are essential to understand when investigating reproductive dysfunction. Both rodents and larger mammals (humans, sheep, cows, and pigs) experience meiosis prenatally. Primordial follicle development, where the total number of oocytes is established for the span of the female's life, occurs prenatally in most female mammals, whereas in rodents, folliculogenesis occurs in the first week of early postnatal life [45, 46].

Similarly to humans, regulation of rodent puberty is through pulsatile release of GnRH from the hypothalamus, which stimulates the pituitary gland to release gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH)

Table 4.2 Common animal models in Developmental Origins of Health and Disease (DOHaD) studies and comparisons of important factors involved in study design

	Mice	Rats	Sheep	Non-human primates	Humans
Cost	Low	Low	High	High	High
Gestation time	Short (19.5 d)	Short (22 d)	Long (145-150 d)	Long (163-185 d, Baboon)	Long (280 d)
Offspring	Multiparous	Multiparous	Singleton/twins	Singleton/twins	Singleton/twins
Age at puberty onset (female)	21-28 d	21-28 d	Varies	3–4 years	12–13 years
Life span	Short (2–4 years)	Short (2–4 years)	Moderate (10–12 years)	Long (35–40 years)	Long
Development	Altricial	Altricial	Precocial	Precocial	Precocial
Folliculogenesis	Early postnatal life	Early postnatal life	Prenatal	Prenatal	Prenatal
Menstruation	No (exception being Spiny Mouse)	No	No	Yes	Yes
Reproductive cycle	Short estrus cycle (4-5 d)	Short estrus cycle (4-5 d)	Seasonal, moderate estrus cycles (16-17 d)	Menstrual cycle (33 d)	Menstrual cycle (28 d)
Ovulation	Polytocous	Polytocous	Monotocous	Monotocous	Monotocous
Placentation	Discoid, hemotrichorial and labyrinthine	Discoid, hemotrichorial and labyrinthine	Cotyledonary	Discoid, hemomonochorial and villous	Discoid, hemomonochorial and villous
Behaviour	Nocturnal	Nocturnal	Diurnal	Diurnal	Diurnal
Housing Size	Small	Small	Large	Large	N/A
Genetic Manipulation	Common; gene knock-out/ over-expression, genotyping	Common; gene knock-out/ over-expression, genotyping	Gene expression	Gene expression	Gene expression

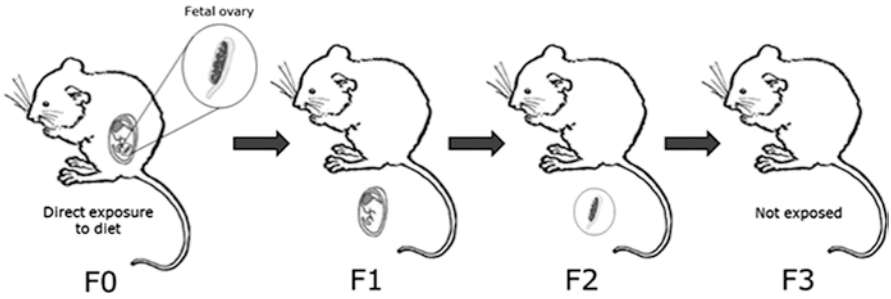

Fig. 4.1 Multigenerational transmission of dietary insults to the mother (F0) influences the female fetus (F1) and her developing oocytes (F2) through exposure to the initial maternal environment during gestation and development. Transgenerational investigation begins at F3 in females

that in turn stimulate the gonads to produce sex steroids and regulate estrus cycles and ovulation [47]. Unlike humans, most rodent species do not menstruate. As previously mentioned, rodent estrus cycles are short (4–5 days) and defined by 4 distinct phases: proestrus (12–14 h), estrus (25–27 h), metestrus (6–8 h), and diestrus (55–57 h). These stages can be recognised by a majority nucleated cells, unnucleated cornified cells, leukocytes, and a combination of leukocytes and nucleated cells respectively [46, 48]. Staging female rodents is a simple and effective method of determining estrus cycle regularity and distinguishing abnormal and prolonged cycles, which offers an advantage in preclinical studies assessing the estrus cycle. Further to this, the spiny mouse (Acomys cahirinus) is a menstruating rodent species with a reproductive cycle marked by uterine and ovarian phases similar to other menstruating species. The spiny mouse may therefore be readily used in the study of menstrual and other related reproductive disorders, as well as neonatal development [49].

Further aspects to consider include differences in contributions of the corpus luteum to hormonal control during pregnancy. In humans, progesterone, the key product of the corpus luteum, is essential in the establishment of pregnancy and survival of the embryo until the development of the placenta [50, 51]. Conversely, the rodent corpus luteum continues to secrete progesterone throughout gestation, before regression of the corpus luteum and the subsequent drop in progesterone acts as a trigger for parturition [52]. Placentation also differs in several aspects. In rodents and humans, placentation is discoid and hemochorial, though rodent placentae are hemotrichorial and labyrinthine, while human placentae are hemomonochorial and villous. Labyrinthine species are often characterised by higher fetal growth rates and shorter gestational times compared to villous placenta [53]. The varying timing and type of placentation are important experimental considerations in any study of fetal nutrition, given the possibility of early life events influencing the transport of maternal nutrients to the developing fetus, and the importance of placental endocrine signals in the control of nutrient supply. While rodent and human placentation share similarities, it is important to note the differences between the species, such as the inverted nature of the rodent yolk sac which remains active

throughout rodent gestation, versus the human yolk sac which precedes placental development, before its function later becomes unnecessary following the first trimester [53].

Rodents are also nocturnal, requiring the use of lighting to alter their natural light cycles. Experiments done during their sleep cycle may change circadian rhythms, with run-on effects to behavioural patterns, hormone production, and metabolic homeostasis [54]. Mice, being smaller in size, may offer more of a challenge in undertaking physiological experiments, though their advantages lie in the ability to genetically alter either whole animal physiology, a specific organ or tissue, or utilise specific gene knock-outs or gene over-expression with relative ease. Similarly, knowledge of the mouse and rat genome offers significant advantages when investigating genetic function [44]. Some inbred rodent strains may remain genetically constant over multiple generations, where genotyping of an individual can effectively represent the whole strain, and allows the creation of a strain-specific genetic profile [55]. Strains of mutant mice have also been regularly used to investigate reproductive dysfunction, where genetic manipulation can alter key genes linked to pubertal initiation and other fertility aspects, allowing researchers insight into how particular genes help regulate reproductive health [47]. For example, knock-outs of G-protein-coupled receptor Gpr54 and Kiss1 in mice have been used to confirm their importance in reproductive health, with loss-of-functions of both markers producing hypogonadism in male and female mice, and suggesting regulatory effects on GnRH secretion [56]. Finally, these characterised genes can act as useful biomarkers of reproductive health when quantifying their expression [47].

4.3.2 Sheep

Ovine models offer numerous advantages when used as an animal model for examining the effects of altered early life nutrition that other, smaller animal models do not. Many experiments investigating developmental programming have been conducted in the sheep, due in part to a number of similarities between sheep and human fetuses, a robust knowledge of the regulation of the reproductive neuroendocrine system, and the ability to use surgical methods to directly study the effects of various insults on the ovine fetus in vivo. Gestational length in sheep is between 145 and 150 days, during which fetal size and developmental rate are similar to human fetuses [57]. The gestational length allows for extended fetal testing and any feto-placental adaptions resulting from the nutritional insult to take effect [57]. This increased size, compared to rodent pups, thus allows for surgical manipulation, improved collection of blood and tissue samples, often over repeated intervals including during gestation, and permits for a larger variety of outcomes to be assessed [58]. Conversely, the larger fetus translates to increases in the size, and therefore expense, of any intervention compounds required. Similarly, due to their size, sheep require larger housing, and for longer in any study investigating disease development or intergenerational effects. Again, this makes the sheep model more

expensive and time-consuming than smaller models [44]. As ruminants, sheep also offer difficulties with regards to nutrition studies, with attention required when creating a suitable diet given their different digestive physiology [58].

While rodent models can produce up to 15 offspring (depending on strain), the smaller offspring number of sheep models requires double the number of dams to acquire adequate numbers of male and female offspring for investigation that is statistically sufficiently powered. Singleton births and the largely similar pre- and postnatal pattern of organogenesis of the fetus/neonate in ovine models are more comparable to the human situation. As previously mentioned, sheep and humans are both precocial species, with folliculogenesis occurring prenatally in both species. These shared developmental stages offer advantages when comparing between the species. Puberty is reached at approximately 7 months, which, as with many other mammals, is induced by increased pulsatile activity of GnRH and the subsequent flow-on effects of the HPG axis leading to first ovulation [59]. However, sheep are seasonal breeders, limiting fecundity [60] and influencing both the adult receptive phases and the initial puberty onset in young females as puberty typically begins during the breeding season, while outside of the receptive breeding season, females are anovulatory [61, 62]. The environmental factors which influence the estrus cycle include the photoperiod, while age, nutritional status, presence of males, temperature and the season can also influence the duration [61]. Typically, cycles in the ewe are 16–17 days in length and marked by the growth of a number of small follicles to ovulatory sizes in both ovaries due to follicle stimulating hormone surges [63]. Introduction of rams, treatment with progestogen, melatonin, or manipulation of the photoperiod in housed ewes, can induce estrus in those previously anovulatory, allowing mating outside of traditional breeding seasons [61]. Placentation in the sheep also differs from humans as it is cotyledonary, where ruminant placentation consists of a number of smaller placentae compared to the single area of contact found in humans [64, 65].

4.3.3 Non-human Primates

While not commonly used in the DOHaD field due to both ethical and high-cost factors, non-human primate models have offered valuable insight into developmental programming and early nutrition due to the many aligning aspects of physiological, neurological and reproductive development between humans and other primates [66]. The comparability between humans and non-human primates is the largest advantage to the model, as they are able to more accurately translate to human pregnancy and development than other models, especially in terms of nutritional studies looking at fetal development.

One species which closely resembles human physiology, developmental programming and reproductive processes, is the baboon. The baboon's life span is relatively long at 35–40 years, with puberty in the female baboon beginning between the age of 3–4 years, and sexual maturity reached between 4–6 years [67]. Puberty

begins after 4 years in the male baboon [68]. Like humans, the baboon is a precocial, monotocous species. The length of gestation is approximately 163–185 days [67], with placentation analogous to humans [69]. The reproductive physiology and internal reproductive tract of the female baboon is similarly comparable to a human female [70], which allows easier comparison between baboon models and human health. The stages of the estrus cycle are easily determinable, due to the estrogen-mediated swelling of perineal skin, and allow non-invasive techniques to determine menstrual cycle stage. Menstruation similarly occurs in Old World monkeys such as the rhesus macaque, with neuroendocrine regulation of the menstrual cycle comparable to that in humans [71].

Other invaluable advantages of non-human primate models include the possible extrapolation of human gene and protein structure to other primates such as the baboon. This has already allowed gene expression and protein phenotyping of possible pathways in fetal studies of reduced nutrition [72, 73]. However, as with the ovine model, non-human primates require larger housing than smaller animal models, and a much longer investigation time if intergenerational effects are to be measured, or if the study aims to investigate later life disease development. These lengthier experimental periods have clear logistical implications as regards cost and time required.

4.4 Models of Reproductive Dysfunction

In animal models of reproductive disease, the importance of a well-characterised reproductive system and cycle are essential, while the comparability to the physiological features of the human cycle also needs to be taken into consideration. The development of models to investigate specific reproductive conditions also requires the development of phenotypes characteristic of the disease. For example, amenorrhea, defined as the absence of menstruation, can be induced by mutations in the FSH receptor (FSH-R) of mice. This null-mutant model illustrates how the resulting loss of estrogen production caused by the loss of FSH-R is associated with the cessation of the ovaries' reproductive actions, such as at the natural end of a woman's reproductive life. These effects can be reversed by exogenous treatment of estradiol-17β, and highlight useful markers which can be used in disease identification [74]. Furthermore, polycystic ovarian syndrome (PCOS), a syndrome characterised by hyperandrogenic chronic anovulation [75], with potential origins in epigenetic changes occurring during fetal development, can be programmed in several species through prenatal exposure to excess testosterone [76]. Rodent models are commonly used for PCOS modelling, where androgen exposure can cause phenotypes associated with the syndrome; anovulation, hyperandrogenism, and polycystic ovaries [77]. Dietary inducement of these phenotypes through high-fat and high-sugar diets in rats has also been shown to cause symptoms commonly associated with reproductive conditions such as PCOS [78]. Ovine and non-primate models, might prove more effective in understanding the primary causes of PCOS given the

similarity in prenatal folliculogenesis to humans (as mentioned in Sects. 4.3.2 and 4.3.3), the impact maternal steroid homeostasis has on fetal reproductive physiology and later vulnerability to PCOS [76].

4.5 Early-Life Dietary Models and Reproductive Dysfunction

Maternal nutrition during pregnancy and lactation plays an important role in the potential phenotype of the offspring in later life. The timing of any dietary intervention has important implications for how the fetus is affected, with many early studies focusing on later stages of pregnancy due to higher fetal nutrient demands [79]. However, influences on fetal development may be induced at any stage from preconception through to the end of lactation, indicating the wide range of mechanisms which may be at work in the development of later metabolic and reproductive disorders.

4.5.1 Undernutrition

The use of maternal undernutrition through global caloric, protein restriction, or specific micronutrient restriction, has long been a popular insult used to assess the developmental programming of offspring. Nutrient availability in utero may be manipulated through maternal intake, placental function, and the metabolic health of the mother, while in the early postnatal period, offspring nutrient availability can be reliant on maternal intake and lactation.

4.5.1.1 Global Undernutrition

Maternal undernutrition through global caloric restriction is the most comprehensively characterised model in the DOHaD field. This diet allows comparisons to human starvation or famine models, and offers unique insight into both gestational undernutrition and, through postnatal diets of improved nutrition, the effect of PARs [1]. Global undernutrition varies from moderate restriction (30% of ad libitum control) [80] to severe (70% of ad libitum control), or as a percentage of total energy required over gestation. While the initial insult may cause later dysfunction in offspring, a second insult postnatally of a HFD has been shown to further the negative effects of the initial challenge [80], suggesting that catch-up growth compounds issues previously initiated by the prenatal environment [81]. Early investigations into the DOHaD hypothesis focused in large part on the induction of reduced birth weight through the use of maternal global nutrient restriction. The large number of

studies in both human cohorts and animal models have detailed the negative health effects caused by the restricted diet; intrauterine growth restriction, reduced birth weight followed by catch-up growth and a higher risk of obesity in later life, increased risk of cardiovascular diseases in later life, insulin resistance, hyperleptinaemia [6, 82], and reproductive health [83, 84].

Many studies have reported reduced ovulation rates and reproductive success of offspring who suffered undernourishment as fetuses in mice [85, 86], rats [87–89], and sheep [79, 90, 91]. An early study in sheep using either a diet 150% (high) or 50% (low) of required energy for pregnancy found reduced ovarian development in fetal ovaries of ewes on the lower restricted energy diet. The failure of early oocyte degradation at day 47 is thought to increase the number of early oocytes, while delay in the onset of meiosis at a later stage of fetal ovarian development is involved in deleterious effects to immature germ cells [91]. These adverse effects were confirmed in a later study, where delays in fetal ovarian meiosis onset were induced following undernutrition before and during ovary formation [92]. These findings have been replicated in rodent models, with undernutrition of pregnant mice over a specific 3 day period of gonad sex differentiation in the fetus (beginning 12.5 days following conception) contributing to the impairment of folliculogenesis [85]. Again, undernutrition is thought to lead to delays in oocyte meiosis onset and thereby increase apoptosis of immature ooctyes, reducing the number of ooctyes and impairing later life reproductive health. At postnatal day 3, reductions in the mRNA protein levels of genes involved in primordial follicle formation, Nobox, Sohlh2, and Lhx8 offer an explanation for the decrease in oocyte reserve in offspring [85]. Bernal et al. demonstrated reductions in primordial, secondary, and antral follicle number in the female offspring of rat dams undernourished during pregnancy, where reduced mRNA levels of ovarian Gdf-9, a key gene in early follicular maturation, may inhibit follicular survival. Estrogen receptor beta (ER-β) mRNA was also decreased in female offspring, and may exert downstream effects on leptin receptor mRNA expression and therefore influence ovarian steroidogenesis and oocyte maturation through leptin-mediated effects [89]. In rats, fetal undernutrition may adversely affect the HPG axis of the developing fetus, leading to reduced concentrations of circulating estradiol, premature reproductive deterioration in later adulthood, and disrupted estrus cycles [87]. Measurements of estradiol and its receptor are useful indicators of reproductive function, enabling investigation into their potential feedback effect on the HPG axis and direct effects on the ovary [74]. Similarly, testosterone, which may disrupt estradiol dynamics, is another valuable marker of reproductive dysfunction, with high concentrations associated with PCOS [93]. Altered timing of puberty onset in mouse female offspring following gestational undernutrition has also been reported, with the suggestion that lowered hypothalamic Kiss1 mRNA concentrations contributed to the delay in vaginal opening [94]. The same study demonstrated that injections of kisspeptin, neuropeptides expressed by the gene Kiss1 which are involved in the excitatory regulation of GnRH, normalised puberty onset, indicating its influence on the reproductive health of undernourished rodents [94]. Furthermore, Chan et al. demonstrated increased ovarian mRNA levels of circadian clock genes Clock, Period (Per1), and

Cryptochrome (Cry1) in rat offspring of undernourished dams [95], where disruption of these genes have been associated with delayed puberty onset and abnormal estrus cycles in clock gene-null mice [96].

In ovine adulthood, this impaired reproductive ability can be illustrated by reductions in the ovulation rate of female offspring, with little effect to reproductive function in male offspring [90], and the reduced lambing rates experienced by female offspring of undernourished mothers [79]. These alterations could be mediated through reduced pituitary sensitivity to GnRH, where early gestational undernourishment may stall development of the HPG axis and reduce pituitary responsiveness to GnRH [97]. However, later studies in sheep have found no evidence of differences in GnRH sensitivity in female offspring, or other hormones such as insulin and insulin-like growth factor-1 (IGF-1) [90, 98], suggesting gonadotropin-independent mechanisms. This might be through variations in thyroid hormones, specifically reductions in triiodothyronine (T3) in undernourished fetuses, and dissimilarities in the pattern of chronic versus acute undernutrition [98].

4.5.1.2 Protein Restriction

Dietary protein is a source of amino acids for the body's protein synthesis, and is involved in regulation of food uptake as well as metabolism of lipids and glucose [99]. The restriction of protein during pregnancy negatively effects the growth and development of the fetus, programming various organs leading to increased risks of metabolic issues such as insulin resistance, hypertension, glucose homeostasis [100], and reproductive dysfunction [101]. Maternal protein restrictive diets also increase the incidence of obesity in male and female offspring when fed a postnatal control diet [100], and raises the risk of metabolic disorders such as T2DM, insulin resistance, and hypertension when fed a HFD postnatally [2].

Control, chow diets for rodents normally consists of 15–20% protein, with restricted diets frequently halving the protein content. Typical sources of protein, such as casein or soy-based, differ in their effects on offspring. Maternal diets of soy-based protein are associated with increased postnatal weight gain, impaired food regulation [102], and increased indicators of metabolic syndrome [103] compared to casein sourced protein in rat offspring. Other aspects of dietary composition need to be taken into account when creating protein restrictive diets, as the ratio of carbohydrates and lipids to protein can alter fetal body composition and adiposity [104]. Similarly, different effects may be observed in a sex-specific manner, with female offspring of rat dams fed a protein restrictive diet of 10% casein displaying increased fat and altered insulin sensitivity compared to male offspring [100].

In rat studies, protein restriction pre- and/or postnatally negatively influences female offspring reproductive health. Delayed puberty onset in female rodent offspring has been reported in numerous studies [37, 105, 106], and associated with lower ovarian and uterine weight, as well as altered steroid synthesis. Maternal malnutrition may hinder maturation of the HPG axis in rodents [106]. High maternal concentrations of glucocorticoids during pregnancy are known to contribute to

delays in female offspring puberty onset [107], a mechanism further implied by delayed vaginal opening in female offspring of rat dams marked by heightened corticosterone concentrations during pregnancy [105]. Pre- and postnatal insults also result in marked reductions in LH receptor expression at day 21 postnatally, while female offspring exposed to protein restriction during lactation only, exhibit decreases in LH and FSH concentrations but no differences in their receptor. Both mechanisms can negatively impact ovarian maturation and puberty onset timing, through interactions at the ovarian or HPG axis level [37], with the inference that low leptin concentrations may lead to the reductions in LH and FSH concentrations and consequent low estradiol serum concentrations [106]. Restriction during lactation also results in reduced numbers of primordial follicles and negatively influences endothelial regulators involved in angiogenesis, with reductions in vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) mRNA receptor expression. Stimulation of follicle growth and primordial follicle development by VEGF and FGF respectively, have been suggested as further mechanisms through which reproductive function is affected [108]. Fetal protein restriction is associated with premature reproductive function ageing, illustrated by alteration of estrus cycles [105]. Further to this, when mismatch between pre- and postnatal diet occurs, deleterious effects to female offspring follicle number occur at increased rates compared to constant dietary restriction across both time points [37].

4.5.2 Maternal Overnutrition

Given the increasing rates of overweight and obese individuals, especially among women of reproductive age, and the easy access to high-fat, high sugar heavily processed foods typically consumed in Western countries, the incidence of excessive maternal and postnatal caloric intake is more common than undernourishment. Overnutrition in early life increases the risk of developing cardiometabolic disease in later life, heightens the incidence of obesity and its associated morbidities [109–111], and is linked with a number of reproductive dysfunctions such as PCOS, anovulation and early onset puberty [112].

4.5.2.1 Single Source High-Fat Diet

In preclinical models investigating obesity and overnutrition, the HFD is commonly chosen for its effective inducement of an obese phenotype in both mothers and offspring. Typically defined as energy dense diets high in fat and sugar, HFD may range from 20% to 60% kcal from fat in rodents. However, given differences in the source and amount of fat a single, standardised diet is lacking. Differences between diets can lead to inconsistencies, with variation in macro- and micronutrient composition and energy density altering energy intake, body composition, and the course of weight-gain. There is also the potential that poorly designed HFDs can lead to

secondary issues such as protein deficiency that may confound results. The fat component of a HFD may come from animal-derived fats (e.g. lard, beef tallow) or plant oils (e.g. corn, palm oil, soybean oil or safflower oil), both of which are known to induce obesity phenotypes in rodents, particularly strains susceptible to obesity such as C57BL/6 mice, and Wistar and Sprague-Dawley rats [113]. Excess calorie consumption seen with HFD, increases the risk of both obesity and related morbidities, and reproductive dysfunction. Work in an ovine model suggests that diet-induced maternal obesity leads to increased fetal growth due to redistribution of excess maternal energy substrate to the fetus, driven in part by the increased insulin resistance in the mother [114].

Fetal exposure to obesity or HFD before conception appears to adversely affect oocytes and later generations of offspring. In mice, the offspring of mothers with pre-conception obesity are smaller at birth than controls, but display marked catch-up growth and are heavier than controls by the time of weaning. Impaired glucose tolerance, dislipidemia, and increased adiposity are also reported in the post-weaning period [115]. Reductions in IGF-1 receptor expression in developing fetuses, as a result of maternal obesity, have been linked to reduced insulin signalling and growth restriction [116]. These actions may have further consequences such as ovarian regulation, puberty onset, and ovulation, induced through the insulin-related ovarian regulatory system, which involves insulin, IGF-1, IGF-II, and their receptors and binding proteins [10]. When maternal obesity is induced by HFD in mice during gestation, there is a decrease in the number of offspring ovarian follicles. This reduction is seen in primordial, antral, and Graafian follicles. Alterations in the expression of genes associated with follicular development and growth, and apoptosis, such as FOXO3a and Gdf9, are related to this reduction in follicle numbers. Furthering this effect are variations in the cyclic expressions of genes responsible for ovarian circadian oscillations; Clock, Bmal1, Cry1, and Per1 [116]. In humans and mice, inhibition of Bmal1 causes reduced fertility, and has been implicated in the regulation of numerous genes related to ovarian folliculogenesis [95]. Maternal overnutrition also increases the risk of abnormal estrus cycles in female offspring and reduces the weight of ovaries compared to offspring of control fed mothers [117]. Dysfunction of metabolic homeostasis in female offspring results in increased adiposity and subsequent increases in leptin concentrations and hepatic IGF-1, a regulator of leptin secretion. The permissive role of leptin in puberty initiation through the HPG axis would therefore enable earlier puberty onset and, in adulthood, estrus cycle irregularity [117]. In humans, maternal obesity is similarly implicated in the earlier onset of puberty in female offspring, which, when combined with GDM, leads to earlier transitions into pubarche for female offspring [112].

Similar to results in mice, a maternal HFD in the rat is associated with earlier puberty onset in female offspring, increased likelihood of irregular estrus cycles and persistent estrus compared to offspring of control diet mothers [118], as well as impaired follicular development and reduced future reproductive health [36]. A postnatal HFD following maternal undernutrition during pregnancy resulted in dysregulation of steroidogenesis in female offspring, with specific to mRNA levels of

3b-hydroxysteroid dehydrogenase (3b-HSD) and ovarian leptin receptor (Ob-Rb). Both factors are involved in ovarian steroidogenesis [89]. Increased apoptosis of early oocyte pools might also be influenced through increased maternal-fetal inflammation, while noted reductions in anti-müllerian hormone (AMH), an inhibitor of primordial follicle assembly, and its receptor AMHRII can dysregulate the normal transition of primordial follicles in neonates [13]. Maternal overnutrition—a high, ad libitum diet of 10.2MJ/kg dry matter—in sheep similarly reduces the primordial follicle pool in female fetuses, with alterations in the normal development taking place prior to gestational day 100, before the initiation of the HPG axis is required for reproductive maturation. This implies placentally produced endocrine hormones, growth factors and estrogen, may exert local influences on ovarian development in the sheep fetus and identifies these factors and their receptors as relevant markers in reproductive assessment [119]. Multigenerational effects can be seen too, where ewes (F1 generation) exposed to maternal overnutrition during gestation of the F0 generation, exhibited higher concentrations of blood glucose and insulin compared to controls during their own pregnancies. The F2 offspring of the F1 mothers had increases in blood glucose, insulin, and cortisol, with marked increases in adiposity compared to offspring of control mothers [120]. As previously mentioned, heightened leptin concentrations predispose women to early puberty onset through actions on the HPG axis, and can inhibit normal ovulation [18]. Inflammation induced by obese states may also contribute to negative reproductive effects. Obesity and pregnancy are states of low-grade, chronic inflammation. Studies of inflammatory cytokine knockouts [interleukin (IL)-1α] concluded that inflammatory environments negatively contribute to reduced ovarian reserve in mice [121].

4.5.2.2 Other Single Nutrient Diets

Given the rise in consumption of sweetened beverages and food, and their association with increased global obesity rates, the investigation of maternal sugar consumption has grown in necessity [122]. Commonly, fructose and sucrose are the sugars of choice. Fructose diets typically consist of a solution where a percentage of the daily caloric intake is derived from sugar, with fructose-fed rodent diets a common animal model of metabolic disease, where rats commonly display insulin resistance, hypertension, and obesity [123]. While larger percentages of fructose have been used in maternal studies, these may not be analogous to a general human diet, instead comparative to a diet in excess of relevant fructose consumption. Fructose intake of 15–20% of daily calories is equivalent to the average consumption in humans of high fructose consumers [124]. In any animal study the composition of a high-sugar diet should relate to a relevant human intake.

One study in rats highlighted elevated glucose and leptin concentrations in female fetuses and neonates of fructose fed mothers, with a reduction in female placental weight. These effects weren't mirrored in male fetuses, illustrating sex-specific effects of the high fructose diet during pregnancy on placental growth and fetal development [125]. Further sex-specific variations have been noted in fructose

diet studies in adult rats, such as a potential protective mechanism by female sex hormones, particularly estrogen, against diet-induced hypertension and insulin resistance [126]. Further investigations in rats fed a 20% fructose solution, identified a reduction in steroidogenic enzymes, such as P450arom, which contributed to inhibited concentrations of estradiol, an important factor in ovarian maturation, and its receptor, estrogen receptor alpha(ERα) [127]. High sucrose and HFD induce heightened insulin concentrations, which in turn act on the ovary to increase the ovary's production of androgens, contributing to the hyperandrogenism characteristic of PCOS. Furthermore, this diet upregulates genes related to ovulation, follicle maturation, steroid signalling, and inflammatory regulation within rat ovaries. Upregulated epiregulin (Ereg), for example, mediates LH and plays a key role in follicular maturation to the primary stage. While upregulation also occurred in genes related to estrogen metabolism such as Ste2, involved specifically in the production of 7α,20β-dihydroxypregn-4-en-3-one and oocyte maturation [78].

4.5.2.3 Cafeteria Diet

The cafeteria diet, also known as the "junk food" diet, simulates the highly processed diet common in Western societies. In preclinical models, the cafeteria diet consists of a nutritionally varied diet of high-energy, palatable food and can include chocolate, condensed milk, cake, deli-style meats, biscuits and bread. In rodent models, extended cafeteria diet induces and models metabolic syndrome, inducing obesity, increased insulin resistance, and glucose intolerance [128, 129]. The cafeteria diet is also associated with increased adipose and liver inflammation in comparison to the single source HFD, and has been argued as a more robust model for modern human obesity inducement than others [130]. While this diet may offer a variation in nutrition more comparable to a human diet than single source (HFD), the lack of standardisation is one reason why the cafeteria diet is used less frequently than diets of single nutrient excess [131].

Despite the less frequent use of the 'junk food' diet approach, studies have illustrated metabolic dysfunction in offspring exposed in utero. Rat offspring following a maternal cafeteria diet during gestation and lactation, had marked upregulation of IGF-1 and other genes related to adipocyte growth [132], which corresponded with increases in adiposity, glucose, insulin, and leptin. There was differential expression seen between female offspring and male offspring, with increased concentrations of plasma leptin and blood glucose in female fetuses of fructose-fed mothers, but not in male foetuses [133]. As with HFD based models, this dysregulation of glucose, insulin and leptin is associated reproductive dysregulation through indirect and direct effects to the HPG axis and the ovaries [10, 18]. In female rats fed a cafeteria diet post-weaning, obesity and metabolic dysfunction were noted at adulthood, as well as a limited reproductive capability associated with reduced numbers of oocytes and prenatal follicles, mediated through the reduction of LH concentrations. Further dysregulation was seen in the loss of progesterone pulses, which in turn failed to induce LH surges, leading to ovulatory dysfunction [134]. Reduced serum levels of

ovarian AMH protein, an inhibitor of follicular recruitment, has also been demonstrated in populations of primordial, primary, and antral follicles in obese rats following the cafeteria diet, suggesting increased progression from primordial pools and possible early exhaustion of oocyte reserve. Further to this, the study demonstrated reductions in estrogen concentrations, leading to abnormal estrus cycles [135], and highlighting again the suitability of estrogen as a reproductive biomarker.

4.5.3 Surgical Interventions

Fetal malnutrition may find its root cause in two over-arching mechanisms, maternal undernutrition and placental insufficiency. During placentation, failure of spiral arteries to transform into wider, low-resistance vessels hinders the supply of maternal blood to the placenta, and therefore the transfer of oxygen and nutrients [42]. This increases risk factors for maternal hypertension, insulin resistance and small-for-gestational-age (SGA) offspring [136], where SGA can influence later life risk of cardiometabolic diseases. In humans, uteroplacental insufficiency is the most common cause of intrauterine growth restriction (IUGR), which arises in a tenth of all pregnancies in the western world. Surgical interventions offer insight into human cases of IUGR, where altered placental efficiency is more common in developed countries than maternal malnutrition.

Bilateral uterine vessel ligation is a common non-dietary model of placental insufficiency in rodents and sheep to induce IUGR characteristics in offspring, with the model mirroring human placental insufficiency. While roughly a third of rodent fetuses die or are partially reabsorbed following ligation [137], this intervention is nevertheless popular, and reduces blood flow to the placenta via surgical ligation of the rodent horn towards the end of gestation. The degree to which blood flow is restricted is dependent on where on the uterine horn the ligation is made [138]. Placental insufficiency also impairs maternal mammary function, compromising milk quality and postnatal offspring growth [139]. In rats, maternal uterine ligation resulted in delayed puberty onset [140] and decreased progesterone concentrations in female offspring, while offspring cross-fostered onto control dams following exposure to in utero ligation, experienced catch-up growth during lactation and entered puberty 2 days earlier than the previously mentioned cohort [141]. Placental restriction in the ovine model can be induced through a number of methods; uterine carunclectomy, which restricts the number of placentae formed at the onset of pregnancy; single umbilical artery ligation, which reduces the flow of nutrients to the placenta; and placental embolisation, a method which reduces the placental transfer surface area through blockage of placental capillaries by microspheres. [142]. These methods cause undernutrition and hypoxemia in the fetus, which can alter IGF-1, insulin, leptin, and lead to IUGR [143, 144]. In humans, IUGR is thought to inhibit ovarian development and impact the HPG axis' control of puberty initiation through the influence of neuropeptide Y and leptin [143].

4.6 Conclusions

Pre-clinical models of early-life altered nutrition are essential for understanding the link between fetal development, nutrition and altered later life disease. While epidemiological studies in humans have offered valuable insight into the DOHaD hypothesis and influences on reproductive health, animal models offer a greater chance to utilise techniques and experiments not possible in humans given ethical and moral constraints, with possible interventions and understanding able to be expanded and explored. The investigation of metabolic dysfunction induced by misaligned early-life diet is a well explored topic, however more emphasis is required on the reproductive diseases influenced through developmental programming. Further to this, the understanding of both female and male reactions to altered early-life nutrients should be essential. This review has explored the use of preclinical models and illustrated potential biomarkers that are important indicators of reproductive function. Understanding the mechanisms associated with these pathways can offer new, valuable insights into today's reproductive diseases, and possibly lead to interventions to mitigate these effects early on.

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Chapter 5
Targeting Mitochondrial Defects to Increase Longevity in Animal Models of Neurodegenerative Diseases

Ester Casajus Pelegay, Francesco Puzzo, Acelya Yilmazer, and Umut Cagin

Abstract Bioenergetic homeostasis is a vital process maintaining cellular health and has primary importance in neuronal cells due to their high energy demand markedly at synapses. Mitochondria, the metabolic hubs of the cells, are the organelles responsible for producing energy in the form of ATP by using nutrients and oxygen. Defects in mitochondrial homeostasis result in energy deprivation and can lead to disrupted neuronal functions. Mitochondrial defects adversely contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease (PD). Mitochondrial defects not only include reduced ATP levels but also increased reactive oxygen species (ROS) leading to cellular damage. Here, we detail the mechanisms that lead to neuronal pathologies involving mitochondrial defects. Furthermore, we discuss how to target these mitochondrial defects in order to have beneficial effects as novel and complementary therapeutic avenues in neurodegenerative diseases. The critical evaluation of these strategies and their potential outcome can pave the way for finding novel therapies for neurodegenerative pathologies.

Keywords Mitochondria · Drug target · Longevity · Animal models · Neurodegenerative disease

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5.1 Introduction

Mitochondria are the double membraned organelles found in eukaryotic cells. They have become part of eukaryotic life following an endosymbiotic event that occurred many years ago to favor more efficient energy production, which has shaped multicellular life [1]. Mitochondria are the central component of cellular metabolism for producing energy in the form of ATP by the consumption of energy sources such as carbohydrates and fatty acids. These organelles play key functions within eukaryotic cells since they are the principal sites of energy production. Catabolic processes such as glycolysis and lipid beta-oxidation are important to provide substrates so that the mitochondria can generate adenosine triphosphate (ATP) through reduction–oxidation (redox) reactions carried out by oxidative phosphorylation (OXPHOS) complexes located on the inner mitochondrial membrane [2]. They have their own ~16.5 kb genome which consists of 37 genes, 13 of which are mRNAs encoding for subunits of OXPHOS complexes together with nuclear DNA encoded ones. Their function is not limited to energy production as they have pivotal roles in Ca^{2+} homeostasis and apoptosis.

One of the principal cause of aging is postulated to be the progressive accumulation of cell damage owed to exceeded production of reactive oxygen species (ROS) from mitochondria [3, 4]. The use of transgenic animal models and in vivo gene transfer for the study of genes involved in the mitochondrial redox reactions have significantly improved the knowledge regarding detrimental effects of the ROS in the context of aging. Mitochondrial dysfunction can cause dramatic changes in the expression of many nuclear and mitochondrial genes. The nuclear genes that are dys-regulated through mitochondrial defects, the so-called 'mitochondrial retrograde signaling' pathway, have been informative for characterization of the genes whose regulation can further normalize mitochondrial function [5].

5.2 Genetic Ways of Boosting Antioxidant Capacity

5.2.1 Central Player of Oxidative Stress: Hypoxia Inducible Factor 1- Alpha

Hypoxia inducible factor-1 (HIF-1) is a heterodimer which consist of a beta constitutive domain and an alpha domain which is regulated by oxygen levels. It is a transcriptional factor whose role is vital for the adaptation to low oxygen tension. HIF-1 further regulates other genes which take part in various physiological processes such as angiogenesis, glucose and iron metabolism, cell proliferation and survival signals. It has also been related with the aging process since supply and availability of oxygen play a key role in neurodegeneration and aging [6]. The gradual decline of oxygen amount supplied to the brain with aging, known as 'hypoxia' can

produce hypometabolism in the brain. Hypometabolism is a pathogenesis factor in Alzheimer's disease (AD) patients and for this reason HIF1-α could be at the central stage in the aging process and specifically in AD pathogenesis [7]. Moreover, recent studies suggest that maintaining HIF1α levels could have neuroprotective effects, and that ATF4 may be responsible for that response [8]. Inhibiting prolyl hydroxylase domain proteins, which hydroxylate HIF1α triggering its degradation, protects against in vivo neurodegenerative effects associated with mitochondria neurotoxin [9]. Accordingly, the iron chelator M30 has neuroprotective effects by activation of HIF1-α, promoting its expression [10]. In contrast, HIF-1 is a significant regulator of neuronal mitochondrial retrograde signaling in Drosophila and when knocked-down it has been shown to improve neuronal functions and longevity in Drosophila models of Leigh syndrome and Parkinson's disease (PD) [11].

5.2.2 Catalase

Catalase is a peroxisomal enzyme which, together with mitochondrial glutathione peroxidase, is involved in antioxidant reactions upon ROS formation. Specifically, it breaks down H_2O_2, a product from beta-oxidation within peroxisomes [12]. In a pioneering work, researchers created transgenic mice in which catalase was either specifically targeted to peroxisomes (pCat), nucleus (nCat), or mitochondria (mCat). Notably, they demonstrated that mice bearing mCat showed increased lifespan and reduced susceptibility to ROS compared to the wild-type counterpart [13]. Moreover, Li and colleagues demonstrated that mCAT improves exercise performance in mice [14]. In fact, injection of mCAT by adeno-associated virus (AAV)-mediated gene transfer in neonatal mice showed that mCAT overexpression could enhance endurance during exercise without any alteration of contractile force. Researchers endorsed this gene therapy approach as a co-treatment for Duchenne muscle dystrophy where muscle fibers present a high level of ROS production that contributes to the worsening of the disease [15]. Furthermore, such a gene therapy strategy may be interesting to apply during the natural process of aging where subjects undergo a general decrease of muscle mass (sarcopenia).

5.2.3 Superoxide Dismutase 2 (SOD2)

SOD2 is the mitochondrial counterpart of the peroxisomal catalase converting superoxide species of oxygen to H_2O_2 and O_2 [16]. In a mouse model heterozygous for SOD2 (SOD2+/−), aged mice presented a significantly increased oxidative stress in their smooth muscle cells (SMC). Those mice showed mitochondrial impairment and pathological stiffness of SMC which is correlated with risk of

atherosclerotic plaque formation during aging [17]. More recently, a genetic study on healthy aged subjects was carried out analyzing polymorphic variants of SOD2 which have been associated with risk of antioxidant process impairments. Salminen and co-workers revealed a suboptimal brain aging in subjects carrying a specific variant of SOD2, demonstrating the detrimental outcome associated with polymorphism in the SOD2 gene [18].

5.2.4 Nuclear Factor Erythroid–Derived 2 (Nrf2)

Nrf2 is a transcription factor that enhances the expression of detoxifying and anti-inflammatory genes [19]. Several recent works have highlighted the key role that Nrf2 plays to reduce the oxidative burden during aging. Interestingly, it has been recently suggested that Nrf2 has a regulatory role for the proper functioning of neural stem/progenitor cells (NSPCs) during aging [20]. Over-expression of Nrf2 has been shown to alleviate the aging-related oxidative stress in aging mice liver [21], while on the other hand its down regulation severely affected cognitive function and increased neuro-inflammation in a mouse model of accelerated senescence [22]. Furthermore, aged Nrf2−/− mice presented a significant reduction in contractile force and muscle mass accompanied by an augmentation in mitochondria ROS production and cellular redox dysregulation [23]. Interestingly, changes in Nrf2 and ER-stress were found to be associated in early stages of AD [24]. Furthermore, adenoviral Nrf2 gene transfer can protect against A-beta toxicity [25]. The requirement of healthy Nrf2 mediated antioxidant signaling is also vital since genetic reduction of Nrf2 was found to exacerbates cognitive decline in AD [26]. Taken together, Nrf2 activation stands out as a promising strategy to slow down AD [27].

In the last decade researchers have also been investigated the role of ROS in the context of ophthalmology. Nrf2 was found as one of the most important components for the vulnerability in aging retinal pigment epithelium (RPE) and identified as a target gene to improve aging-related ophthalmology diseases [28]. Recently, the Cepko's group tested the eye-mediated expression of antioxidant genes such as Nrf2, SOD2, catalase and also the peroxisome proliferator–activated receptor γ coactivator 1-α (PGC1 α) which is master transcriptional regulator of genes involved in mitochondria biogenesis and respiration [29]. They found that only an AAV-mediated gene transfer of Nrf2 was able to significantly improve the prognosis in two different mouse models of severe neuro-ophthalmic diseases [30]. Moreover, a further demonstration of successful antioxidant gene therapy comes from a recent work in which age-related macular degeneration (AMD) and acute liver damage were treated by overexpressing Nrf2. In a first set of experiments, mice were injected with AAV expressing Nrf2 and after the induced-liver damages the AAV-treated mice showed no lethality associated with the acute liver failure. Next, mice affected by AMD were treated by intravitreal injection of AAV-Nrf2, and they presented a significant enhancement of protection from severe light damage [31].

5.2.5 Sirtuin 3 (Sirt3)

Sirt3 is a protein belonging to the NAD + −dependent deacetylase family and it is considered to have a crucial function in delaying the biological loss of function during aging [32]. It has been showed to be a target of PGC1α and SOD2 and play an important role in mitochondrial biogenesis and suppression of ROS [33, 34]. Recently, two different studies using transgenic animal models further demonstrated the tight link between Sirt3 and the process of aging. A mouse model lacking the p66 adapter protein revealed that low levels of acetylation and nitration of proteins could increase the lifespan due to an improved metabolic homeostasis by the regulation of Sirt3 activity [35]. Moreover, the study of myocardial senescence in a myocardial specific knockout mouse (Sirt3−/−) showed an increase of oxidative stress, mitochondrial protein dysfunction, and deficiency of Parkin-mediated mitophagy [36]. A recent report linked decreased Sirt3 expression, both at mRNA and protein levels, to AD pathogenesis and further demonstrated that Sirt3 over-expression can restore mitochondrial oxygen consumption [37].

However, the beneficial role of overexpressing Sirt3 is still controversial and it might depend on its tissue specific expression. Indeed, Sirt3 has been shown to be upregulated in some type of cancer and designed as target for the development of anti-tumor drugs [38, 39]. The pleiotropic functions of Sirt3 could be better defined in future studies harnessing new technologies such as viral vector delivery or gene editing to either overexpress or knockout Sirt3 gene in vivo. Moreover, a tight tissue specific regulation might be useful to dissect the role of Sirt3 and eventually use this as a target to counteract ROS formation in aging. Taken together, these results pave the way to gene therapy products for the treatment of age-related diseases in which an imbalance of oxidative stress is predominant.

5.3 Pharmacological Anti-oxidant Therapies Directed to Mitochondria

5.3.1 Coenzyme Q (CoQ, CoQ10, Ubiquinone)

CoQ has a key role in the mitochondrial electron transport (mETC) chain since it works as an electron transporter between oxidative phosphorylation complexes, specifically from complexes I and II to complex III, granting ATP synthesis. But its functions go further as it is also an important antioxidant factor in many tissues, protecting from oxidative damage (Fig. 5.1) [40]. Because of its role in oxidative stress regulation and ROS production by the mETC, it has been associated with age related-diseases. CoQ supplementation has been used in the treatment of some diseases including cardiovascular disease, metabolic syndrome and diabetes, kidney disease, neurodegenerative disease, inflammation and fertility [41]. An antioxidant environment is crucial for the cells, in particularly for stem cells. It has been

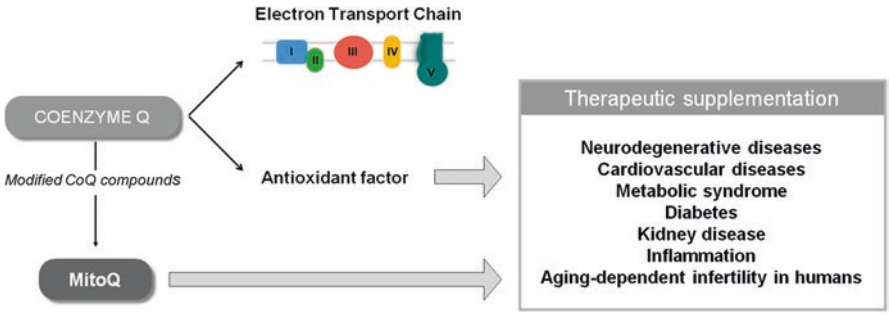

Fig. 5.1 Coenzyme Q and MitoQ as therapeutic agents. CoQ and mitochondrial targeted antioxidant MitoQ can work against oxidative stress to diminish its adverse effects. CoQ and MitoQ were shown to be effective in treatment of common diseases including aging related neurodegenerative diseases

demonstrated that CoQ can improve senescence in mesenchymal stem cells, which were aged-induced by D-galactose, and this protective mechanism acts via the Akt/mTOR signaling pathway [42]. CoQ has also been studied as a senescence therapy in an accelerated-senescence mouse model [43, 44], in old mice [45] and in aged rats where CoQ supplementation prevented oxidative stress and fibrosis in the development of cardiac remodeling [46]. CoQ is highly linked with female fertility since mitochondrial homeostasis is closely related with oocyte aging. Therefore, this can be an important complementary therapy for aging-dependent infertility in humans [47], since CoQ can restore oocyte fertility during reproductive aging by maintaining mitochondrial function [48].

5.3.2 Mitochondrial Targeted 'MitoQ'

Although CoQ has been successful as a therapy, modified CoQ compounds have been developed to improve its therapeutics effects, such as the mitochondrial targeted 'MitoQ'. There are growing number of studies which demonstrate the protective effect of MitoQ in many disorders including neurodegenerative and vascular diseases. MitoQ prevented cognitive decline, oxidative stress, synaptic loss and brain health in a mouse model of AD [49] and it also extended lifespan in a transgenic C. elegans AD model [50]. This mitochondria-targeted molecule has been shown to be protective against synaptic damage and mitochondrial toxicity in affected neurons in Huntington's disease [51]. Many other studies have demonstrated that MitoQ can mitigate aged induced mitochondrial dysfunction in the nervous system of mice [52] and rats [53]. The beneficial effects of MitoQ are not limited to nervous system as it has been shown to be effective in ameliorating age-related arterial endothelial dysfunction in mice [54] as well as aortic stiffening in old mice [55]. These promising results in model organisms have paved the way for the clinical translation of the MitoQ treatment in humans. Although more work

needs to be done, the safety and efficacy of MitoQ was recently tested on twenty healthy older adults (Clinical Trial NCT02597023). Rossman and colleagues proved that chronic supplementation with MitoQ improved vascular function in healthy older adults [56].

5.4 Modulating Mitochondrial Dynamics: PGC1α, Drp1 and Mitofusins

Mitochondria are dynamic organelles that change their structure and function in order to meet the energy demand of cells. They continuously divide and fuse together, move back-and-forth and damaged mitochondria are cleared by the autophagy machinery. The so-called 'master-regulator' of mitochondrial biogenesis, PGC-1α, is a widely studied target gene in order to ameliorate the defects caused by mitochondrial dysfunction. PGC-1α is known to be a co-activator of many transcription factors, such as the ones belonging to the PPAR family. This variety of connections make PGC-1α involved in many cellular processes. It is not only a key regulatory factor in mitochondrial biogenesis but also plays relevant roles in other processes including anti-oxidative mechanisms, metabolic and genetic control of β-oxidation, lipogenesis, gluconeogenesis and thermogenesis (Fig. 5.2) [57]. Such a wide range of functions make PGC-1α an interesting therapeutic avenue also for its involvement in the aging process [58, 59]. However, there are conflicting data about how it is involved and shapes aging. Some authors have shown beneficial effects whereas others obtained opposite results upon up-regulation of mitochondrial biogenesis via over-expression of PGC1α. One of the results shows neuroprotective effects upon PGC-1α over-expression in an AD mouse model. PGC-1α has been reported to regulate the enzyme BACE1, involved in Aβ generation and the expression of this enzyme is decreased in Alzheimer's disease patients [60]. On the contrary, prolonged over-expression of PGC-1α has also been shown to result in a selective loss of dopaminergic markers and increased dopamine catabolism induced by α-synuclein over-expression in the adult rat nigrostriatal system [61]. Interestingly, it has been reported that the down-regulation of PGC-1α can be responsible for blunted antioxidant response in Friedreich's ataxia and its reactivation, along with AMPK and PPARγ agonists, can restore a proper response induced by hydrogen peroxide treatment in patient cells [62]. Ablation of PGC-1α has also been related to telomere shortening and dysfunction and DNA damage which produces an accelerated vascular aging and results in atherosclerosis [63]. Apart from these discrepancies, a recent study showed that gene expression patterns in skeletal muscle of aged mice over-expressing PGC-1α in muscle resemble those of younger mice, supported also by a decrease in aged-related changes in neuro-muscular junctions [63]. These findings suggest that PGC-1α may be regulated in a tissue-specific manner and this may account for the observed discrepancies.

Mitochondrial dynamics has been described as an important process during aging and neurodegenerative diseases. Drp1, Mnf1/2 and OPA1 are proteins which

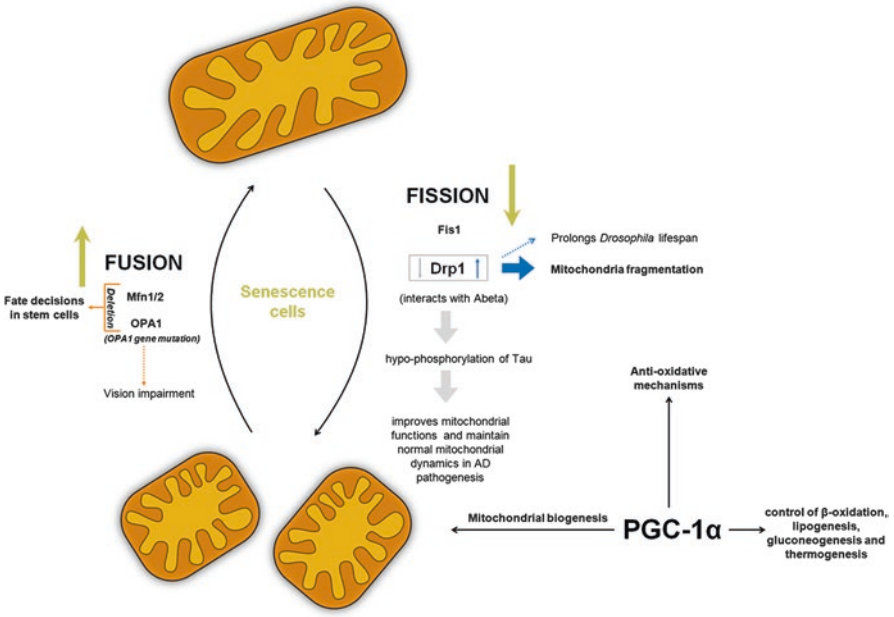

Fig. 5.2 Mitochondrial dynamics and age-related diseases. Aberrant mitochondrial dynamics has been widely observed in neurodegenerative diseases such as AD and PD. Mitochondria are dynamic organelles that constantly divide and fuse together. The balance between the fission factors (Drp1 and Fis1) and the fusion factors (Opa1 and Mfn1/2) determines the mitochondrial morphology. PGC1α is the master regulator of mitochondrial biogenesis and has roles in anti-oxidant and metabolic pathways. Their possible roles in neurodegenerative diseases and therapeutic strategies are indicated with arrows

regulate cycles of fission and fusion and are vital to maintain tissue homeostasis (Fig. 5.2). Around 60% of cases of Autosomal Dominant Optic Atrophy (ADOA), which is a common inherited optic atrophy, are due to mutations in OPA1 gene, leading to vision impairment. A study demonstrated defects in mitochondrial ATP synthesis and reduced respiration rates in patients with poor vision compared with patients with normal vision. Moreover, a compensatory mechanism was observed in the distal complexes of the respiratory chain leading to maintained vision in these patients [64]. Fission factors (Fis1 and Drp1) are down-regulated while fusion factors (Mfn1/2 and OPA1) are up-regulated in cell cultures of senescent endothelial cells which makes them elongated and stationary [65]. However, similar to the observations in PGC-1α studies, the scientific community has shown contradictory results about the therapeutic effects of fusion and fission proteins. OPA1 or Mfn1/2 deletion causes impaired mitochondrial dynamics which further affects fate decision in stem cells. Disruption of mitochondrial morphology is also a hallmark of aging and neurodegenerative diseases in adults [66]. Furthermore, aged mice normally exhibit a hyper-fragmented mitochondrial network, due to increased Fis1 and Drp1 levels, which can be normalized upon exercise training in a PGC-1α dependent manner. However, mitochondrial fusion protein content (MFN1/2 and OPA1) was unaffected by aging and exercise [67].

Mitochondrial fragmentation and ultrastructure has been described as key factors in PD and AD pathogenesis. Proteomics studies in the context of PD revealed that mitochondrial shaping proteins, mainly those related to cristae organization, were altered. Those alterations in cristae have direct consequences in OXPHOS organization within the inner mitochondrial membrane and the process of ATP production [68]. In AD neurons, Drp1 was described as a key protein as its up-regulation is related to excessive mitochondrial fragmentation, which leads to functional abnormalities and synaptic degeneration [69]. Drp1 interacts with amyloid beta (Aβ) and this interaction is involved in AD progression and pathogenesis. In this way, it has been suggested that a partial reduction of Drp1 may reduce Aβ production and consequently result in amelioration of mitochondrial dysfunction [70, 71]. Phosphorylation of Tau has also been studied in order to evaluate the protective effects of Drp1 reduction, as Tau phosphorylation is also involved in pathogenesis of AD. It has been shown that the reduction in Drp1 results in hypo-phosphorylation of Tau which improves mitochondrial function and maintains normal mitochondrial dynamics [72]. Furthermore, protective effects of mitochondria division inhibitor 1 (Mdivi1) has been recently shown as it improves mitochondrial and synaptic functionin AD [73]. Moreover it is worth mentioning that, not only Aβ accumulation, but also accumulation of mutant amyloid precursor protein (APP) can affect mitochondrial dynamics in AD [74, 75]. Interestingly, APP can accumulate in mitochondria-associated endoplasmic reticulum membranes (ER-MAM contact sites) and contribute to bioenergetic impairments [76].

Drp1 is a tightly regulated protein. Specific phosphorylation of Drp1 by GSK3β increases GTPase activity and its mitochondrial localization, thereby promoting mitochondrial fragmentation [77]. Recent studies showed that S-nitrosoglutathione reductase (GSNOR), a protein whose expression is reduced in senescent cells, is a modulator of mitochondrial function. GSNOR deficiency promotes an excessive S-nitrosylation of Drp1 and Parkin, thereby impairing mitochondrial homeostasis in mice [78]. However, an up-regulation of Drp1 in midlife prolongs Drosophila lifespan and health span, by promoting mitochondrial fission and autophagy since, in this case, mitophagy has an anti-aging effect [79]. Finally, there are evidences that impaired mitochondrial fission, mainly involving Fis1 and Drp1 proteins in aged Rhesus monkeys, present a similar phenotype as in patients with AD [80]. One has to take into account that Drp1 can be regulated differently at various times of development and can show species-specific regulation, which may explain the observations mentioned above.

5.5 Caloric Restriction (CR): A Dietary Approach

Nutrient consumption should be carefully evaluated since the main role of mitochondria is to produce energy from various sources such as carbohydrates and fatty acids. The energy expenditure is the amount of energy a person needs to maintain organismal homeostasis and carry out physical functions. Thus, this concept could be a key in the regulation of mitochondrial performance, since mitochondria can

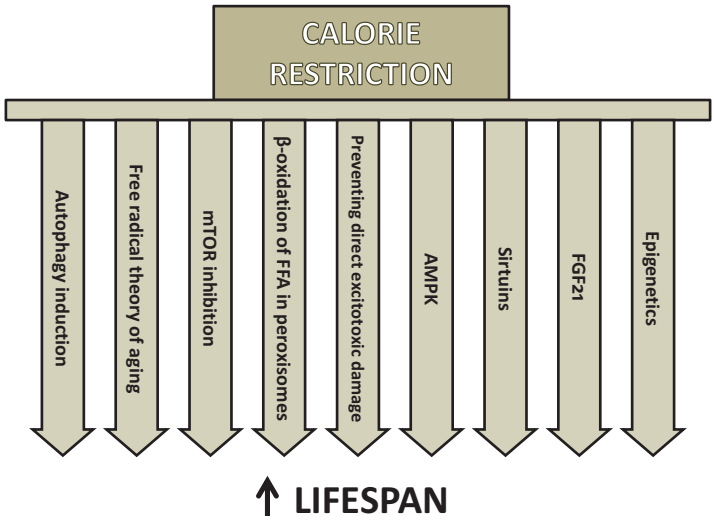

Fig. 5.3 Calorie restriction (CR) can extend lifespan through various mechanisms. Possible pathways are indicated which have been described to be involved in the mechanisms by which CR can extend lifespan in different species including flies, worms, mice and rats

modulate their activity depending on the available source of energy [81]. Benefits of CR in age related diseases have been shown in many species, ranging from worms and flies to non-human primates, and recently in humans. Although the molecular pathways regulated by CR have not been fully uncovered yet, several studies have started to discern these, some of which having primary importance (Fig. 5.3).

The beneficial effects of CR on lifespan supports the 'free radical theory of aging', first postulated by Denham Harman in 1956 [4]. CR, without malnutrition, may lead to lower O_2 consumption, therefore less ROS generation and less oxidatively-damaged proteins. This is tightly connected to mitochondria (as they are primary sites of O_2 consumption and ROS generation), since the observation of decreased H_2O_2 levels, and reduced levels of mtDNA damage in many tissues have beneficial effects [82].

CR is an inducer of autophagy, a cellular response activated upon nutrient deprivation. Autophagy inhibition impairs the beneficial effects of CR, thus preventing its antiaging capacity [83, 84]. However, the full depth of the molecular connection between these two processes remains unclear. It has been demonstrated that macroautophagy (MA) and chaperone-mediated autophagy (CMA) are up-regulated after starvation [85, 86]. Moreover, both are in constant cross-talk to compensate possible impairment of each other. CMA impairment can be compensated by up-regulation of MA, as -was shown in taupathy, which can ameliorate neurodegeneration [87, 88]. Examples of involvement of MA and CMA in neuronal homeostasis and neurodegenerative diseases includes AD and alpha-synuclein induced neurotoxicity [89, 90]. Moreover, there is evidence suggesting that MA is decreased in aging [91]. Defects in mitophagy due to mutations in PINK1 or Parkin have also

been observed in PD, suggesting a more direct effect of autophagy and mitochondrial homeostasis in neurodegenerative diseases [92, 93]. Taken together, these findings suggest that CR can be a therapeutic strategy promoting autophagy to treat aging-related diseases.

Recent studies have led to the discovery of key genes such as the ones in the mammalian target of rapamycin (mTOR) pathway and the sirtuin family as underlying factors of the pathological conditions in age-related diseases. mTOR is mainly located in cytoplasm but is also found in association with other organelles such us mitochondria [94]. Two complexes of mTOR, mTORC1 and mTORC2, are involved in many important processes, but primarily morpho- and organo-genesis. Deregulation of mTOR has been implicated in many diseases, including age-related pathologies [95]. It has been reviewed that one of the consequences of mTOR inhibition is the extension of lifespan, as demonstrated in several species such as yeast, worms [96], flies [97], mice [98] and also human fibroblasts [99]. There are other direct lines of evidence linking mTOR and CR. It has been demonstrated that the lack of mTOR does not produce an improvement of lifespan under CR without malnutrition in yeast [100]. In other words, mTOR is required for the lifespan extension upon CR. The same outcome was observed in C. elegans when mTOR was inhibited [101]. It is known that mitochondria are regulated by mTOR [102] since mTOR is able to form a complex with the transcription factor YY1 and PGC-1α [103], and this transcriptional complex is involved in the regulation of genes controlling the mitochondrial oxidative function.

Yeast are able to remodel their metabolism under CR conditions, by matching the level of ATP produced as in non-CR conditions, and this specific remodeling results in prolongation of lifespan [104]. It has been studied how a low-calorie diet alters age-related dynamics of cellular processes in yeast. These processes range from carbohydrate and lipid metabolism, to peroxisomal and mitochondrial functionalities, stress response control and cell cycle regulation. Therefore, the open question is: 'How can CR coordinate this huge variety of processes?' β-oxidation of fatty acids in peroxisomes is a longevity assurance process in yeast cultures under CR-conditions [105]. Acetyl-CoA goes to mitochondria from peroxisomes and in CR yeast most of the ATP generated is due to peroxisomalAcetyl-CoA and not the one produced in mitochondria. Thereby, peroxisomal fatty acid β-oxidation might influence chronological lifespan in yeast. It is further postulated that the mitochondrial oxidation of Acetyl-CoA from peroxisomes in CR yeast may shift the balance of mitochondrial fusion/fission towards fusion. Otherwise, mitochondrial fission leads to fragmentation of the mitochondria, consequently promoting apoptosis and resulting in age-related regulated cell death. After all, CR decrease toxic levels of ROS production, preserves mitochondrial respiration and ATP synthesis and prevents mitochondrial fragmentation by shifting a balance towards mitochondrial fusion (see previous section for a more detailed discussion on targeting mitochondrial dynamics) [95].

CR has been considered as one of the most effective antiaging therapies in mammalian model organisms. In laboratory mice, the biosynthesis and degradation of triacylglycerols (TAGs) in white adipose tissue and mitochondrial fatty acid oxida-

tion in skeletal muscle play an important role in longevity regulation [106]. Some researchers have tried to find interactions between longevity genes by using Ames dwarf mice, which live longer under CR conditions [107].

Benefits of CR have been linked to rodent models of age-related neurological disorders [108]. This has been shown by CR assays in SAMP8 mice, a model of pathological accelerated aging. Deep analysis of transcriptomic profiles confirmed that the age-related profile of SAMP8 astrocytes recovered healthy levels of mitochondrial and ribosomal mRNAs which were initially decreased in control conditions (i.e. normal diet). Moreover, a reduction of oxidative damage after CR was observed, tending to normalize pathologically-aged astrocytes [109]. More recent studies in mice have shown that CR is effective at preventing direct excitotoxic damage, a pathological process related to many age-related neurological disorders such as AD and PD. Furthermore, CR can significantly change mitochondrial function mainly by affecting the capacity of calcium buffering. This can underlie a different pathway by which CR can be beneficial, suggesting that under CR conditions, Sirt3-mediated de-acetylation and inhibition of cyclophilin D promote the inhibition of mitochondrial permeability transition. Thus, it results in enhanced mitochondrial calcium retention, or a better buffering, and avoids cell death caused by an overwhelmed buffering capacity [110]. The relationship between CR, aging and mitochondrial function, and neuronal activity has also been studied in rats, in which CR prolongs lifespan. This suggests that CR is beneficial in the aging brain by preserving metabolic homeostasis and thus maintaining normal neuronal function [111].

Another group of key molecules for a better understanding the mechanistic action of how CR is related to lifespan is the family of sirtuins. Sir2, a NAD + − dependent protein deacetylase, mediates lifespan extension in response to restriction of nutrients in yeast [112, 113]. Furthermore, Sirt1, the orthologue of Sir2 in mammals, has produced considerable interest, becoming a great candidate to explain beneficial effects of CR, since it has been reported to be involved in neuroprotection [114], reducing fat storage by repressing PPARγ activity [115, 116] and insulin secretion in β-pancreatic cells [117]. Sirt1 is the main deacetylase of PGC-1α which positively regulates genes involved in mitochondrial biogenesis and fatty acid utilization, thereby leading to increased rates of fatty acid oxidation in response to low glucose, allowing mammalian cells to switch from glucose to a fatty acid oxidation metabolism in nutrient deprivation conditions. Thus, this metabolic adaptation might be a conserved mechanism of lifespan extension through improved efficiency of energy intake [118, 119]. The aim for a potential application of CR in gerontology is caloric restriction mimetics. This strategy is focused on searching for molecules which can activate sirtuins and eventually mimic some beneficial effects of CR [120, 121]. Some of the most studied molecules as dietary activators of Sirt1 are resveratrol and melatonin, two deeply investigated ‘anti-aging’ molecules [122]. Sirt3 has also been considered as one of the major mitochondrial deacetylases [123] and it is able to deacetylate FOXO3 to protect mitochondria against oxidative damage [124]. SIRT3 plays an essential role of enhancing the mitochondrial glutathione antioxidant defense system under CR, suggesting that there are mitochondrial adap-

tations which depend on Sirt3 and they might be pivotal for slowing down aging-related processes in mammals [125]. This has been supported by other authors who have related CR to Sirt3 and showed that these molecules are able to trigger a global reprogramming of the mitochondrial protein acetylome [126].

CR is also tightly related to various other metabolic features of aging such as hepatic fat metabolism. It has been shown that lifelong CR leads to a beneficial metabolic adaptation in mice, mainly reducing lipogenesis and enhancing lipolysis and ketogenesis. The authors further described a putative starvation master regulator, fibroblastic growth factor 21 (FGF21), which is associated with the aforementioned metabolic reprogramming [127]. It has been demonstrated that FGF21-transgenic mice have an extended lifespan compared to wild-type mice [128], and the anti-aging mechanism of FGF21 is thought to be attenuation of the oxidative stress damage and decrease of advanced glycation end-products in D-galactose-induced aging in mice brain [129]. FGF21 also protects against angiotensin-induced cerebrovascular aging by improving mitochondrial biogenesis and inhibiting p53 activation in an AMPK-dependent manner [130].

Drosophila melanogaster is also a widely used model for aging research and lifespan assessment [131], and beneficial effects of CR have also been observed in flies. The maintenance of energy homeostasis by adenosine nucleotide ratios has been described as a key point in CR since dietary adenine manipulation can alter metabolism to influence animal lifespan. As it was previously observed in worms [132], the AMP:ATP ratio appears to be predictive of lifespan in flies. The activity of AMPK plays a relevant role in this process, considering that an extended lifespan was observed after increasing its activity. In line with this observation, no improvements were seen upon inhibition of AMPK activity [133]. Pathways previously described to be involved in modulation of the longevity under CR, such as insulin/IGF signaling, mTOR pathway, AMPK activity and sirtuins, take part in the response to dietary restriction in flies also, suggesting that this response is evolutionarily conserved (Fig. 5.3) [134].

Furthermore, epigenetics has recently been described as a possible mechanism involved in CR response to extend lifespan in several species. The potential epigenetic mechanisms suggested to be triggered in response of CR are DNA methylation and histone modifications such as acetylation, methylation, phosphorylation and biotinylation [134]. These modifications have been directly related to aging in some species such as C. elegans [135, 136], Drosophila [137] and mice [138]. Lian et al. [134] suggested that the deficiency of methyl-donor enzyme may have an effect on histone markers and could thereby lead to breakage and chromatin remodelling which, in turn, affects lifespan.

Following evidence of the relation between CR and extended lifespan in small model organisms, researchers explored the potential benefits of CR in larger mammals such us non-human primates and humans. Although delayed aging effects of CR was clear in some species, there were no direct evidences in rhesus monkeys until some cornerstone studies were performed with those populations. These studies concluded that CR can reduce the incidence of diabetes, cancer, cardiovascular disease and brain atrophy, and consequently slow aging in non-human primate

species [139]. More recent studies demonstrated that CR reduces age-related and all-cause mortality in rhesus monkeys on a long-term restricted diet since young adulthood [140]. A recent study in humans, which consisted of a 2-year CR trial in healthy, non-obese subjects, found evidence that long-term CR can enhance resting energy efficiency, resulting in decreased systemic oxidative damage [141]. Another group carried out a study in healthy elderly subjects demonstrating that the CR group showed beneficial effects on memory performance [142].

CR has also described as a promising complementary treatment in cancer immunotherapy. One of the reasons the immunotherapy has not been fully successful in elderly patients is that in these subjects the immune system is severely compromised as a result of the aging. Therefore, complementary therapies to enhance the effect of the immunotherapy are currently under investigation including CR which may improve the therapeutic efficacy due to its anti-aging properties. Moreover, Farazi and colleagues found out that CR may maintain immunological fitness of CD4 T cells by priming the immunological environment which is critical for efficacy of the treatment [143].

5.6 Conclusions

Currently there is no cure for commonly observed age-related diseases such as AD and PD. Pathogenic mechanisms contributing to neuronal defects in age-related diseases are complex. However, mitochondrial defects are widely observed in many neurodegenerative diseases. Therefore, novel therapies ameliorating the mitochondrial defects are promising and can be beneficial. Genetic and pharmacological targeting of the ROS caused by defective mitochondria showed normalization of various disease-related phenotypes in many species. Furthermore, dietary CR has been successful and recently reported to slow-down human aging. On the other hand, genetic manipulation of mitochondrial dynamics showed contradictory results, possibly due to cell-, tissue- and disease-specific differences in neurodegenerative diseases.

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Chapter 6
Computational Approaches for Identification of Pleiotropic Biomarker Profiles in Psychiatry

Han Cao and Emanuel Schwarz

Abstract The discovery of biomarkers is considered a critical step towards an improved clinical management of psychiatric disorders. Despite the availability of advanced computational approaches, the lack of strong individual predictors of clinically relevant outcomes, combined with the usually high dimensionality, significantly hamper the identification of such markers. Consistent with the often observed lack of diagnostic specificity of biological alterations, research suggests an underlying genetic pleiotropy between psychiatric illnesses and frequently comorbid conditions, such as type 2 diabetes or cardiovascular illnesses. As research is transitioning away from conventional diagnostic delineations towards a dimensional understanding of psychiatric illness, gaining insight into such pleiotropy and its downstream biological effects bears promise for identification of clinically useful biomarkers. In this review, we summarize the computational methods for identifying biological markers indexing pleiotropic effects and discuss recent research findings in this context.

Keywords Computational approaches · Genetics · Biomarkers · Psychiatric disorders · Metabolic disorders

6.1 Introduction

Psychiatric disorders are severe illnesses, which account for a large portion of the global disease burden (32% of years lived with disability and 13% disability-adjusted life-years) [1]. The clinical burden of these disorders is compounded by a young age of onset, a frequently chronic course and somatic comorbidities that contribute to an increased morbidity and mortality [2]. It is widely accepted that the clinical management of psychiatric disorders is hampered by the insufficient

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understanding of the biological underpinning of these illnesses. Diagnostic guidelines such as the fifth edition of the Diagnostic and Statistical Manuel of Mental Disorder (DSM5 [3]) do not contain any biological measures to aid in the diagnostic process. As a consequence, the currently accepted diagnostic criteria likely do not delineate illnesses with high biological specificity, and genetic, neuroimaging and other biological alterations frequently cross diagnostic boundaries. A reflection of this biological heterogeneity is the substantial comorbidity with somatic illnesses that significantly contribute to mortality [2]. For example, type 2 diabetes, has a two to three-fold increased prevalence in patients with schizophrenia (SZ) [4, 5] and has historically been considered a secondary consequence of antipsychotic treatment and life-style effects. Weight gain is induced by second generation antipsychotics [6], leading to frequent medication discontinuation and relapse. However, there is substantial evidence that metabolic dysfunction is a partially treatment-independent comorbidity of psychotic disorders. As examples, a metabolic profile involving elevated levels of insulin has also been found in medication-naïve patients with SZ [7, 8], first-episode patients show impaired insulin resistance [9] and relatives of SZ patients have a higher diabetes risk [10]. Risk variants have been implicated in both conditions [11], and at a systems level, mitochondrial dysfunction has been suggested as a unifying etiological theme [12, 13]. Recently, an oxidative stress-related expression signature has been identified in SZ that predicted a peripheral T2D biomarker [14], supporting the existence of a genetically influenced biological dimension that mediates comorbidity risk. Another prominent example of an important somatic comorbidity of psychiatric illness is cardiovascular disease (CVD). SZ patients show a 50% increased risk of dying from CVD compared to the general population and a CVD incidence that accounts for more premature deaths than suicide [15]. Besides an unhealthy lifestyle [16], the CVD incidence is attributed to risk factors believed to be comorbid with SZ and genetics studies suggest an underlying pleiotropy that supports shared mechanistic effects between both conditions [17]. These CVD risk factors have further been shown to be equally prevalent in bipolar disorder (BPD) [18]. Antipsychotic treatment compounds CVD risk through increased abdominal adiposity, as well as through direct, adiposity-independent effects [19]. These examples demonstrate that disentangling the biological heterogeneity of psychiatric illnesses needs to go hand-in-hand with an exploration of pleiotropy, and genetic or molecular signatures that elevate risk for multiple psychiatric conditions and/or somatic comorbidities.

Pleiotropy exists widely across human traits, with 4.6% of SNPs and 16.9% of genes showing cross-trait effects in the genome [20]. Disease-specific estimates have yielded substantially higher values. In autoimmune diseases, for example, more than 44% of SNPs were found to be shared between at least two traits [21]. Substantial efforts have been made to elucidate the pleiotropy underlying mental illness as well as that of the comorbidity with somatic conditions. Specifically, a genetic correlation exceeding 60% has been repeatedly reported between SZ and BPD [22, 23]. Similarly, a correlation exceeding 40% was observed between BPD and major depressive disorder (MDD) as well as between SZ and MDD [23]. In addition, some neurodegenerative diseases were recently found to be associated

with SZ, such as amyotrophic lateral sclerosis (genetic correlation 14.3% [24]). Moreover, pleiotropic effects were found between SZ and somatic illnessessuch as cardiovascular disease [17], lung cancer [25] and T2D [11].

As psychiatric illness research is moving away from conventional diagnostic delineations towards a dimensional understanding, these results suggest that a deeper insight into etiological processes will require an understanding of the factors underlying pleiotropy and comorbidity. This may in turn aid in clinically meaningful, biological stratification of mental illnesses and result in novel avenues to improve the clinical management of these conditions. In this review, we give a non-comprehensive overview of computational methods that can be used for the identification of pleiotropic markers and highlight interesting results from their application in psychiatry.

6.2 Genetic Overlap and Correlation

Genetic pleiotropy is typically quantified using two related measures: genetic overlap and genetic correlation. Although the term "genetic overlap" is frequently used to characterize pleiotropy in general, we use it here to specifically denote a set of specific single nucleotide polymorphisms (SNPs), which is associated with more than one trait. Genetic correlation refers to the relationship between two traits across the whole genome [22] and is quantified as the correlation between the effect sizes of all variants determined for two given traits. Early studies on pleiotropic effects were performed on family studies [26, 27] by observing multiple traits simultaneously on each given subject. However, the limited number of traits and the expensive longitudinal measurements make such studies challenging to perform. Genome-wide association studies (GWAS) enabled the efficient estimation of the association strength between a given genetic variant and a specific trait, allowing the exploration of genetic cross-trait relationships in large samples.

Here, we categorize the methods for identifying genetic pleiotropy into two broad classes depending on whether the objective of the analysis relates to sets of individual SNPs or to the entire genome. The first class of methods is frequently applied to characterize the genetic relationship between traits by joint exploration of the respectively genome-wide significant variants. One of the commonly applied methods, Mendelian randomization [28], identifies the association between the genome-wide significant variants (instrumental variables) and the respective phenotypes [29, 30]. The second class of methods is frequently applied to genetically complex conditions, such as psychiatric disorders, that are thought to be influenced by large numbers of genetic variants with each variant accounting for only a small fraction of heritability. These methods explore the genetic relationship between conditions considering all SNPs across the genome regardless of significance. In this review, we limit our focus to the latter category. Depending on the availability of individual genotype data, two classes of methods can further be differentiated among such methods to those using: (1) individual-level data or (2) summary

statistics. In the former class, the cross-phenotypic effect can be identified using case-control data by comparing the genetic similarity at the individual subject level across phenotypes, allowing the prediction of individual-level pleiotropic risk. The methods part of the latter class only requires the summary statistics of SNPs for estimation and can therefore be easily applied to large-scale studies, such as in the simultaneous estimation of the pairwise genetic correlations of 24 traits [23]. As genotype data are not required for this analysis, such methods are more computationally efficient than those of the former class, and even a real-time online web service is available (such as LD hub [31]). However, since the cross-phenotypic effect is calculated based on per-SNP effect sizes, the methods do now allow inferences at the individual subject level.

6.3 Methods Using Individual-Level Data

This section discusses two common strategies for estimating the genetic overlap and correlation using individual-level data: determination of polygenic risk scores and linear mixed models.

6.3.1 Polygenic Risk Score Methods

Polygenic risk scores are determined by aggregating small individual effects of common variants across the genome into an individual-level 'risk score'. The effect of each SNP is calculated using single-variant analysis, i.e., using logistic (binary trait) or linear (continuous trait) regression. A measure of the effect-strength is then multiplied with the observed genotype in an independent sample and the resulting estimates summed across the genome. To explore the pleiotropic effect between traits A and B, the per-SNP effect of trait A is computed and used for the risk score calculation of trait B. The subset of SNPs explaining the most phenotypic variance of trait B is then identified by varying the P-value threshold used to combine individual SNP effects into the polygenic score. Note that the observations of traits A and B have to be independent (i.e., having no shared controls) since otherwise the result is biased and spurious significance is observed.

One early study [32] explored the genetic overlap between SZ and BPD, and SZ and non-psychiatric illnesses using polygenic risk scores. This demonstrated that risk for SZ was associated with that for BPD but relatively independent of that for non-psychiatric illness. Specifically, the discovery dataset included 3322 patients with SZ and 3587 healthy controls after filtering, quality control and population stratification analysis. The effect sizes were estimated using this dataset and then tested on the datasets comprising patients with SZ (for validation), BPD and non-psychiatric illness (negative control). The risk score yielded the most significant association for SZ ($P = 2 \times 10^{-28}$, $R^2 = 0.032$), followed by

BPD($P = 1 \times 10^{-12}, R^2 = 0.014$), and no significant evidence was found for the genetic relationship between SZ and non-psychiatric illnesses. Regarding the identification of a shared set of SNPs, the thresholds yielding the best performance for prediction of BPD also differentiated patients with SZ from controls, implying a shared risk pattern among psychiatric disorders. A similar approach was used in another test exploring the predictability of cross-population PRS. The authors tested the polygenic risk scores of European patients with SZ on a cohort of African patients with SZ. This indicated that only 0.4% (P = 0.008) of phenotypic variance was explained, highlighting the accumulated impact of differential linkage disequilibrium patterns.

Due to the simplicity and intuitive nature of polygenic risk scores, such methods have been applied in numerous studies to explore the genetic association between psychiatric and non-psychiatric phenotypes, including creativity [33], cognition [34], addiction [35] and smoking behavior [36]. For creativity, the PRS of SZ and BPD were both significantly associated with creativity [33]. The maximum variance of creativity explained by the SZ polygenic risk score was 0.24%, and a similar estimate was obtained for the BPD score (0.26%). These results imply a non-negligible shared heritability between SZ and creativity. Furthermore, to explore the contribution of educational attainment (e.g., number of years in school) to the observed association, the psychiatric PRS was regressed against the combined predictors of educational attainment and creativity, yielding non-attenuated significance of the creativity association. This indicated that the shared heritability between psychosis and creativity was unlikely to be driven by the level of education. Another study [36] explored the genetic pleiotropy underlying SZ and addiction as well as smoking in an Icelandic population. Clinical evidence strongly indicates that patients with SZ show a higher rate of substance use disorders [37]. To explore a possible underlying genetic pleiotropy, a SZ polygenic risk score was tested on 11 (3 quantitative and 8 binary) phenotypes of substance use disorders. This demonstrated a significant positive association of SZ and all phenotypes of addiction as well as smoking. Stratifying subjects according to sex showed that females carried higher shared heritability ($R^2 = 0.77$ for alcohol and $R^2 = 1.35$ for cocaine use disorder) than males ($R^2 = 0.51$ for alcohol and $R^2 = 0.41$ for cocaine use disorder). Considering that smoking and addiction behaviors are more prevalent among males, the results may suggest a stronger environmental influence for males with downstream impact on prevalence.

Another interesting line of research regarding polygenic risk scores is the incorporation of additional knowledge to improve predictability [38–40]. For example, "LDpred" [38] incorporates the linkage disequilibrium (LD) information into the score. A previous study [41] demonstrated a strong implication of the LD structure in the genetic heritability of polygenic phenotypes, supporting the utility of integrating such information into the risk score. For this, LDpred takes the original effect sizes as priors and then adjusts these according to the LD effect using a Bayesian framework. Subsequently, the adjusted effect sizes are used for risk score calculation. The method was shown to improve the explained variance in a large SZ dataset

by 5%. Another study [39] integrated risk scores of multiple potential pleiotropic conditions for predicting a target phenotype. The authors utilized regularized multiple regression to explore the association of 81 candidate phenotypes and the target phenotype. A "sparse structure" was introduced (using LASSO and elastic net [42]) for automatically filtering out unrelated phenotypes. The authors applied this approach on three developmental traits (educational achievement, body mass index and general cognitive ability) and observed an improvement (1.1%~1.6%) of the explained variance. Similarly, another study [43] considered the improvement of cross-population polygenic risk score prediction using the same methodology. The authors combined the scores of multiple populations using regularized multiple regression to explain the trait in the target population and demonstrated an improvement of the explained variance compared to conventional methods.

6.3.2 Linear Mixed Model Methods

In the polygenic risk scores framework, the effects sizes of all SNPs have to be estimated from the data. Thus, the accuracy of the estimation for a given SNP depends on the number of available subjects, and a certain level of estimation error remains. During the estimation, these uncertainties accumulate and affect the risk score. To address this, a linear mixed model approach has been developed to estimate genetic heritability [44, 45] and genetic correlation [22, 46], and was integrated into the software toolkit GCTA [47]. To estimate the genetic correlation between traits A and B, the bivariate linear mixed model was used:

$$\mathrm{y}^{A} = \mathrm{x}^{A}\beta^{A} + \mathrm{Z}^{A}\mathrm{u}^{A} + e^{A}, e \sim \mathrm{N}\left(0, \mathrm{I}\sigma^{2}_{e^{A}}\right), \mathrm{u}^{A} \sim \mathrm{N}\left(0, \mathrm{I}\sigma^{2}_{u^{A}}\right)$$

$$\mathrm{y}^{B} = \mathrm{x}^{B}\beta^{B} + \mathrm{Z}^{B}\mathrm{u}^{B} + e^{B}, e \sim \mathrm{N}\left(0, \mathrm{I}\sigma^{2}_{e^{B}}\right), \mathrm{u}^{B} \sim \mathrm{N}\left(0, \mathrm{I}\sigma^{2}_{u^{B}}\right)$$

where for both phenotypes A and B, x refers to the design matrix containing the covariates, Z = n × p is the standardized genotype matrix with n individuals and p SNPs, y = n × 1 refers to the phenotype vector, β is the fixed effects for covariates, e = n × 1 is the residuals, and u = p × 1 is the vector of random effects. For any SNP m, $u^{A}{}_{m}$and $u^{B}{}_{m}$ describe the corresponding random effects, and the variance components are

$$\begin{bmatrix} \mathrm{u}^{A}{}_{m} \\ \mathrm{u}^{B}{}_{m} \end{bmatrix} = \begin{bmatrix} \sigma^{2}_{u^{A}} & \rho\sigma^{2}_{u^{A}}\sigma^{2}_{u^{B}} \\ \rho\sigma^{2}_{u^{A}}\sigma^{2}_{u^{B}} & \sigma^{2}_{u^{B}} \end{bmatrix}$$

where $\sigma^{2}_{u^{A}}$ and $\sigma^{2}_{u^{B}}$ are the genetic variance of traits A and B, while $-1 \leq \rho \leq 1$ refers to the genetic correlation. Since the E(u) = 0, this formulation bypasses the

challenge of accurately estimating small effect sizes. The genetic correlation is then estimated based on the covariance of subjects across traits. To solve the equation, the restricted maximum likelihood (REML) method is conventionally applied [46]. The results of such linear mixed model analysis need to be carefully interpreted. A positive correlation indicates the patients of trait A are more similar to the patients of the trait B than they do to their own controls, and the patients of trait A being more similar to the controls than patients of the trait B leads to a negative correlation.

Using this method, one study [22] analyzed the genetic correlation between each pair among five common psychiatric disorders: SZ, BPD, MDD, attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorders (ASD), and Crohn's disease (CD) was used as the negative control. This indicated substantial genetic correlations between SZ, BPD and MDD (0.43~0.68), and less pronounced correlations between ADHD and MDD (0.32) as well as ASD and SZ (0.19). Moreover, CD was genetically independent from all psychiatric disorders indicating the specificity of the psychiatric risk pattern. The identified genetic relationship was further consistent with a previous study using polygenic risk scores [32]. To identify the genetic overlap, the authors first partitioned the genome into distinct subsets using functional annotations and subsequently tested the genetic correlation using these. Among three possible subsets, central nervous system (CNS) explained a higher proportion of heritability than baseline, indicating that the per-SNP heritability was higher for CNS-related SNPs compared to other SNPs in the genome. Moreover, using the CNS subset, the correlation between SZ and BPD (0.37) was higher than the genome based correlation (0.30 for SZ and 0.32 for BPD), suggesting that the shared genetic variation associated with psychiatric phenotypes converged in CNS-related variants. In addition, the authors randomly separated BPD subjects into three disjoint subsets, calculated the genetic correlation between subset pairs, and subsequently compared the results to the cross-disorder genetic correlation. The similar level of within-BPD correlation (0.55~0.88) and cross-disorder correlation (0.68) further supported a substantial genetic similarity of SZ and BPD.

Due to the unbiased estimation and adaptability to small sample sizes, linear mixed models have been commonly applied to explore the genetic relationship between various conditions. One study [34] explored the genetic relationship between SZ and cognitive phenotypes, and found that SZ was negatively associated with the cognitive score "performance IQ". Another study [48] explored the specific and shared genetic architecture of obsessive-compulsive disorder (OCD) and Tourette syndrome (TS) using linear mixed models. The significant heritability (OCD: 0.58 and TS: 0.37) and genetic correlation (0.41) supported results from previous studies. To integrate the potential risk variants, the genome was partitioned according to chromosome, minor allele-frequency (MAF) and functional annotations. The MAF-based partition yielded different results between OCD and TS. SNPs of low MAF regions (<5%) explained a large proportion of variance for TS (21%), but 0% heritability for OCD. However, 31% of the variance for OCD was explained using high MAF regions (40–50%). Such differences in effects related to MAF regions may reflect the different genomic architecture of these phenotypes.

For functional annotations based partition, the disorders showed a high similarity. For example, the expression quantitative trait locis significantly associated with parietal lobe and cerebellum were tested for TS and OCD, and found to explain 29% and 35% variance, respectively.

6.4 Methods Using Summary Statistics

In this section, we describe two commonly applied methods using summary statistics instead of individual-level data: LD score regression (LDSC) and conditional false discovery rate (FDR).

6.4.1 LD Score Regression

For a polygenic phenotype, SNPs with higher LD scores (measured as the amount of genetic variation tagged by a given SNP [41]) will obtain higher X^2 scores in general than SNPs with low LD scores [41]. Therefore, the heritability of a polygenic disorder can be estimated by examining the linear dependence between the X^2 scores and the LD scores across all SNPs [38, 41]. Similarly, in the two-phenotype scenario, the pleiotropic SNPs should demonstrate stronger linear dependence between LD scores and statistics in both phenotypes than non-pleiotropic SNPs. Therefore, by examining the relationship between the correlation of statistics of phenotypes and the LD scores, the cross-phenotype effect can be quantified.

$$\mathbb{E}\left[z_{1,i}z_{2,i}\right] = \frac{\sqrt{N_1N_2}\rho_c}{M}\ell_i + \frac{\rho_s N_s}{\sqrt{N_1N_2}}$$

The cross-trait LD score regression model is shown above, where N_1 and N_2 are the sample sizes and $z_{1,i}$, $z_{2,i}$ are the z-scores of SNP*i* of two given traits. ρ_s is the phenotypic correlation among N*s* overlapping subjects. ρ_c is the genetic covariance and can be transformed into the genetic correlation (with a range between -1 and 1) using the heritability ($\rho = \frac{\rho_c}{\sqrt{h_1h_2}}$). The intercept accounts for the shared observations between traits, such that the result is not biased by the shared controls. To train the model, weighted linear regression is typically used to account for inconsistent variances of residues. Intuitively, the model can be interpreted as the quantification of the linear dependency between $\mathbb{E}\left[z_1z_2\right]$ and LD scores across all SNPs where $\mathbb{E}\left[z_1z_2\right]$ refers to the correlation of the best-estimated effect sizes for both traits. Therefore, a low genetic correlation is obtained if the risk pattern of one trait is uncorrelated to that of the other trait or the correlation is independent from the LD scores. A negative correlation should be interpreted as the genetic correlation of the

first trait and the "inverse of the second trait". For example, the negative correlation between anorexia nervosa and obesity should be interpreted as the correlation between anorexia and "not being obese". Due to the algorithmic simplicity and genetic interpretability, LDSC has been applied in large-scale analyses such as the determination of genetic correlations across common brain disorders [49] as well as common human traits [23].

A study using LDSC for estimating genetic correlations [23] explored 276 genetic correlations among 24 traits indicating several novel genetic relationships of epidemiologically-associated phenotypes. The authors first replicated a previously reported genetic correlation between psychiatric disorders based on LMM [22]. Subsequently, the genetic correlation between 24 traits (including 4 psychiatric disorders: SZ, BPD, MDD and ASD) were calculated. For example, the negative correlation of 'age at menarche' and T2D [50], 'anorexia nervosa' and obesity [51], and 'college attendance' and ASD [52] were consistent with results from previous studies. Moreover, a novel positive correlation between SZ and anorexia was identified, potentially supporting a comorbid relationship between the two conditions [53]. Notably, some clinically comorbid conditions, such as Crohn's disease and Rheumatoid arthritis [21], did not show genetic associations, which may be due to the difference between genetic correlation and pleiotropy. As an example, suppose a set of SNPs variants are genome-wide significant for both disorders but the directionality of associations are opposite. Then the correlation ($\mathbb{E}\left[z_1 z_2\right]$) is low despite the significance. This would result in a weak genetic correlation and strong pleiotropy.

Another study [49] applied the LDSC analysis on common psychiatric and neurological disorders. The authors analyzed the genetic correlation between 41 traits in total, including 10 psychiatric disorders (n = 524,021), 15 neurological disorders (n = 525,840) and 12 behaviors-cognitive traits ($n \approx 10^6$, 4 cognitive traits, 6 personality traits, 2 behavioral traits). The authors first explored the relationship of the heritability and the age of onset of each disease, which indicated that illnesses with earlier onset tended to have higher heritability than those with later onset. Analysis of pairwise genetic correlations between brain disorders suggested a shared genetic architecture between psychiatric illnesses. For neurological disorders, few significant correlations were observed, implying greater genetic specificity for each given neurological disorder. Regarding the genetic correlation across neurological and psychiatric disorders, few significant correlations were found, indicating a lack of evidence for substantial genetic pleiotropy.

6.4.2 Conditional FDR

Using summary statistics data, LDSC can efficiently quantify the genetic correlation, but not easily identify the pleotropic variants. For this purpose, conditional FDR methods have significant utility.

The conditional FDR method was developed by extending the conventional FDR towards a Bayesian framework [54–56]. FDR correction can be considered as an approximation $\mathrm{FDR}(p) \approx \frac{p}{q}$, where p is the nominal p-value and q is the empirical quantile accounted by the proportion of SNPs with p-values smaller than p. Similar to polygenic risk scores, to integrate the potential risk variants, SNPs are grouped by p-value thresholds [32, 54]. For pleiotropy-informed analysis, the SNPs are grouped according to the p-values determined for another disorder. For example, to control the FDR for SZ, the SNPs are stratified (conditioned) on the nominal p-values obtained for BPD associations. Because of the shared genetic architecture, the potential SZ risk variants are integrated by "filtering out" the SNPs not associated with BPD. This yields a pleiotropy-informed FDR control ($\mathrm{FDR}(p_1|p_2) \approx \frac{p_1}{q_{p_1|p_2}}$), where p_1 is the nominal p-value of the SZ association and $q_{p_1|p_2}$ is the empirical quantile of the SZ-associated SNPs in each strata (stratified by association with BPD). To quantify the performance of conditional FDR, the true discovery rate (TDR) is estimated as 1- FDR($p_1|p_2$).

The method was applied to datasets comprising patients with SZ (9379 cases and 7736 controls) and BPD (6990 cases and 4820 controls) with no shared controls [17]. By utilizing BPD as the auxiliary phenotype, the authors observed an increased TDR for SZ. Fifty-eight significant variants were identified for SZ. Among these, eight SNPs were consistent with a previous large-scale GWAS study [57] and cross-disorder analysis [58]. In comparison, the standard Bonferroni method failed to identify any significant SNPs and conventional FDR identified four SNPs. By setting SZ as the auxiliary phenotype, 35 significant BPD-associated SNPs were identified. Among these, four were identified in previous BPD GWAS studies [57–60]. These results highlight the improved possibility of susceptibility-variant identification when considering pleiotropic effects. Finally, to identify the pleiotropic SNPs associated with both SZ and BPD, the authors developed conjunction statistics by considering FDR($p_{SZ}|p_{BD}$) and FDR($p_{BPD}|p_{SCZ}$) simultaneously, yielding 14 significant pleiotropic SNPs.

Due to the methodological simplicity and lacking requirement of individual-level genotype data, the conditional FDR has been used to explore the shared variants of SZ and other phenotypes, including CVD [17], cancers [25] and educational attainment [61]. For SZ and CVD, one study [17] explored the genetic overlap using eight CVD phenotypes, including systolic blood pressure, triglycerides and body mass index. The authors demonstrated the improvement of detection power for SZ risk variants. For example, using triglycerides risk for stratification, the TDR of SZ-associated SNPs improved approximately 100-fold compared to the conventional procedure. Another study [25] explored pleiotropic effects between SZ and lung, breast and prostate cancer. Among these, only lung cancer showed a strong improvement of the TDR pointing to potentially shared genetics. At a p-value threshold of 0.01, the conjunction FDR identified three pleiotropic SNPs (rs7749305, rs2081361 and rs8042374) after removing the MHC region, corroborating results from the previously performed cross-phenotype analysis of lung cancer and smoking [62], as well as blood triglycerides [63].

6.5 Combined Analysis

In practice, the different methods have specific advantages and drawbacks. Therefore, it might be effective to apply multiple methods simultaneously for the analysis of pleiotropy. For example, cross-trait LDSC has apparent advantages in that it efficiently adapts to large-scale problems and is robust to spurious correlations caused by shared controls. However, cross-trait LDSC also has several limitations. First, a larger sample size is needed to achieve the same standard error compared to the individual-level data-based methods (e.g., LMM). Second, the method could be biased by an unknown population structure, and is thus challenging to apply to cross-population studies. Furthermore, since the method primarily aims to identify the genetic correlation, it has limitations for identifying specific variants simultaneously associated with multiple traits. Similarly, the conditional FDR also has several drawbacks, including the lack of capacity to deal with shared controls, potentially leading to spurious significance and an inability to accurately quantify the genetic correlation between disorders. However, joint application of multiple methods may aid in compensating for these drawbacks. For example, the simultaneous application of cross-trait LDSC and conditional FDR was shown to allow estimation of genetic correlation and overlap based on summary statistics data.

For example, one study [24] analyzed the pleotropic effect between eight psychiatric disorders and amyotrophic lateral sclerosis (AML). Using cross-trait LDSC, a significant genetic correlation was found between SZ and AML and used for downstream analysis. Combined with the individual-level genotype data, the SZ polygenic scorewas applied to AML and identified a set of common variants $p_T < 0.2$ explaining up to 0.12% variance. Subsequently, using conditional FDR, nine pleiotropic SNPs were found, among which four were novel. In this work, the combination of techniques is noteworthy, where authors used LDSC for rapid screening of potentially correlated phenotypes and then applied PRS for estimating the explained variance and conditional FDR for identification of the pleiotropic SNPs.

6.6 Shared Biological Markers in Non-genetic Modalities

In the above section, we reviewed the literature concerning the identification of pleotropic effects in the field of psychiatric genetics. Despite the encouraging findings, it is worthwhile highlighting several aspects that complicate the application of the presented methods. First, there is a risk of spurious genetic correlation due to the LD structure (e.g., two SNPs in strong LD that are harbored by biologically unrelated genes). The genetic data-driven methods may identify a strong pleotropic effect of each SNP for a given set of traits, but are unlikely to identify the true pleotropic variant without including additional information. In this case, incorporation of functional annotation may aid in identifying such pleiotropic variants. Second,

limitations of available sample sizes may limit the ability to identify pleiotropic effects due to uncertainties in the estimation of risk associations. For example, analysis of the genetic correlation [23] demonstrated a lack of genetic pleiotropy between SZ and T2D, a rather unexpected finding considering results from other clinical and molecular studies [8, 10, 11, 14]. Future studies may indeed aim to incorporate multiple data modalities for exploration of pleiotropy, which may further aid in elucidating the functional consequences of genetic effects.

From a computational perspective, knowledge mining on non-genetic data modalities is easier than that on genetics data due to of several factors, including the relatively larger effect sizes, the often lower data dimensionality and a more direct biological relationship between predictors that is, for example, captured in biological pathway databases. These aspects make general data-analysis methods effective in non-genetic modalities for exploring the pleotropic effects, i.e., machine learning [14], canonical correlation analysis (CCA) [64] and statistical regression methods [65]. Here, we discuss a selection of interesting applications of these methods.

6.6.1 Aggregate Measures of Individual Predictors

Similar to the polygenic risk score framework, mass-univariate analysis can be performed on other modalities (such as transcriptomics data) and biological similarity can be explored by comparing the transcriptomics profiles of two phenotypes. To identify such overlap, previous studies have utilized approaches incorporating ontological information or co-expression networks.

A recent study [65] combined the LMM approach (determined per gene) and weighted gene co-expressionnetwork analysis (WGCNA) [66] to explore the shared transcriptomic pattern between five common psychiatric disorders (SZ, BPD, MDD, ASD and alcohol abuse (AA)). The discovery data contained 700 patients with psychiatric disorders and approximately 300 healthy controls. LMM was applied to handle the controls shared across datasets. The spearman's rank correlation coefficient was then used to estimate the transcriptomic correlation between disorder pairs and identified significant positive correlations between the disorder pairs of the two sets: (ASD, SZ and BPD) and (SZ, BPD and MDD). To explore the potential confounding caused by antipsychotic medication, the authors compared the transcriptomic profiles of non-human primates treated with antipsychotic medications with those identified from the discovery data. The near independence suggested that the identified transcriptomic overlap was unlikely driven by antipsychotic medication effects. Subsequently, it was tested whether or not transcriptomic similarities of disorder-pairs were also reflected in genetic association data. This analysis identified a strong correlation, supporting a potential genetic effect on the transcriptomic similarities [22]. To identify the shared and distinct gene modules between disorders, WGCNA was performed. The eigengene, as representative of each module, was used in LMM to identify the model-level profile for each disorder. As a result, a dimension formed by eight biologically plausible gene models was

identified to delineate the biological variation of five psychiatric disorders. In addition, the rare variant analysis linked neuronal-related gene modules to rare mutations identified in ASD and SZ, suggesting a converging comorbidity effect between ASD and SZ based on both common and rare risk variants.

6.6.2 Machine Learning

Machine learning has been widely used for biomarker discovery [67] because of its capacity for mining high-dimensional data and the ability to perform individual-level predictions. Different from the mass-univariate analysis, machine-learning considers the interaction between predictors, increasing the model's flexibility to adapt to complex data distributions. However, such flexibility easily leads to the well-known overfitting problem [42], which is particularly noteworthy for pleiotropic analyses as it limits the cross-disorder generalizability of the model.

For example, one study [14] explored the shared transcriptomic architecture between SZ and T2D using a machine learning strategy (iterative LASSO [68] with bootstrapping). This work applied a cross-tissue prediction procedure, which learned a polygenic transcriptomic SZ profile using cortical expression datasets and subsequently applied this model on pancreatic expression data to test associations with a molecular measure of glycemic control (HbA-1c level). This strategy utilized the restricted feature selection procedure to prevent the model from overfitting. Aggregating individual genes using ontological information, two ontological categories ("kidney development" and "respiratory electron transport chain") were identified as fundamental for HbA-1c prediction. Application of WGCNA within these categories further narrowed down the list of important predictors to a small number of genes driving the transcriptomic overlap between SZ and T2D. The finding suggested a systemically shared molecular alteration between SZ and T2D, and highlighted a potential role of kidney, mitochondrial dysfunction and oxidative stress-related processes underlying this overlap.

6.6.3 Sparse CCA

CCA has been repeatedly applied to integrate brain-imaging and other data modalities, such asgenetic association data [69]. CCA attempts to find linear combinations of predictors (canonical vector pairs) in individual datasets that show maximal correlation across datasets. A typical challenge is that the number of predictors in a given datasets frequently exceeds the number of observations by far. As a consequence, CCA may easily detect variable combinations that are unrelated to illness or that are challenging to interpret biologically. To address this problem, sparse CCA has been developed, which introduces the sparsity (i.e., a LASSO penalty) into the predictor space of each dataset [70]. In sparse CCA, the canonical vectors

are created using a subset of predictors in each dataset, where the subsets are identified by maximizing the correlation across datasets. Another advantage of sparse CCA results from the orthogonality of the canonical vectors. Similar to PCA, where the first principle component is orthogonal to the second, in CCA the first canonical vectors pair is orthogonal to the second. Considering the sparsity, the predictors used for creating the first canonical vectors are therefore non-overlapping with the predictors used for further canonical vectors. This allows sparse CCA to stratify two set of vectors (such as genetic and imaging data for same subjects) depending on their cross-modality correlations.

One successful application [64] of this strategy was to stratify psychiatric symptoms according to distinct patterns of brain functional connectivity. The discovery datasets included the connectivity network (BOLD signal) of 264 brain regions and a set of clinical ratings (111 items based on K-SADS [71]) for 663 subjects. Sparse CCA was then applied to identify the association between the two data modalities. As a result, due to the imposed sparsity, the symptom-connectivity predictors were stratified into four orthogonal dimensions (each related to a canonical vector pair) and annotated as mood, psychosis, fear and externalizing behavior. In the mood dimension, for example, symptoms (e.g., depressive symptoms, suicidality, and irritability) were linked to the salience network and ventral attention network. Moreover, using resampling and network analysis, a strong correlation between the default mode and the executive network was identified as a common feature across all dimensions pointing towards a neural mechanism shared across psychiatric symptoms.

6.7 Conclusions

The analysis of biological signatures underlying comorbidity is becoming increasingly important in psychiatric research. This review presented a selection of computational methods for identifying pleiotropic and other biological signatures overlapping across conditions. Application of such methods to an ever increasing resource of available data will aid in untangling the shared and specific biological processes underlying psychiatric disorders and frequent comorbidities. We hope that these efforts will result in clinically-useful diagnostic and predictive tools and ultimately aid in refining diagnostic delineations based on insight into the pathomechanisms of psychiatric disorders.

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Chapter 7
miRNA Regulation of Glucose and Lipid Metabolism in Relation to Diabetes and Non-alcoholic Fatty Liver Disease

Tanapa Suksangrat, Phatchariya Phannasil, and Sarawut Jitrapakdee

Abstract Glucose and lipids are important nutrients because they provide most of the energy for the cells. A pre-translational regulation by microRNAs (miRNAs) plays a pivotal role in cellular metabolism by targeting the key rate-limiting enzymes of relevant pathways to fine-tune control of metabolic homeostasis. Aberrant expression of these miRNAs can result in an over or under expression of those key enzymes, contributing to the etiology of diabetes and non-alcoholic fatty liver disease (NAFLD). Here we discuss recent studies of various miRNAs that control insulin sensitivity, hepatic glucose production and de novo lipogenesis and how aberrant expression of these miRNAs contributes to the pathophysiology of diabetes and NAFLD in animal models. We also review the current application of circulating miRNAs as biomarkers for diagnosis or disease monitoring in diabetes and NAFLD.

Keywords Diabetes · Non-alcoholic fatty liver disease · Metabolism · miRNA

7.1 Introduction

Glucose is an important energy source for most living organisms. In mammals, brain and red blood cells are dependent on glucose as the primary energy source while skeletal muscle can use both glucose and fatty acids as the energy source. In contrast, liver and adipose tissue can convert excess glucose and fatty acids to triglycerides which are important form of energy storage during a prolonged starvation period [1, 2]. Alterations of glucagon and insulin levels influence metabolism

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of these two nutrients to maintain energy homeostasis. In mammals, during fasting periods, glucagon is secreted into the plasma to maintain glucose concentrations within a narrow range, protecting the brain from hypoglycemia-induced organ damage. Glucagon achieves this by stimulating hepatic glycogen breakdown and hepatic gluconeogenesis [3]. In adipose tissue, glucagon stimulates lipolysis, generating free fatty acids and glycerol. Free fatty acids are transported to liver and skeletal muscle where β-oxidation occurs. However, during feeding periods, elevated plasma glucose stimulates insulin release, resulting in a metabolic shift from catabolic to anabolic metabolism [4]. Under this physiological condition, insulin acts on skeletal muscle and liver by promoting glycogen synthesis and stimulating de novo lipogenesis both in adipose tissue and liver, preventing overproduction of glucose during a postprandial period [5, 6].

7.2 Regulation of Glucose and Lipid Metabolism by Insulin and Deregulation

Failure of insulin action, known as insulin resistance, can perturb metabolic homeostasis in insulin responsive tissues. Insulin resistance in skeletal muscle and adipose tissue results in failure of glucose uptake, causing the retention of glucose in plasma. In contrast, hepatic insulin resistance results in the over-stimulation of hepatic glucose production, aggravating plasma glucose level [7]. In response to insulin resistance, pancreatic β-cells secrete more insulin to compensate for insulin resistance, causing elevated levels of plasma insulin known as hyperinsulinemia. Hyperinsulinemia can result in overstimulation of de novo lipogenesis and excessive fat deposition in liver, causing hepatic steatosis or non-alcoholic fatty liver disease (NAFLD) [8, 9]. Furthermore, excessive de novo lipogenesis in liver increases triglyceride secretion as the very low-density lipoprotein (VLDL) to the plasma (Fig. 7.1). Hyperlipidemia can further induce systemic insulin resistance, resulting in elevated plasma glucose due to the failure of glucose uptake by peripheral tissue and increased hepatic glucose production. The elevation of both glucose and lipid causes glucotoxicity and lipotoxicity which trigger β-cell apoptosis and lower insulin secretion [10] (Fig. 7.1).

The insulin signaling pathway plays a key role in communication with post-translational modification of the key enzymes or proteins that regulate glucose and lipid metabolism. Binding of insulin to its receptor on the target cells triggers autophosphorylation, resulting in phosphorylation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2). The phosphorylated insulin receptor substrates then bind and activate phosphoinositol-3-kinase which further converts phosphatidylinositol 4,5-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate [11]. Phosphatidylinositol 3,4,5-trisphosphate then activates Akt/PKB, which in turn phosphorylates AS160 protein, an essential cytoskeleton protein that stimulates GLUT4 translocation to the plasma membrane into skeletal muscle and adipose

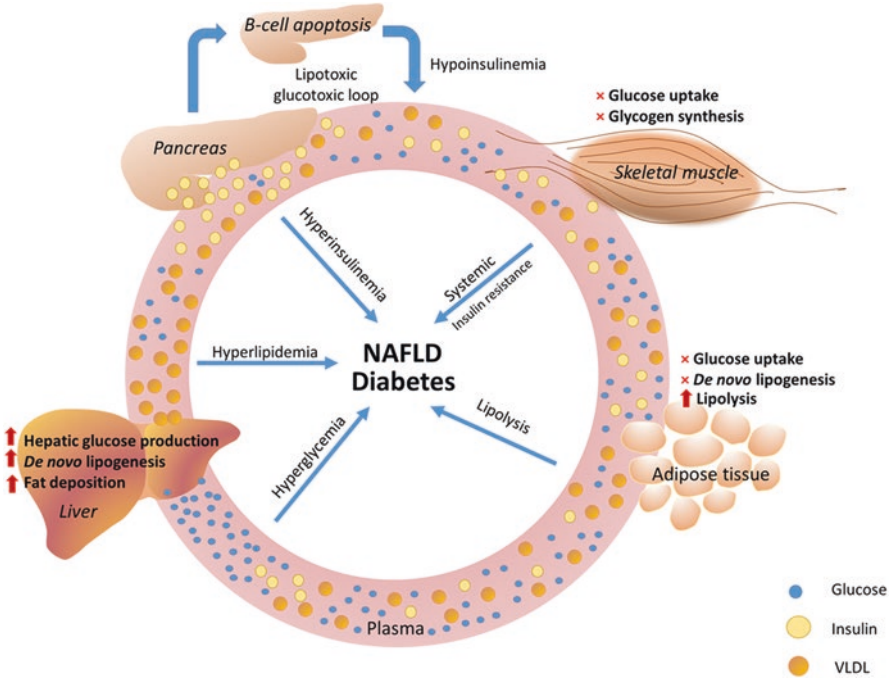

Fig. 7.1 Metabolic hallmarks of type 2 diabetes and NAFLD. Insulin resistance results in hyperinsulinemia which stimulates de novo lipogenesis and ectopic fat deposition in liver, causing NAFLD. Excessive de novo lipogenesis overspills triglyceride as VLDL in the circulation and causes systemic insulin resistance. Failure of glucose uptake in peripheral tissue and excessive hepatic glucose production result in an overt hyperglycemia. Adipose tissue insulin resistance also increased lipolysis, resulting in elevated level of non-esterified fatty acids. This situation combined with elevated levels of VLDL and glucose creates glucotoxicity and lipotoxicity which progressively deteriorate pancreatic beta cells, aggravating T2DM and NAFLD. (X = inhibit. ↑ = increase)

tissue [12, 13]. Akt/PKB phosphorylation also stimulates glycogen synthase through phosphorylation of GSK3, increasing glycogen synthesis in skeletal muscle and liver. In addition, Akt/PKB signaling activates expression of key de novo lipogenesis enzymes including acetyl-CoA carboxylase 1 (ACC1), and fatty acid synthase (FASN), resulting in increased triglyceride synthesis in liver and adipose tissue. In liver, Akt/PKB signaling inactivates FoxO1 transcription factor of the gluconeogenic enzyme genes by phosphorylation, inhibiting gluconeogenesis during postprandial periods [14]. Insulin resistance therefore prevents FoxO1 inactivation, resulting in a constitutive expression of gluconeogenic enzymes and contributes to excessive hepatic glucose production. Although deregulation of the above metabolic enzymes occurs at the transcriptional or post-translational levels, growing evidence indicates that deregulation can occur epigenetically through the microRNAs (miRNAs) [15–17].

7.3 miRNAs Mediating Abnormal Glucose and Lipid Metabolism

The miRNAs are small non-coding RNA, comprising of 20–30 nucleotides in length. They regulate expression of genes by hybridizing to their complementary sequences, located mostly at the 3′-untranslated regions (UTR) of the target mRNAs [18]. The area of base complementary between miRNA and target sequence known as "seed match" can be nearly 100% or partially complementary. This less stringent binding allows miRNAs to bind to any mRNA containing a 3'-UTR sequence partially similar to the seed match region thereby generating a variety of mRNA targets for one species of miRNA [19, 20]. Furthermore, one particular mRNA may be regulated by multiple miRNAs, adding a more complex regulatory network [19, 21]. Binding of a miRNA to its target mRNA can affect mRNA translation in two ways. If the complementary base pairing between the seed match and target sequence is perfect, this binding will result in complete degradation of mRNA target. However, if the complementarity between the seed match and target is not perfect, this would partially inhibit protein synthesis [19, 20]. Recent studies demonstrate that miRNAs can act locally within the cell or they can be transported in extracellular vesicles or exosomes that can be taken up by adjacent or remote cells or tissues [22]. Currently there are more than 2500 miRNA genes that have been identified in the human genome [23]. All of these appear to control a variety of biological and biochemical processes such as growth and development, stress response, cell death and apoptosis, and metabolism [24].

In this review, only the miRNAs that regulate key metabolic enzymes related to diabetes and NAFLD will be emphasized.

7.3.1 miRNA Regulating Insulin Sensitivity and Glucose Transport

miR-103 and miR-107 were the first two miRNAs shown to regulate insulin sensitivity in liver and adipose tissue in mice [25]. These two miRNAs were found to be elevated in leptin deficient (ob/ob) mice and in high fat diet-induced insulin resistant mice. Over-expression of miR-103 and miR-107 in the above mouse models down-regulate the expression of caveolin-1, a component of caveolae lipid raft required for insulin receptor signaling [26]. The decreased expression of caveolin-1 impairs the downstream insulin signaling cascade, disrupting glucose metabolism such as by the reduction of GLUT4 translocation in adipose tissue and increased hepatic gluconeogenesis. Inhibition of miR-103 or miR-107 expression ameliorates hyperglycemia and insulin sensitivity, demonstrating their potential roles as the therapeutic miRNAs [25]. Zhou et al. [27] reported miR-181a as a regulator of hepatic gluconeogenesis, acting through sirtuin-1, a positive regulator of hepatic insulin signaling. Mechanistically, insulin resistance induces expression of

miR-181a which down-regulates expression of sirtuin-1. The decreased sirtuin-1 level in turn causes a hepatic insulin resistance, resulting in the over-expression of two gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), in parallel with increased hepatic glucose production. Inhibition of miR-181a over-expression in hepatocytes of high fat-diet induced diabetic mice ameliorates hyperglycemia. In addition to the first three miRNAs, miR-802 was also reported to regulate hepatic insulin sensitivity because its expression was found to be elevated in mice bearing leptin receptor deficiency (db/db) and in high fat diet (HFD)-induced diabetic mice [28]. Mechanistically, elevated miR-802 decreases expression of HNF1β while increasing expression of the insulin suppressors, SOCS1 and SOCS3. Increased expression of both SOCS1 and SOCS2 in turn desensitizes insulin signaling, resulting in increased hepatic glucose production in these mouse models. miR-291b-3p has also been reported to regulate hepatic gluconeogenesis through the insulin signaling pathway. Over-expression of miR-291b-3p in normal mice impairs activation of Akt/PKB signalling, leading to a constitutive activation of FoxO1 transcription factor. This results in increased expression of PEPCK and G6Pase, contributing to hyperglycemia. In contrast, inhibition of miR-291b-3p expression was found to improve insulin sensitivity and ameliorate hyperglycemia [29].

7.3.2 miRNA Regulating Glucose Uptake and Glycolysis

The solute carrier family 2 facilitated glucose transporter member 4 (GLUT4) is the glucose transporter that is exclusively expressed in skeletal and cardiac muscles and adipose tissue. GLUT4 is an insulin-responsive glucose transporter which controls the overall rate of glucose uptake. Insulin signaling stimulates translocation of GLUT4 to the plasma membrane where it facilitates glucose uptake into the cells. Decreased GLUT4 expression or translocation causes elevated plasma glucose in patients with type 2 diabetes (T2DM) [30–32]. In a T2DM mouse model, re-expression of GLUT4 in skeletal muscle ameliorates insulin resistance and hyperglycemia [33]. Several miRNAs have been implicated in the direct regulation of GLUT4 expression via binding to the 3'-UTR of GLUT4 mRNA or indirectly via regulation of GLUT4. In this review we only emphasize the mRNAs that directly regulate GLUT4 expression.

miR-93 was first reported to directly regulate GLUT4 expression in adipocytes, through binding to the 3'-UTR of GLUT4 mRNA. Over-expression or inhibition of miR-93 has been shown to decrease or increase expression of GLUT4, respectively. Furthermore, miR-93 expression was elevated in women with polycystic ovary syndrome and insulin resistance [34]. miR-106b was reported to negatively regulate GLUT4 expression in L6 rat myoblasts through direct binding at the 3'-UTR of GLUT4 mRNA. The inhibitory effect of miR-93 on GLUT4 mRNA expression was accompanied by decreased glucose uptake and consumption while this inhibitory effect was lost in insulin resistant L6 myoblasts [35]. miR-29a-3p and miR-29c-3p

have been shown to regulate expression of GLUT4 in skeletal muscle. Silencing miR-29a expression increases insulin-induced glucose uptake in C1C12 mouse myocytes [36]. Over-expression of either miR-29a-3p or miR-29c-3pin primary human skeletal muscle lowers glucose uptake under both basal and insulin-stimulated conditions, and is accompanied by decreased glucose oxidation and muscle glycogen synthesis [37]. miR-29 indirectly regulates GLUT4 expression by inhibiting expression of the secreted protein acidic rich in cysteine (SPRAC) protein, a regulator of GLUT4 expression, which is often dysregulated in T2DM patients [38]. The expression levels of both miR-29a-3p and miR-29c-3p were also found to be elevated in plasma of the ob/ob mice and in humans with T2DM, reinforcing their involvement in the pathophysiology of T2DM [37]. Using miRNA-qualitative trait analysis (miQTL), Gottman et al. [39] showed that the expression of miR-31 was elevated in association with low GLUT4 expression in white adipose tissue of T2DM patients. In support of this association, ectopic expression of miR-31 in human primary preadipocyte culture lowered expression of GLUT4 in parallel with impaired adipocyte differentiation. Although the negative effect of miR-31 on GLUT4 expression was apparent in human white adipose tissue, the expression levels of this miRNA in sera of healthy and T2DM patients were found to be indistinguishable [39].

7.3.3 miRNA Regulating Hepatic Glucose Production

During a fasting period, glucagon signals the activation of several transcription factors and co-activators such as such as FoxO1, PGC-1 α and cAMP-responsive element binding protein (CREB), that drive transcription of gluconeogenic enzymes [40, 41]. Insulin signaling activates Akt/PKB which subsequently inactivates activity of the above transcription factors/coactivators, thereby inhibiting hepatic glucose production. Hepatic insulin resistance blunts the Akt/PKB signaling cascade, resulting in constitutive activation of gluconeogenesis and glycogenolysis. There appears to be the aberrant expression of miRNAs which link Akt/PKB signaling and hepatic glucose production.

miR-23a was first reported as a regulator of gluconeogenesis. Expression of miR-23a was found to be elevated in hepatocytes of hepatocellular carcinoma mice where gluconeogenesis is attenuated [42]. miR-23a inhibits gluconeogenesis through direct binding at the 3'-UTRs of both G6Pase and PGC-1α mRNAs. The latter is the transcriptional co-activator of gluconeogenic genes. Ectopic expression of miR-23a in normal primary mouse hepatocytes decreases expression of G6Pase, concomitant with reduced hepatic glucose production [42]. Liang and co-workers [43] reported miR-29a-c as a regulator of gluconeogenesis by targeting both G6Pase and PGC-1α mRNAs through the direct binding to their 3'-UTRs. Over-expression of miR-29a-c was found to reduce expression of these two genes, accompanied by attenuation of hepatic glucose production and alleviation of hyperglycemia in primary hepatocytes of both normal and db/db mice. miR-33 was reported to control

hepatic glucose production by direct targeting of the expression of PEPCK and G6Pase in human primary hepatocytes [44]. Insulin induces the expression of miR-33 in hepatocytes where it directly inhibits expression of PEPCK and G6Pase via binding to the 3'-UTRs. In addition to a direct regulation of these two gluconeogenic enzymes, miR-33 inhibits expression of CREB, and the retinoid-related orphan receptor-α (RORα) transcription factors as well as the SRC-1 transcriptional coactivator, all of which control transcription of gluconeogenic genes. In addition to gluconeogenesis, miR-33 also regulates glycogen breakdown by inhibiting expression of glycogen phosphorylase and phosphoglucomutase. Overexpression of miR-33 in human hepatocytes resulted in the inhibition of PEPCK, G6Pase, glycogen phosphorylase and phosphoglucomutase, accompanied by reduced glycogen content and hepatic glucose production [44].

miR-26a was reported as a regulator of insulin sensitivity, gluconeogenesis and lipid metabolism in mice [45]. Although miR-26a regulates gluconeogenesis by down-regulating PEPCK, fructose-1,6-bisphosphatase 1 (FBP1) and G6Pase mRNAs, only the PEPCK mRNA contains a miR-26a binding site in its 3'-UTR. Expression of miR-26a was down-regulated in livers of ob/ob mice and high fat diet (HFD)-induced insulin resistant mice. In humans, expression of miR-26a is inversely proportional to the body mass indices of overweight and obese subjects with T2DM. Over-expression of miR-26a in HFD-induced insulin resistant mice and db/db mice was found to improve insulin sensitivity while it reduced lipogenesis and hepatic glucose production [45]. Although miR-26a regulates the overall program of gluconeogenesis, only PEPCK contains a miR-26a binding site in its 3'-UTR.

In addition to miR-26a, miR-214 has been reported to control gluconeogenesis through inhibition of the ATF4 transcription factor [46]. Mechanistically, cAMP/PKA signaling or fasting down-regulates expression of miR-214, resulting in expression of ATF4 which further interacts with FoxO1 and stimulates expression of both PEPCK and G6Pase genes. Over-expression of miR-214 in primary hepatocytes of HFD-induced insulin resistant mice inhibited ATF4 expression, resulting in down-regulation of PEPCK and G6Pase, and amelioration of hyperglycemia [46].

Zhuo et al. [47] identified miR-451 as a negative regulator of hepatic glucose production in mice through inhibition of gluconeogenesis. Mechanistically, a low level of miR-45 expression in diabetic mice blocks Akt/PKB signaling and FoxO1 phosphorylation, resulting in over-expression of PEPCK and G6Pase. miR-451 also inhibits expression of glycerol kinase, an enzyme that feeds glycerol into the upper part of the gluconeogenic pathway. Ectopic expression of miR-451 in hepatocytes of diabetic mice was found to decrease expression of gluconeogenic enzymes and hepatic glucose production, ameliorating hyperglycemia. Langlet and colleagues [48] identified miR-205-5p as a regulator of Akt/PKB signaling and FoxO1. Over-expression of miR-205-5p in normal mice decreases hepatic glucose production and increases glucose tolerance while inhibition of miR-205-5p expression in high fat diet-induced insulin resistant mice improves glucose intolerance and hyperglycemia. Collectively these data indicate the protective role of miR-205-5p against hyperglycemia. The miRNAs which regulate hepatic glucose production either through gluconeogenesis or glycogenolysis are summarized in Fig. 7.2.

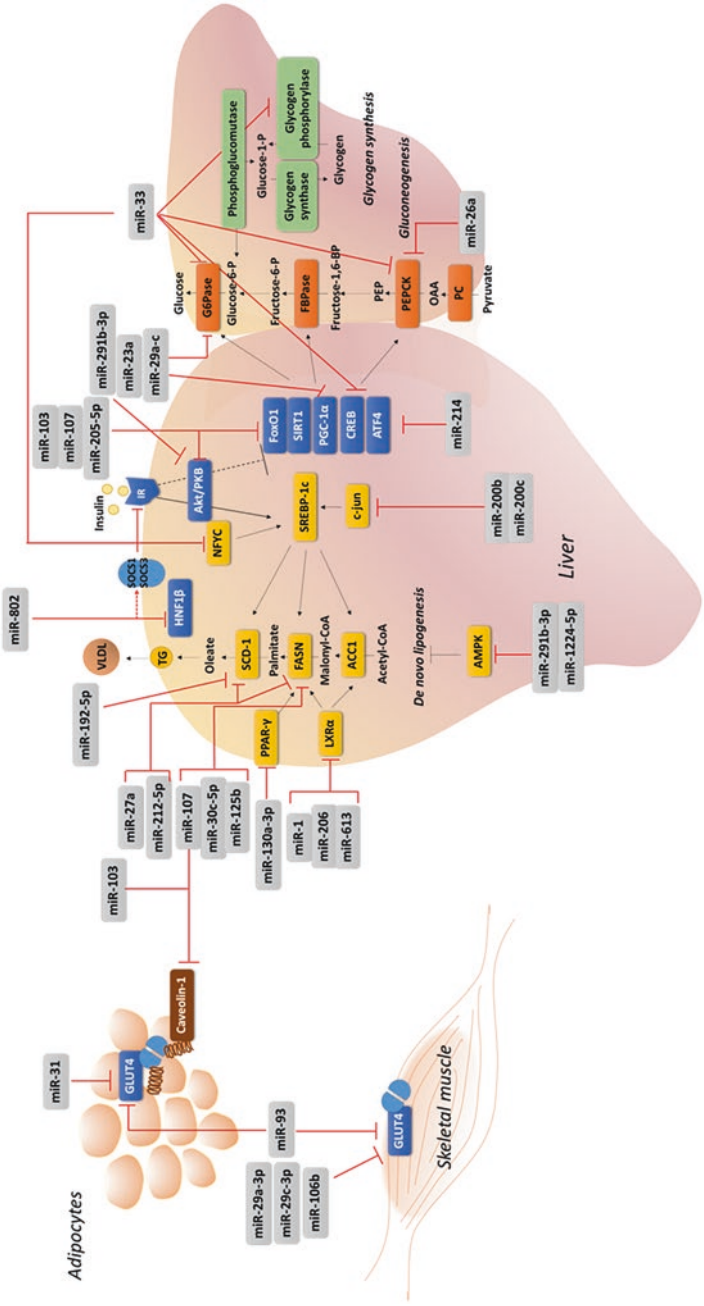

Fig. 7.2 miRNAs regulating insulin sensitivity, glucose transport, hepatic glucose production and de novo lipogenesis in mouse model. The metabolic pathways of de novo lipogenesis, gluconeogenesis and glycogen synthesis with key metabolites and enzymes (bold letters), and the pathways in liver are shown while the regulators of metabolic enzymes are boxed in blue. The miRNAs are boxed in grey and their targets are shown by a red line with a vertical bar

7.3.4 miRNA Regulating De Novo Lipogenesis

De novo lipogenesis is the synthesis of lipids from glucose and occurs in liver and adipose tissue. This process starts from the conversion of citrate-derived acetyl-CoA to long chain fatty acids, catalyzed by a series of lipogenic enzymes. Acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN) are the first two rate-limiting enzymes which regulate the overall de novo lipogenic program. ACC1 catalyzes the carboxylation of acetyl-CoA molecules to form malonyl-CoA which is then used as the carbon donor during each cycle of chain extension by FASN. Once completed, the long chain acyl-CoA is esterified with glycerol to form triglyceride which can be used as a storage nutrient or exported as the VLDL [49]. Over-stimulation of de novo lipogenesis can contribute to the pathophysiology of hepatic steatosis or NAFLD. Ectopic fat deposition in liver also causes hepatic insulin resistance and T2DM [49] (Fig. 7.1). Growing evidence indicate that deregulation of several miRNAs is associated with an aberrant expression of ACC1 and FASN in liver and adipose tissue in rodents and humans, contributing to the development of NAFLD. Some miRNAs directly regulate expression of FASN or ACC1 while some miRNAs regulate these enzymes through their transcription factors, liver X receptor (LXR), farsenoid X receptor (FXR), sterol regulatory element binding protein 1c (SREBP1c) or peroxisome proliferator-activated receptor-γ (PPAR-γ).

Bhatia and co-workers [50] first reported miR-107 as a direct regulator of FASN expression via direct interacting with 3'-UTR of FASN mRNA. Over-expression of miR-107 inhibits expression of FASN accompanied by decreased palmitate synthesis. Fang et al. [51] showed that miR-30c-5p directly binds to the 3'-UTR of FASN mRNA and represses its expression. In db/db mice, the expression of miR-30c-5p was down-regulated, resulting in an elevated level of FASN mRNA. Ectopic expression of this miRNA in these mice lowers expression of FASN, ameliorating excessive fat synthesis and deposition in their livers. Guo et al. [52] identified miR-212-5p as a negative regulator of de novo lipogenesis via binding to the 3'-UTRs of FASN mRNA. Ectopic expression of miR-212-5p in mouse hepatocytes down-regulates expression of FASN in parallel with decreased triglyceride synthesis and accumulation. Remarkably, over-expression of this miRNA in livers of mice can protect high fat diet-induced hepatic steatosis. Zhang et al. [53] found that miR-27a was a direct regulator of FASN expression. In normal mouse liver, ectopic expression of miR-27a reduced expression of FASN accompanied by reduced lipid accumulation. The expression of miR-27a was lowered in livers of ob/ob mice bearing NAFLD and administration of mimic miR-27a to their hepatocytes lowered expression of FASN concomitant with decreased triglyceride accumulation, ameliorating hepatic steatosis. Interestingly, alteration of miR-27a levels in liver was correlated with hepatic and plasma triglyceride, suggesting that miR-27a is a potential biomarker for detecting hyperlipidemia [54].

With regard to indirect regulation of FASN, some miRNAs regulate FASN expression by inhibiting expression of FASN transcription factors or other regulators. Zhong et al. [55, 56] reported that miR-1, miR-206 and miR-613 negatively regulated expression of both FASN and ACC1 in HepG2 cells by inhibiting expression of LXRα, concomitant with reduced fat accumulation. In addition to the above three miRNAs, miR-130a-3p can also down-regulate expression of FASN by inhibiting expression of PPAR-γ [57]. miR-130a-3p expression was found to be decreased in db/db mice with hepatic steatosis. Over-expression of miR-130a-3p in this mouse model inhibited FASN expression, concomitant with reduced hepatic steatosis and serum triglycerides, suggesting its role as an anti-hepatic steatotic miRNA. Goedeke and co-workers [58] identified miR-33 as an inhibitor of FASN expression by blocking expression of the nuclear transcription Y subunit gamma (NFYC), a transcription factor of SREBP1-responsive genes such as FASN and ACC1. Inhibition of miR-33 expression in primary mouse hepatocytes increases expression of FASN and ACC1, concomitant with increased hepatic lipid accumulation and circulating triglycerides. Although its direct targets are unknown, miR-185 negatively regulated expression of FASN, HMG-CoA reductase (one of rate-limiting enzymes in cholesterol biosynthetic pathway), sterol regulatory element binding proteins, SREBP2 and SREBP1c, ameliorating insulin sensitivity and hepatic steatosis [59]. miR-291b-3p is also implicated in the pathophysiology of NAFLD as its expression level was elevated in livers of high fat diet-induced hepatic steatotic mice [60]. Mechanistically, miR-291b-3p down-regulates FASN expression by inhibiting expression of the AMP-activated protein kinase (AMPK), a negative regulator of de novo lipogenesis, increasing lipogenesis and circulating triglycerides. Similar to miR-291b-3p, miR-1224-5p also inhibits expression of AMPK expression in mice. The expression of miR-1224-5p was elevated in livers of high fat diet-fed mice with hepatic steatosis and the inhibition of miR-1224-5p expression led to increased expression of AMPK and decreased expression of FASN [61].

Other miRNAs which indirectly regulate FASN expression include miR-200b and miR-200c which target c-jun, a transcription factor for SREBP1c expression. The decreased expression of c-jun in turn down-regulates expression of SREBP1c, lowering FASN expression. The expression of these two miRNAs was decreased in livers of high fat diet-induced steatotic mice while over-expression of these two miRNAs reduced FASN expression and lipid accumulation in their livers, indicating their roles in protecting against hepatic steatosis [62]. miR-125b also plays an important role in protecting high fat diet-induced hepatic steatosis in mice by inhibition of FASN expression and reduction of circulating triglycerides [63]. Interestingly miR-125b expression was induced by estrogen while this protective effect was lost in ERα-knockout or ovariectomized mice.

In addition to ACC1 and FASN, over-expression of stearoyl-CoA desaturase-1 (SCD-1), an enzyme that catalyzes the formation of monounsaturated fatty acids such as oleate and palmitoleate, hasalso been implicated in NAFLD. Deficiency of SCD-1 can protect mice from high-fat diet induced hepatic steatosis [64]. Liu et al.

[65] identified miR-192-5p as a direct regulator of SCD-1 through binding to the 3'-UTR of SCD-1 mRNA. The expression of miR-192-5p was decreased, concomitant with increased expression of SCD-1 in livers of high fat diet-induced steatotic rats. Using human Huh7 hepatocytes as a model, exposure of this cell line to a high concentration of palmitate was found to down-regulate expression of miR-192-5p, concomitant with increased SCD-1 expression and triglyceride accumulation. Moreover, over-expression of miR-192-5p decreased SCD-1 expression and alleviates triglyceride synthesis. In addition to regulating FASN expression, miR-27a and miR-212-5p were also found to regulate expression of SCD-1 via direct interaction with the 3'-UTR of SCD-1 mRNA [52, 53]. Over-expression of either of these miRNAs in ob/ob mice bearing hepatic steatosis down-regulates expression of SCD-1 concomitant with reduced lipid accumulation, alleviating liver steatosis. The miRNAs which regulate expression of FASN, ACC1 and SCD1 either directly or indirectly are summarized in Fig. 7.2.

7.4 Circulating miRNAs as Biomarkers for Diabetes and Non-alcoholic Fatty Liver Disease (NAFLD)

Circulating miRNAs are either released in the extracellular vesicles, exosomes, or bound to high density lipoprotein or argonaute protein [66–68]. The expression levels of circulating miRNAs under different pathophysiological conditions are varied, reflecting abnormal metabolism. Detecting circulating miRNA in the biological fluids also provides a non-invasive means to diagnose or monitor disease progression [17, 68]. Although the evidence above clearly indicates that aberrant expression of several miRNAs is associated with diabetes and NAFLD, not all miRNAs are released from the cells as circulating miRNAs [68]. Even if they are, their abundance or stability may be too low to be detected by the current technical means which limits their use as biomarkers [67]. Over several years, numerous efforts have been made to identify the circulating miRNAs as biomarkers for diabetes and NAFLD. As noted below, some of the above miRNAs implicated in hepatic insulin sensitivity, glucose and lipid metabolism in mouse models may not be applicable in humans.

7.4.1 miRNA Markers for Diabetes in Humans

Among various miRNAs that have been implicated in diabetes in animal models, only a few of the above miRNAs are associated with diabetes in humans. With regard to insulin sensitivity, miR-103 and miR-107, which regulate hepatic insulin resistance in mouse models, were also found to be elevated in sera of patients with

T2DM [69–71]. Of particular interest, the levels of miR-107 together with miR-33, miR-150 and miR-122 were increased in sera of patients with T2DM, suggesting that these miRNAs may act in concert, contributing to insulin resistance in humans [71]. miR-143 is another miRNA that was previously been shown to regulate insulin sensitivity in mice by targeting the oxysterol-binding-protein-related protein 8 (ORP8), a component required to initiate Akt/PKB phosphorylation [72]. Although the expression level miR-143 was high in three independent insulin resistant rodent models (i.e., high fat diet-induced insulin resistant mice, ob/ob mice or T2DM rats), its abundance in sera of normal and patients with prediabetes or T2DM was not found to be different [72–75]. This suggests that miR-143 may not be a good biomarker. The miR-23a which down-regulates gluconeogenesis by inhibiting expression of G6Pase and PGC1α in primary mouse hepatocytes was also found to be decreased in sera of patients with prediabetes and T2DM, suggesting its potential role as an early biomarker for T2DM [76].

Among other circulating miRNAs, miR-126 has been extensively studied in various cohorts of both T1DM and T2DM. Mechanistically, miR-126 promotes growth of endothelial and vascular tissues by inhibiting the expression of sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-beta), both negative regulators of the vascular endothelial growth factor (VEGF) signaling pathway. In diabetic patients, loss of miR-126 expression in their plasma is attributed to the impaired vascular tissues [77–79]. Furthermore, the decrease of circulating miR-126 in serum was also associated with T1DM and gestation diabetes [80]. In addition to being a serum biomarker, miR-126 was also released in the urine which can be used as a biomarker for an early onset of T1DM in pediatric patients [81].

Glucotoxic- and lipotoxic-induced β-cell death can progressively exacerbate diabetes. Some miRNAs have been associated with the maintenance of islet β-cell mass. For example, miR-375 was recently ascribed as a biomarker for pancreatic β-cell destruction [82, 83]. Due to its high abundance, acute or profound destruction of pancreatic β-cells results in the elevated levels in sera of patients with T1DM and T2DM [84–87]. Likewise, miR-197-3p and let-7 g have also been described as biomarkers for monitoring deterioration of pancreatic β-cells in children with T1DM. The expression of these two miRNAs was found to be positively correlated with C-peptide during the progression of diabetes [88, 89].

Instead of using only one or two miRNAs as potential biomarkers, miRNA profiling has been used as an alternative approach to identify groups of miRNA that may be responsible for the onset or progression of the disease. Erener et al. [90] performed an extensive study of miRNA profiles using a large cohort of children with T1DM and identified 6 miRNAs (miR-454-3p, miR-222-3p, miR-144-5p, miR-345-5p, miR-24-3p and miR-140-5p) which were elevated in serum during the early onset of T1DM. The authors also identified two miRNAs (let-7c-5p and let-7a-5p) which were positively correlated with HbA1c in this cohort. Likewise, Yang et al. [76] performed a miRNA profiling study of sera from patients with prediabetes and T2DM compared with healthy individuals, and were able to identify a

group of miRNAs including miR-23a, let-7i, miR-486, miR-96, miR-186, miR-191, miR-192, and miR-146a which were down-regulated in both pre-diabetic and T2DM individuals.

7.4.2 miRNA Markers for NAFLD in Humans

Among other miRNAs, miR-122 which targets de novo lipogenesis [91] has been extensively studied by several investigators. Yamada et al. [92] found that the levels of miR-122 together with miR-21, miR-34a, and miR-451were elevated in sera of patients with NAFLD. Another study [93] reported that miR-122 together with miR-17, miR-20a and miR-20b were elevated in T2DM patient with NAFLD. In addition, Becker et al. [94] reported that miR-122 was increased in sera of patients with both NAFLD and the non-alcoholic steatohepatitis. Specifically, the expression level of miR-122 was found to be positively associated with total cholesterol and triglyceride levels in plasma of NAFLD patients [92, 95–97]. The level of miR-122 was also elevated in plasma of children with NAFLD in German and Italian cohorts [98]. In contrast to these reports, the expression of miR-122 was reported to be lower in sera of patients with T1DM, T2DM, maturity onset of diabetes (MODY) or mitochondrial mutation-induced diabetes [99].

The elevation of miR-21 in serum of NAFLD patients initially reported by Yamada et al. [92] was supported by two functional studies in which ablation of miR-21 expression ameliorated hepatic steatosis of high fat-diet induced fatty liver mice [100, 101]. The elevated level of miR-34a was also repeatedly demonstrated in sera of patients with NAFLD [92, 95, 102]. Mechanistically, miR-34a exerts its inhibitory effect on expression of the sirtuin 1/AMPK/PPARα axis which is a negative regulator of the de novo lipogenesis pathway [103–105]. miR-33a and miR-33b, which down-regulate expression of FASN indirectly through NFY-C, were found to be up-regulated in plasma of children with hypercholesterolemia. In addition, the expression levels of these two miRNAs were positively correlated with total cholesterol in the serum [106]. miR-181a, which controls insulin sensitivity in mouse models, was also found to be elevated in sera of patients with NAFLD. However, a larger cohort is required in further studies for conclusive data [106]. miR-103 controls insulin sensitivity and is associated with NAFLD, suggesting its potential as a non-invasive biomarker for diagnosis of NAFLD and prognosis of disease severity [68]. Geng et al. [107] showed that the expression of miR-98 may be used as a biomarker for detecting metabolic syndrome as its expression was low in patients with hypercholesterolemia. Mechanistically, miR-98 targets the SREBP2 transcription factor of enzymes involved in cholesterol biosynthesis. Animal studies showed that over-expression of miR-98 decreased total cholesterol in HepG2 cells and serum cholesterol levels in mice. Table 7.1 summarizes selected circulating miRNAs which are associated with diabetes and NAFLD.

Table 7.1 Selected circulating miRNAs which are associated with diabetes or non-alcoholic fatty liver disease

miRNA	Target	Expression level in serum of patients	Reference
DM			
miR-23a	Hepatic gluconeogenesis	Decreased	[76]
miR-103, miR-107	Insulin sensitivity	Increased	[70, 71]
miR-126	VEGF pathway	Decreased	[77]
miR-143	Insulin signaling	Not changed	[75]
miR-197-3p	Islets cell integrity	Increased	[88]
miR-375	Islets cell integrity	Increased	[85, 87]
let-7 g	Islets cell integrity	Increased	[89]
let-7c-5p and let-7a-5p	Positively associated with Hb1Ac	Increased	[90]
NAFLD			
miR-21	DNL	Increased	[92]
miR-33a, miR-33b	FASN	Increased	[105]
miR-34a	DNL	Increased	[92, 95, 102]
miR-98	SREBP2	Decreased	[107]
miR-103	Insulin sensitivity	Increased	[70, 69]
miR-122	DNL	Increased	[92–94]

7.5 Conclusions

Aberrant expression of miRNAs contributes to deregulation of glucose and lipid metabolism, contributing to the etiology of diabetes and hepatic steatosis. Using mouse and cell lines as preclinical models, many attempts have been made to identify and understand the miRNAs which regulate the key enzymes involved in glucose metabolism and de novo lipogenesis. Modulating the expression of these miRNAs may provide a means to reprogram aberrant metabolic pathways that lead to disease. From the clinical view point, serum miRNA profiling of patients with diabetes and NAFLD in different cohorts have been performed, aiming to identify circulating miRNAs which could be used as biomarkers for an early diagnosis or monitoring disease progression. This could be lead to improved outcomes of individuals suffering from metabolic disorders.

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Chapter 8
Hypercaloric Diet-Induced Obesity and Obesity-Related Metabolic Disorders in Experimental Models

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Abstract Overnutrition and obesity have developed into a major public health problem across different parts of the world. Epidemiological studies have shown that excessive intake of dietary components, such as fatty acids and/or sugars, can promote obesity. In this context, the use of dietary intervention in animal models that respond to a diet similar to humans is useful to understand this preventable, multifactorial disease. The aim of this chapter is to aid researchers in choosing specific nutritional interventions and animal strains to induce obesity and obesity-related morbidities in experimental models.

Keywords High-fat diet · Western diets · Fatty acids · Animal model · Obesity

8.1 Introduction

Obesity is a global public health problem that remains the main risk factor for non-communicable diseases, which are responsible for numerous deaths worldwide [1]. In order to understand obesity better, different epidemiological approaches have been adopted including population-based studies, such as cohort, case-control and clinical trials, which have identified some of the disease risk factors, metabolic impacts and treatments [2, 3]. However, these human studies present important limitations that include underreporting and the impossibility of isolating the impact of specific dietary components [4, 5].

Thus, experimental models represent a valuable strategy complementary to human studies. Animal-based studies allow controlled interventions and the isolation of different tissues for metabolic and molecular analysis [6]. Similarly to

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humans, animals such as mice and rats present a positive correlation between dietary fat ingestion and weight and adiposity gain [4]. In one of the first reports in which obesity was successfully induced with diet alone, different strains of mice were fed ad libitum diets with fat content ranging from 5% to 47.5% [7]. Since this time, several experimental models have been developed to induce obesity and obesity-related metabolic disorders through high fat and/or high sugar dietary interventions. The objective of this chapter is to present an overview of these models.

8.2 Dietary Approaches to Induce Obesity and Related Metabolic Alterations

8.2.1 High-Fat Diet (HFD)

There is a broad definition for the term "HFD" [8]. Researchers have used diets with as much as 30–85% [9] of the calories being derived from fat to induce obesity and promote insulin resistance [4, 8]. In addition, the fat component of the HFD varies from plant oils, such as sunflower seed and soy oils, to animal-based fats, such as lard and beef tallow. In some HFD protocols, carbohydrates and proteins are replaced with the fat component and in other protocols fat is simply added to a standard chow.

A successful intervention with a HFD should begin with animals at a young age [8]. In addition, a longer intervention period can promote obesity even with 13–20% of calories originating from fat. As sedentary-caged lab animals become older they have lower energy requirements and will become heavier even with a lower dietary fat content as compared to the standard HFD used to promote obesity. Differences in body weight between control and HFD-fed animals can be observed with at least 2 weeks of feeding, but weight differences are more pronounced with at least 4 weeks of dieting [8]. While weight gain in comparison to the control group is frequently used to evaluate the successfulness of a HFD intervention, a more sensitive form of estimation, such as body composition, is recommended [4]. Woods et al. [10] have shown that Long-Evan rats fed with 40% of the energy coming from fat for 10 weeks presented an increase of 10% in body weight, whereas there was a gain of 35–40% in body fat compared to control animals.

Another factor that directly affects weight, adiposity gain and metabolic disturbances of laboratory animals is the profile of fats in a HFD. In a study from our research group [11], Sprague Dawley male rats were fed three different diets: (1) a control diet, with 16% of energy from soy bean oil; (2) 60% of energy from a lard-based HFD; and (3) 60% of energy from a corn oil-based HFD. Animals fed the corn oil-based diet gained a similar amount of weight compared to the control group at the end of the 8-week intervention, whereas the lard based group gained more weight than rats on the control and corn oil diets. Buettner et al. [8] observed that Wistar rats fed with 42% of the energy coming from different HFDs (lard, olive oil, coconut fat and fish oil) presented higher weight gain than rats fed a standard chow,

with the exception of rats fed a fish oil-based HFD, which had lower weight gain than the controls.

To test the hypothesis that a highly saturated fat-rich diet is more obesogenic than diets with lower saturated fat content, Hariri et al. [12] fed Sprague-Dawley female rats with a HFD, containing 67% of the energy from different fat sources. During 50 days, rats were fed with either: (1) a HFD containing canola, which has a low amount of saturated fatty acid; (2) a HFD containing lard with moderate quantities of saturated fatty acid content; or (3) a HFD containing butter, which has a high saturated fatty acid content. As expected, animals fed with a butter-based diet presented greater daily body weight, body weight gain, and final weight compared to the lard and canola oil groups.

Because fat is the highest energy component of the diet, at 9 kcal/g, animals need less of a HFD diet on a gram basis to become satiated in comparison to the control group. Reports of obesity models involving HFD interventions frequently show that animals from the intervention and control groups consume the same amount of energy daily [13, 14]. However, when energy expenditure is measured in the dark (the active phase for rodents) and light (the lower activity phase) cycles, obese animals were shown to have a lower active metabolic rate in relation to the control group. Additionally, obese animals also presented low resting energy expenditure in the light and dark cycles, which suggests a lower muscle oxidative capacity [15]. Another study has shown that there is less energy dissipated after the digestion of a HFD compared to diets containing lower amounts of dietary fats [16]. This could explain the observations of body weight and fat mass gain in the HFD intervention diets, even when caloric consumption is similar to the control group.

Although a HFD is successful in producing obesity and its related disorders, other diets are just as, or even more, effective. The cafeteria diet (CAFD) and the high fat and high sugar-diet (HFHSD) are among these diets. The preference towards these diets is due to the fact that they usually aim to mimic food consumption of obese individuals. A high-sugar diet is also used to induce metabolic disorders, although its effects are limited.

8.2.2 CAFD

The CAFD is defined as a palatable and caloric dense diet frequently observed in Western society [17], which has been associated with increases in obesity rates [18]. The CAFD is also known as a Western diet, which encompasses elevated intake of processed grains, sugars, dairy products, dietary fats and meats. The procedure in administering a CAFD consists in offering animals a standard chow with free access to cafeteria-style foods, which are given in excess and consumed ad libitum [19]. CAFD foods include biscuits, cheese, processed meats, cake, chocolate, peanut butter or other processed foods [20, 21]. It has been shown that the CAFD causes hyperphagia [22] because it is hedonistically pleasant [23]. One possible mechanism is that such diets provoke over-ingestion by activating reward processes [24].

Interestingly, when Sampey et al. [25] compared CAFD to a 45% lard-based HFD, the authors observed that control and HFD-fed Wistar rats consumed approximately 100 kcal/day/rat, while CAFD-fed rats consumed 30% more calories than the other groups. Differently from CAFD-fed rats, by week two, HFD-fed rats decreased their dietary intake. This effect is frequently seen in rodents when they are placed on energy dense diets. These rats stop eating when the energy requirements have been met. This effect is not seen in CAFD-fed rats, probably because palatable foods can bypass the homeostatic energy control by triggering the reward system [26, 27].

In relation to body weight and metabolic outcomes in rodents, it was demonstrated that both the HFD and CAFD were associated with increased adiposity in male Wistar rats [25]. However, consumption of the CAFD seems to induce higher weight and abdominal fat gain in adult male BALB/c mice compared to the effects seen with the HFD. In addition, the CAFD promotes more damage in the heart, kidney and liver [17], and increases inflammation, and induces hyperinsulinemia, hyperglycemia and glucose intolerance in male Wistar rats [25], when compared to the HFD group.

Although the CAF and Western diets are generally HFDs, both are less standardized than HFDs [28]. Particularities of the CAFD include the choice of snacks to represent diets from different regions of the world. Animals self-select certain types of foods instead of others, which also accounts for variation of phenotypes within and between studies using a CAFD [29]. A common criticism of the CAFD is the variability of the macro and micronutrient contents of the snacks used. Aside from being rich in saturated dietary fatty acids, snacks are frequently rich in sugars, additives and low in protein, vitamins and minerals [18, 23, 29, 30]. These factors can interfere with observed results.

8.2.3 HFHSD

A diet combining sugar and lipids seems to be more efficient in triggering metabolic alterations and obesity when compared to the HFDs [31]. One of the models using a HFHSD approach consists of feeding animals with a chow containing lard, simple sugars, polysaccharides and protein amounting to 4.5 kcal/g of diet, accompanied by sweetened condensed milk [32]. The obesogenic effect of the HFHSD is credited to the increased content of saturated fatty acids, which are less available as an energy source and as a result are acetylated into triacylglycerol and stored in adipose tissue [12, 33]. Additionally, rapidly absorbed carbohydrates, such as simple sugar from sweetened condensed milk, cause a rapid elevation in serum insulin levels resulting in a rapid decrease in blood sugar levels [34]. These shifts signal to the mesolimbic system to modify dopamine concentrations, triggering neurochemical responses similar to those seen for addiction, which elicits a craving response [35]. This may contribute to obesity by evading the homeostatic energy intake of the rodent [26].

There are different animal models of HFHSD in the literature [13, 27, 36, 37] and the dietary formula may change depending on the nutritional composition [38]. Therefore, it is important to consider macronutrients in the diets, because of their role in metabolism and insulin regulation, and this is possibly more important than caloric intake [39]. Recently in the study of Masi et al. [13], it was observed that a high sugar diet (control diet offered with sweetened condensed milk) was more proinflammatory than the HFD. In addition, the high-sugar diet induces a hepatic fibrosis phenotype, whereas the HFD alone is more deleterious to insulin metabolism and may provoke liver steatosis. Although feeding with the HFD and HS diet alone induces metabolic disturbances, the combination of both nutritional components as a HFHSD intensifies the isolated effects of each diet. In this case, an exacerbation of insulin resistance and adipose tissue inflammatory state is observed. In addition, while isolated HSD and HFD diets cause an approximate five-fold increase in body weight, a HFHS diet was shown to increase body weight by approximately nine-fold in comparison to the control group.

A model of HFHSD suggested by Panchal et al. [40] is based on the introduction of fructose in the diet together with sweetened condensed milk as a source of sugar and beef tallow as a source of fat. When fed this diet for 16 weeks, Wistar rats showed greater body weight and abdominal fat gains, along with a higher increase in abdominal circumference compared to the control group. Aside from inducing the metabolic syndrome, the HFHS diet also altered the function of vital organs, such as the heart, liver, kidneys and pancreas [40].

If the HS component of a HFHS diet is fructose, a more pronounced deleterious metabolic effect is observed in rodents [41], especially when associated with the metabolic effects of a diet rich in saturated fatty acids [12]. As observed by Kohli et al. [42], adult male C57Bl/6 mice assigned for 16 weeks to a high-fat high-carbohydrate (HFHC) diet with high-fructose corn syrup in the drinking water, developed obesity, presented increased hepatic reactive oxygen species and non-alcoholic fatty liver disease phenotype, with fibrosis. Different from a HFD alone, fructose consumption is necessary to induce liver fibroses and not only fat deposition [42].

Wistar rats fed a HFHSD for 12 weeks, with a modification of the fat source to 20% ghee, presented increased blood pressure, abdominal circumference, glucose intolerance and dyslipidemia compared to the control group. Despite these metabolic disturbances, HFHS fed animals had a distinct outcome form previous studies by being leaner than the control group, and this was attributed to the modification of the fat source [43].

The composition of HFHSDs vary between research groups, but one point that seems to be common is that a HFHSD causes prominent metabolic disturbances [13, 40, 43]. Also, as outcomes can change depending on the choices of macronutrients source, it is important to select the macronutrient component according to the objectives of the study [44]. In addition, it has been discussed that human diets that cause metabolic disorders are complex and are not only characterized by a high fat content. Thus, the use of a HFHSD is important to understand the role that dietary components have and their impact on metabolic diseases in humans [13].

8.2.4 HSD

Diets rich in sugar have also been used to induce obesity and metabolic disorders in animals [45, 46]. To induce the metabolic syndrome, there are two models commonly used: (1) a high-sucrose diet offered to rats leading to their development of spontaneous hypertension; and (2) a high-fructose diet offered to Sprague Dawley rats leading to their development of the metabolic syndrome [47].

Feeding Sprague-Dawley rats with a standard diet with free access to water containing either glucose, sucrose or fructose in different concentrations has been described in the scientific literature [45, 48]. When given free access to solutions of sugar, animals tend to drink 60% of total energy from the sugary solution and obtain 40% of the energy from the standard diet [45]. In the study of Castonguay et al. [45], it was shown that Sprague-Dawley rats with unrestricted access to sugary solutions (either sucrose, fructose or glucose) and a standard chow consumed less food than the control group. There was also a preference towards a solution containing sucrose rather than glucose or fructose, in that sequence [45]. Differences in animal body weight were not observed after 1 week on the diets. Other researchers also reported that a high sucrose diet was linked to reduction in weight gain [49].

There are conflicting results in the literature about the effectiveness of a high-sucrose diet on inducing obesity [50]. There is evidence of a positive relationship between a HSD and obesity development and insulin resistance [51]. However, it is reported that successful intervention with a HSD depends on sucrose administration. Sucrose must be offered separately from the standard chow, such as being mixed in with drinking water [52] or as a supplement [53]. If mixed in with the diet [49], animals might reduce their food intake and maintain their weight.

Although many factors can influence food intake, the taste of sweetness is an incentive that plays a role on increased ingestion of chow in animals [54]. The macronutrient composition of diets can also have a role in inducing obesity [55]. Lomba et al. [50] observed that animals fed a diet rich in sucrose presented increased weight and fat mass compared to the starch-fed diet control group.

Thus, when choosing a high sugar diet model to induce obesity or metabolic disorders, it is relevant to consider that the outcomes may change depending on the sugar source [45, 50]. In addition, the way in which sugar is administrated (in solution, or mixed in the diet or as a supplement) can also be a factor [49, 52, 53].

8.3 Animal Models of Obesity

Studies relying on animal models have significantly improved the knowledge of human obesity and obesity-related metabolic diseases. Currently, the most employed models in this field could be divided into two major groups: (1) monogenic models which have a spontaneous or genetically engineered mutation that lead to an

obesity-prone phenotype; and (2) polygenic animals, which are diet-induced obese models and mimic human gene-environment interactions more precisely. Other obesity-prone categories such as chemically or mechanically induced are available but are less employed [56, 57].

Monogenic models have provided information on the role of allelic variants and gene mutations in energy homeostasis, fat-storage regulation and other mechanisms underlying obesity. Spontaneous mutations in the leptin pathway, such as those seen in the ob/ob mouse [58], are the most common models, however there are nearly 250 genes that are related to obesity in monogenic models when mutated or expressed as transgenes, such as the melanocortin receptor 4 (MC4R) knockout mouse [59] and the overexpression of the fat mass and obesity-associated (FTO) protein mouse [60] models. Despite significant progress made using monogenic models and the considerable role of single-gene mutations in obesity mechanisms and associated metabolic disorders, there are cumulative evidences revealing that human obesity is a polygenic trait with complex gene interactions and marked differences in inter-individual susceptibilities. Therefore, polygenic diet-induced obesity (DIO) models capture more precisely human gene-environment interactions and resemble the human condition more precisely [61, 62]. In this section, experimental models for obesity and obesity-related metabolic diseases will be discussed, focusing on rodent monogenic and polygenic models.

8.3.1 Rodent Experimental Models: Diet-Induced Obesity, Obesity-Resistant and Monogenic Models

When inducing obesity in experimental models it is important to consider that diet effects can vary greatly depending on diet composition, model type and strain. In this field a wide range of animal models can be applied, including rodents, domestic animals and primates [57]. According to the European Union (EU) Statistical Reports on the Use of Animals for Scientific Purposes [63], in 2011 mice were the most used species, representing approximately 60% of the total used in the EU, followed by rats with a use of around 13%. Some reasons for mice being more extensively used are: (1) there are many different strains with genetic variances; (2) there are more molecular tools available for genomic analysis and standardized methods in the mouse rather than in rats; and (3) mice have relatively short reproductive cycles and larger numbers of pups in a shorter period of gestation [8, 64].

The first reported mouse model of monogenic obesity was the agouti lethal yellow mutant mouse (Ay), characterized by mature-onset obesity and obesity-related metabolic diseases such as type II diabetes [65]. Interestingly, the agouti overexpression in mice adipose tissue was reported to generate increased adipose tissue growth, however, when transgenic models expressed agouti in skin, obesity was not induced. From this information, the authors suggested that agouti has a tissue

dependent obesogenic role [66]. The ob/ob and db/db mice are other examples of widely known monogenic mutations leading to obesity and diabetes phenotypes, respectively [61]. These models have spontaneous mutation in the leptin pathway. The ob/ob mutation affects leptin production and the db/db mutation affects the leptin receptor. However when both mutations occur in mice sharing the same genetic background, the phenotype can be similar [67], highlighting the importance of strain background. In some investigations, monogenic mutations have been introduced into polygenic backgrounds. For example introduction of the Ay mutation into KK insulin resistant and obesity-prone polygenic strain, or ob/ob leptin mutation into C57B/6 DIO strain, led to a more pronounced obesity or metabolic disorder phenotype [28].

The C57BL/6 inbred mouse strain is the most used polygenic model in studies of human obesity and metabolic syndrome. These mice are prone to obesity development, altered insulin secretion levels, glucose intolerance and elevated adiposity when fed ad libitum with HF diets [8]. Other less employed models such as the KK, AKR/J, and DBA/2J inbred obesity-prone mouse strains can also be useful in human metabolic syndrome studies. If the research proposal is to understand obesity resistance, the inbred obesity-resistant strains SWR/J and A/J are appropriate models. This is because they were reported to have reduced adiposity and insulin levels despite over-consumption of a HF diet [68].

Rat models, although less employed, give advantages such as their larger size, which helps during routine procedures, such as gavage and blood drawing, besides the larger amount of tissues available for analysis. The outbred DIO strains Wistar and Sprague-Dawley are the most prevalent in obesity and obesity-related metabolic disorders studies. However, it should be taken into account that outbred animals have greater genetic variability when compared to inbred ones. For example, such strains could show individual variations in insulin resistance and obesity phenotypes [69]. In these cases, a higher number of biological replicates may be required [8, 61]. For obesity-resistance, the carbohydrate preferring S5B/Pl rat is an available option [70]. Concerning the monogenic models, the Obese Zuker rat [71] is analogous to (db/db) mouse, with a missense mutation in the fatty gene (fa), homologous to (db) gene in mouse. The (fa/fa) genotype leads to a defective leptin receptor and generates an early onset of a severe obesity phenotype associated with hyperphagia and low energy expenditure. Simillary, the Koletsky rat [72] shares the same phenotype with the Zucker rat, due to a nonsense point mutation in the same gene, lacking a functional leptin receptor [28, 61]. In contrast to the polygenic strains, monogenic phenotypes are often more pronounced and severe, representing good models in understanding single-gene effects in obesity and for developing pharmacological drugs.

Also, it is important to be aware of the impact of age and sexual dimorphism when choosing obese animal models. As in humans, adipose tissue distribution is influenced by sexual hormones, generating different patterns in the male (more visceral fat) and female (more subcutaneous fat). In addition, the prevalence of obesity is higher in females and that of type-2 diabetes is higher in males. Another important point to consider is that important parameters in obesity and metabolic-related

disease change during life cycle, such as metabolism, fat and lean mass distribution, making the age of the animal an important variable in obesity studies [61].

8.3.2 Mammalian Non-rodent and Non-mammalian Experimental Models

Domestic mammals share similar anatomic and physiologic profile to humans and have been used as experimental models for obesity research. Like humans, dogs and cats are found to have increased prevalence of obesity, suggesting that these pets share etiologic similarities to human obesity, and that environmental conditions are relevant in the context of obesity onset. Although dog models are historically related to metabolic studies [73], and overweight can easily be induced [74], the genetic basis of obesity in the dog is still poorly understood [75]. Non-human primates (NHPs) have direct translational relevance to human features. The shared factors between the species include the clinical and pathological development of diabetes, obesity and metabolic disorders. NHP models include, beside others, the species Macacamulatta (rhesus macaques) and Macaca fascicularis (cynomolgus monkeys), which are particularly useful models due to their spontaneous development of obesity. Studies with NHPs and other non-rodent models are submitted to rigorous analysis for ethical approval and the shortcomings of using these models include the long life cycle, high cost and, especially for NHPs, the low offspring number [76, 77].

Concerning the non-mammalian models such as Caenorhabditis elegans, Drosophila melanogaster and Danio rerio, these are known for their short lifespan (except to D. Rerio [78]) and low maintenance cost. On the other hand, they have the main disadvantage of having a different anatomy and physiology compared to humans, which narrows extrapolation to the human disease context [61]. However, the importance of such models has been demonstrated based on the understanding of conserved pathways in fat-storage and adiposity regulation, gene-gene/environment interactions, and the general mechanisms underlying obesity [79–82].

8.4 Conclusions

Obesity is a main public health problem. In order to establish more effective preventive and treatment interventions, a better understanding of diet-induced obesity and obesity-related metabolic alterations should be gained. There are several valuable experimental models based on different dietary interventions (i.e. HFD, HFHSD and CAFD) and animal strains (monogenic or polygenic; rodents, mammalian non-rodent and non-mammalian). Each model presents advantages and limitations. Thus, when planning an animal experiment all of these aspects should be taken into account. This will help to increase the translatability of the findings in clinical studies of obesity and other metabolic disorders.

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Chapter 9
Metabolic Biomarkers in Nematode *C. elegans* During Aging

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Abstract Changes in energy metabolism occur not only in diseases such as cancer but also in the normal development and aging processes of various organisms. These metabolic changes result to lead to imbalances in energy metabolism related to cellular and tissue homeostasis. In the model organism *C. elegans*, which is used to study aging, an imbalance in age-related energy metabolism exists between mitochondrial oxidative phosphorylation and aerobic glycolysis. Cellular lactate and pyruvate are key intermediates in intracellular energy metabolic pathways and can indicate age-related imbalances in energy metabolism. Thus, the cellular lactate/pyruvate ratio can be monitored as a biomarker during aging. Moreover, recent studies have proposed a candidate novel biomarker for aging and age-related declines in the nematode *C. elegans*.

Keywords *C. elegans* · Energy metabolism · Aging · Metabolic change · Mitochondrial ROS · Cancer

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9.1 Introduction

In mammalian p53-mutated cancer cells, glycolysis is used predominantly for energy production while aerobic mitochondrial respiration is downregulated, a secondary metabolic change known as the Warburg effect [1]. Recent studies have revealed that the mammalian tumor suppressor p53 directly regulates oxygen consumption through transcriptional targets such as the *SCO2* gene, which encodes an assembly protein for the synthesis of cytochrome *c* oxidase (COX) in the mitochondrial respiration chain complex and is associated with changes in glycolytic activity in mice and human cancer cells [2]. Moreover, mammalian p53 regulates the glycolytic enzyme phosphoglycerate mutase (PGM) and the mitochondrial gatekeeper pyruvate dehydrogenase kinase (Pdk2) through post-transcriptional control [3, 4]. Likewise, we recently investigated whether the mammalian p53 ortholog CEP-1 in a model organism *Caenorhabditis elegans* (*C. elegans*) is associated with metabolic transition in the cells [5, 6]. Unlike tumorigenesis in mammals, impaired p53/CEP-1 extends the lifespan through an age-related imbalance in energy metabolism in *C. elegans* [7, 8]. The age-related imbalance in energy metabolism shows that lactate levels and consequently the lactate/pyruvate (L/P) ratio decrease during aging in wild-type adult. However, this phenomenon is different in *cep-1* mutants. Interestingly, changes in the L/P ratio during aging have also been observed in a mutant premature aging model in *C. elegans* [9]. Thus, classical energy metabolism and the inherent changes in metabolite levels are re-evaluated based on the cellular balance during aging and age-related diseases, and some metabolites could be highlighted as novel biomarkers [9, 10]. Here, we review the change in energy metabolism during aging in *C. elegans* and discuss the potential biomarkers in aging and age-related disorders in organisms.

9.2 Metabolism During Development and Aging in *C. elegans*

In the life cycle of *C. elegans*, there are normally four larval (L1-L4) and adult stages, as well as a facultative diapause ‘dauer’ larval stage. Dauer larvae do not feed despite being active [11, 12]. In the energy metabolism of *C. elegans*, the tricarboxylic acid (TCA) cycle is preferentially used for cell growth and proliferation during the L2 to L4 stages; subsequently, both higher tolerance to anoxia and greater protection against reactive oxygen species (ROS) are observed in young adult worms [12–14]. It is likely that the developmental characteristics of energy metabolism are associated with an invariant number of somatic cells, except the adult germ line, after somatic cell division. Marked reductions in oxygen consumption and metabolic rate have been seen in wild-type animals of 10-day-old and above [15]. These reductions are consistent with the gradual decay of muscle function seen in the adult stage, as revealed by pharyngeal pumping and locomotion rates [16, 17].

Recently, we showed that expression of the encoding mammalian COX assembly protein *SCO2* gene homolog *sco-1* gene increases according to age in wild-type *C. elegans* [8]. However, as previously reported, adenosine triphosphate (ATP) and oxygen consumption levels are significantly reduced during aging in the adult stages [15, 18, 19]. These results suggest that mitochondrial components, such as COX, are damaged by mitochondrial ROS during the aging process, and consequently aerobic glycolysis is utilized preferentially rather than the TCA cycle [20].

On the other hand, we showed the age-dependent increases in the expression levels of *pck-1* gene encoding a phosphoenolpyruvate carboxykinase (known as PEPCK or PCK1 in mammals), which regulates gluconeogenesis [21], and *sir-2.1* gene encoding a *C. elegans* sirtuin (also known as NAD^+-dependent histone deacetylase), which is induced upon caloric restriction [22, 23] in wild-type *C. elegans* [8]. These observations indicate an acceleration of gluconeogenesis and calorie restriction during normal aging. Indeed, lactate levels and the consequent L/P ratio decreased in aged wild-type *C. elegans*.

9.3 Hypoxia-Induced Metabolism and Aging in Nematode

The *pck-1* gene is related to an important role involved in unique energy production even in anaerobic environments during various life cycles of many parasitic invertebrates. Although *C. elegans* is a free-living nematode, it also possesses the PEPCK-succinate pathway, which is an anaerobic mitochondrial fermentation pathway for energy production during the parasitic stage of *Ascaris* species, which are mammalian intestinal roundworms [10, 24, 25]. In fact, starved and incubated *C. elegans* can normally survive for a few hours by utilizing carbohydrate stores under anoxic conditions [10, 26]. *C. elegans* PCK-1 regulates several metabolic processes associated with cataplerosis, which is the removal of intermediate metabolites from pathways, such as gluconeogenesis and PEPCK-succinate pathways in anaerobic environments [21, 27]. Our previous report also found the upregulation of gluconeogenesis rather than the anaerobic metabolic pathway during aging in *C. elegans* due to the increased expression of *pck-1* and *sir-2.1* genes, reduction in mitochondrial respiration, and decreased L/P ratio [8, 28].

9.4 Metabolic Changes in a Nematode Model of Premature Aging

According to the free radical theory of aging, the accumulation of ROS as by-products of mitochondrial metabolism is associated with lifespan determination and aging in various organisms [29, 30]. Almost all mitochondrial oxygen consumption is efficiently coupled to the production of ATP; however, a small part of the oxygen

consumed is reduced by wayward electrons to produce potentially toxic ROS. Indeed, mitochondria are the primary source of ROS in cells [31]. It is estimated that ~0.1% of the oxygen utilized by cells is only partially reduced and is leaked as a kind of ROS, e.g., superoxide anion ($O_2^{\cdot-}$) [32]. In *C. elegans*, lifespan is closely related to the concentration of environmental oxygen [33] and the continuous exposure to the hyperoxia accelerates senescence so that the levels of intracellular ROS increase in animals [34–36].

The *mev-1* gene encodes a large subunit of the enzyme succinate dehydrogenase cytochrome *b*, which is a component of complex II in the mitochondrial electron transport chain. Mutation of the gene causes an increase in mitochondrial $O_2^{\cdot-}$ production and consequently shortenes the lifespan of *C. elegans* [37]. Mitochondrial electron transport chain transfers reducing equivalents from NADH (in complex I) and $FADH_2$ (in complex II) in the form of electron flow through complexes III and IV. In the four complexes, which are connected with CoQ and cytochrome *c* (Cyt *c*), protons (H^+) are pumped from the matrix into the intermembrane space to establish an electrochemical gradient and subsequently drive ATP synthase to generate ATP. When flow to oxygen as the final acceptor of electrons is restricted because of a higher H^+ gradient or inhibition at the complexes, the chance of electron leakage increases. The leaked electrons from complexes I and III are transferred to molecular oxygen in the mitochondria and consequently cause the generation of $O_2^{\cdot-}$. Mitochondrial manganese superoxide dismutase (Mn SOD) catalyzes the conversion of $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2) in the matrix (Fig. 9.1) [38]. In *mev-1* mutant, $O_2^{\cdot-}$ production increases at complex I rather than at complexes II and III, and the lactate levels and L/P ratio are markedly higher than in the wild-type [9].

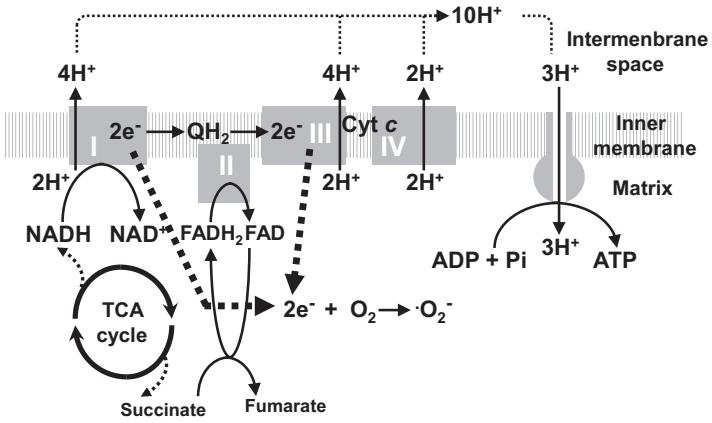

Fig. 9.1 Schematic of electron and H^+ transport in the electron transport chain of mitochondrial OxPhos. Four complexes are connected to CoQ, Cyt *c* and other molecules included in the chain. Electrons leak mainly from complexes I and III during the higher H^+ gradients

These observations suggest that *mev-1* animals preferentially utilize glycolysis instead of using the TCA cycle and subsequently the electron transport chain in mitochondria for production of cellular energy. Interestingly, a recent study has demonstrated that p53/CEP-1 inactivation rescues the shortened lifespan of the *mev-1* mutant [39]. Despite overproduction of ROS by mitochondria in the *mev-1* mutant, however, we observed increases in the expression of COX assembly protein-encoding *sco-1* gene, which is a target of p53/CEP-1 and associated with regulation of mitochondrial respiration (unpublished data). Thus, the levels of cellular metabolites such as lactate and pyruvate, which are key intermediates in the cellular energy metabolic pathways, correlate with a switch from mitochondrial respiration to glycolysis in energy metabolism during aging [8, 9].

9.5 Metabolism of Mutants Related to the Longevity in *C. elegans*

9.5.1 Metabolism in Reduced ins/IGF-1 Signaling

Intracellular ROS levels are regulated via an insulin/insulin-like growth factor-1 (ins/IGF-1) signaling pathway [40, 41], which determines longevity and resistance to oxidative stress in *C. elegans* [42–44]. Through the DAF-16 transcription factor [45, 46], which is the *C. elegans* homolog of the mammalian forkhead transcription factor class O (FoxO) and activated downstream of the ins/IGF-1 signaling pathway, target genes such as those related to antioxidants, mitochondrial respiration, and protein repair systems are regulated during normal aging (Fig. 9.2) [47, 48]. Thus, not only antioxidant systems containing SOD and catalase but also intracellular ROS levels containing mitochondrial $O_2^{\cdot-}$ are modulated via the ins/IGF-1 signaling pathway and are related to determining the lifespan of *C. elegans* [35, 43, 44, 52].

Many previous studies have demonstrated that the quantity of ATP production in both long-lived mutants with genes *age-1* (encoding a homolog of the catalytic subunit of mammalian phosphatidylinositol 3-OH kinase) and *daf-2* (encoding a homolog of the mammalian insulin receptor) [40, 41, 53], which inactivates ins/IGF-1 signaling, is increased compared with wild-type *C. elegans* [45, 54–57]. In addition, the mutants consistently show lower respiratory rates despite increased ATP concentrations [58–62]. That is, a reduction in the metabolic rate is required to extend the lifespan of *C. elegans* [60]. These observations suggest the possibility that worms with reduced ins/IGF-1 signaling preferentially utilize aerobic glycolysis and gluconeogenesis through glyoxylate shunt, which is not present in mammals, rather than mitochondrial respiration in the cells [20]. Intracellular ROS as by-products of the ATP production process in the mitochondrial respiratory chain are efficiently removed due to the higher activities of antioxidant enzymes such as SOD and catalase in *age-1* and *daf-2* mutants [58, 63].

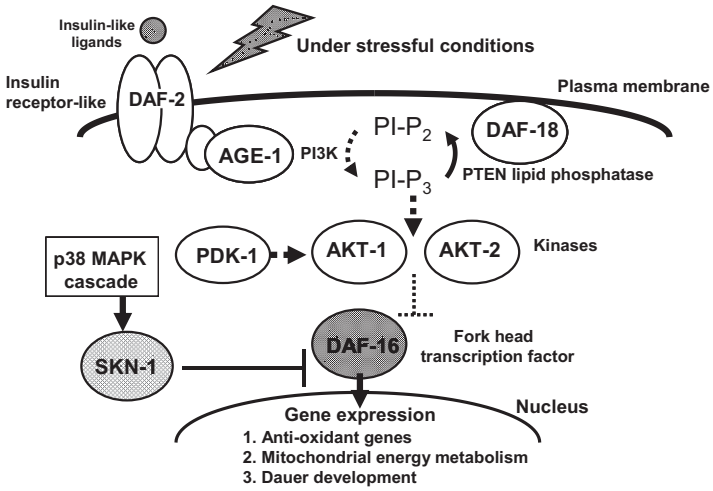

Fig. 9.2 Schematic model of the ins/IGF-1 signaling pathway associated with aging in *C. elegans*. As shown in the schematic, other signals, for instance mammalian NF-E2-related factor (Nrf) ortholog SKN-1 up-regulated by the p38 mitogen-activated protein kinase (MAPK) signaling pathway, plays an important role in fine-tuning molecular compensation among *sod*-genes during normal aging [49–51]

9.5.2 Metabolism Related to Mitochondrial Respiratory Chain

Mutations of the *clk-1* gene (encoding a homolog of the yeast COQ7/CAT5, a component of mitochondrial respiratory chain) in *C. elegans*, show slowing of developmental and physiological processes, including growth, pharyngeal pumping rate, defecation cycle and aging [64]. In the yeast *Saccharomyces cerevisiae*, mutations of the *coq7* gene prevent the biosynthesis of ubiquinone (coenzyme Q or CoQ), a lipid-soluble component of the electron transport chain required for mitochondrial respiration and gluconeogenesis [65, 66]. The mildly reduced mitochondrial respiration of long-lived *clk-1* mutants in *C. elegans* suggests that longevity is promoted by an age-dependent decrease in mitochondrial function [55, 67]. However, aged *clk-1* mutants also retain substantial elevation in ATP levels compared with wild-type animals. Interestingly, energy production and oxygen consumption appear to be uncoupled in *clk-1* mutants [55]. Dietary withdrawal of coenzyme Q from *Escherichia coli* (*E. coli*) extends the lifespan of not only wild-type but also *clk-1* mutant adults [68]. Similar results were reported in COQ7-deficient mice [69]. These findings suggest that mitochondrial coenzyme Q regulates the coupled mitochondrial respiration and generation of ROS that substantially contribute to the lifespan extension in various aerobic organisms.

9.5.3 p53/CEP-1 Dependent Energy Metabolic Regulation

In contrast, the *C. elegans cep-1* mutant shows increased ATP levels throughout aging without increased oxygen consumption compared with wild-type, as well as increases in lactate levels and the consequent L/P ratio depending on age. These results suggest the compensatory and preferential use of glycolysis to generate ATP rather than mitochondrial oxidative phosphorylation (OxPhos) related to the impaired p53/CEP-1, which resembles the energy metabolism seen in mammalian cancer cells. In addition, this implies that the unique anaerobic metabolism related to the PCK-1 in *C. elegans* activates the generation of ATP, as described in Sect. 9.3 [8]. Wild-type p53/CEP-1 supplements a component in COX, which is damaged by mitochondrial ROS during normal aging, through the target *sco-1* gene encoding a COX assembly protein in *C. elegans*. Moreover, a recent report shows that p53 with SIRT6 regulates gluconeogenesis by the promoting of nuclear exclusion of FoxO1 transcription factor, which mediates the activation of PCK1 [70]. Therefore, we conclude that impaired p53/CEP-1 leads to a metabolic imbalance during the aging process and mainly involves PCK1-mediated gluconeogenesis. It also has the potential for metabolic regulation of lifespan in mammalian post-mitotic cells after differentiation, for example, in somatic cells of *C. elegans* [8].

9.6 Homeostatic Control of Energy Balance in Caloric Restriction

Caloric restriction (CR) and fasting has been shown to extend lifespan and postpone age-related decline in various organisms from yeast to mammals. Indeed, *C. elegans* mutants with a slower pharyngeal pumping rate (e.g., *eat-2*, *clk-1* mutants) and a reduced bacterial food intake live significantly longer than the wild-type. The long lifespans of animals with mutations in the *eat-2* gene, which encodes a nicotinic receptor subunit, do not require the activity of DAF-16 transcription factor downstream of ins/IGF-1 signaling, and show no reduction in metabolic rate [71, 72]. Moreover, several recent studies revealed that a few transcription factors in *C. elegans* are closely associated with these phenomena (Fig. 9.3) [73, 74]. CR activates a mammalian basic leucine zipper transcription factor NF-E2-related factor 2 (Nrf2) homolog SKN-1, which signals the peripheral tissues to increase metabolic activity in a pair of ASI neurons in the head of *C. elegans* [73]. The transcription factor PHA-4, which is a homolog of the mammalian FoxA transcription factors family, has an important role in regulating the expression of superoxide dismutases-encoding *sod*-genes in head and tail neurons and intestinal cells, particularly in response to fasting, and lead to the regulation of glucagon production and glucose homeostasis [74]. Thus, mitochondrial ROS production as a trade-off for a temporary higher metabolic rate in peripheral cells consequently induces the expression of

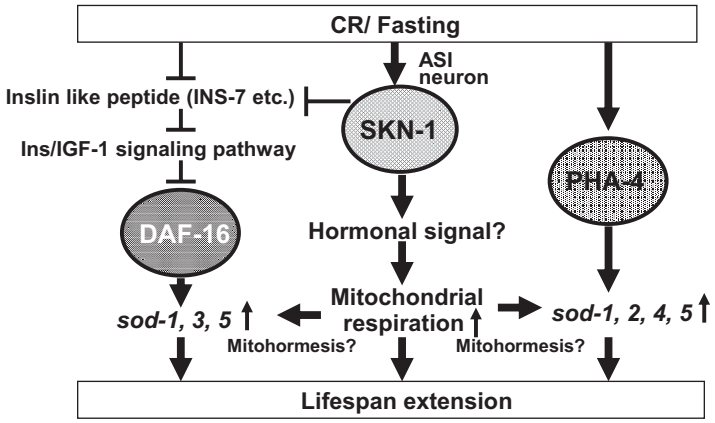

Fig. 9.3 Schematic model of crosstalk of transcriptional factors related to caloric restriction in *C. elegans*

sod-genes, and ultimately enhances longevity-mediated 'mitohormesis' in *C. elegans*.

Mitochondrial hormesis, or more simply mitohormesis, is a hypothetical concept that involves hormetic extension of lifespan [75]. In *C. elegans*, reduced glucose availability promotes ROS production as a side effect of the mitochondrial respiration, and causes induced antioxidant activity, thus increasing oxidative stress resistance and lifespan. The lifespan extension due to reduced glucose is abolished in the disruption of the *aak-2* gene, which encodes a homolog of mammalian AMP-activated protein kinase (AMPK) in *C. elegans*. AMPK activation leads to a decrease in the mammalian target of rapamycin (mTOR) activity [76]. Therefore, AAK-2 activated by a higher AMP/ATP ratio in cells under CR conditions, such as reduced glucose, functions independently of the ins/IGF signaling pathway to extend lifespan due to the decrease in mTOR signaling [77].

On the other hand, recent studies have proposed the hypothesis that CR extends lifespan at least in part by increasing the levels of ketone bodies in various organisms, including nematodes and rodents [78]. The ketone bodies, β-hydroxybutyrate (βHB) and the oxidized forms, which were first found in the ketonuria in diabetes mellitus, are produced by a reversal of the β-oxidation pathway in the metabolism of fatty acids and also during reduced carbohydrate intake such as starvation and fasting. Feeding βHB, which is a histone deacetylase (HDAC) inhibitor, extends the lifespan of *C. elegans* depending on the longevity signals of ins/IGF-1 and p38 MAPK cascade [79]. Thus, reduced glucose intake induces activation of alternative energy metabolic pathways and subsequently changes in the levels of cellular metabolites containing ketone bodies, which are related to longevity. Therefore, the cellular levels could act as metabolic biomarkers to enable understanding of individual energy conditions associated with aging and age-related decline.

9.7 Conclusions

Several genetic mechanisms of aging and lifespan have been clarified to date using the nematode *C. elegans* and are consistent, at least in part, among various organisms. However, the aging process is highly complex due to crosstalk between genetic signaling pathways. Recently, the various roles of mitochondria in the several signaling pathways associated with age-related functions, including energy metabolism, free radical production and apoptosis in aerobic organisms, have been specifically highlighted. In addition, mitochondrial dysfunction during aging is a trigger that induces many age-related changes in energy metabolism. Therefore, identification of these changes among cellular metabolites could help to estimate the condition of an individual and classify age-related disorders such as cancer, diabetes, sarcopenia, and neurodegenerative diseases in aging humans. The cellular levels of these metabolites could be used as genetic-dependent metabolic biomarkers to understand the individual energy conditions associated with aging and age-related declines. The unique ability of energy metabolism pathways in *C. elegans* might function under both aerobic and anaerobic conditions during aging. The use of model organisms contributes to our understanding of not only the mechanisms of aging but also cellular metabolic changes during aging and age-related decline in health.

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Chapter 10
Circular RNAs as Potential Biomarkers and Therapeutic Targets for Metabolic Diseases

Mohamed Zaiou

Abstract Epidemiological studies provide evidence of a continuous rise in metabolic diseases throughout industrialized countries. Metabolic diseases are commonly associated with different abnormalities that hold a key role in the emergence and progression of frequent disorders including diabetes mellitus (DM), nonalcoholic fatty liver disease (NAFLD), obesity, metabolic syndrome and cardiovascular diseases. The burden of metabolic diseases is believed to arise through complex interaction between genetic and epigenetic factors, lifestyle changes and environmental exposure to triggering stimuli. The diagnosis and treatment of metabolic disorders continue to be an overwhelming challenge. Thus, the development of novel biomarkers may enhance the accuracy of the diagnosis at an early stage of the disease and allow effective intervention. Over the past decade, progress has been made in exploring the potential role of noncoding RNAs (ncRNAs) in the regulation of gene networks involved in metabolic diseases. A growing body of evidence now suggests that aberrant expression of circular RNAs (circRNAs) is relevant to the occurrence and development of metabolic diseases. Accordingly, circRNAs are proposed as predictive biomarkers and potential therapeutic targets for these diseases. As the field of circRNAs is rapidly evolving and knowledge is increasing, the present paper provides current understanding of the regulatory roles of these RNA species mainly in the pathogenesis of DM, NAFLD and obesity. Furthermore, some of the limitations to the promise of circRNAs and perspectives on their future research are discussed.

Keywords Circular RNAs (circRNAs) · Metabolic Diseases · Diabetes · NAFLD · Obesity · Epigenetics · Noncoding RNAs (ncRNAs)

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10.1 Introduction

Metabolic diseases refer to different disorders including diabetes mellitus (DM), obesity, metabolic syndrome, dyslipidemia, and non-alcoholic fatty liver disease (NAFLD) [1]. These generally occur when metabolism processes fail. The pathogenesis of metabolic diseases and their chronic complications involve multiple molecular processes and pathways. Early studies using different models revealed that metabolic diseases arise through a complex interplay between genetics, epigenetics, environment, and/or lifestyle factors (nutrition, lack of exercise, etc.) and obesity [2–4]. However, their exact etiology remains partially elucidated.

Despite intensive research into most aspects of metabolic diseases, their causes are still poorly known and only a few effective drugs are available for accurate treatment. Nonetheless, the effectiveness of the current therapy could be improved if it could be implemented at early stage of the disease and targeted to the right subjects who may actually benefit from it. Such an ideal therapy cannot be achieved unless it is combined with predictive biomarkers to guide the treatment. Hence, the search for additional clinically relevant drugs as well as potential biomarkers with precise prognostic and diagnostic value is becoming increasingly important in the field of metabolic diseases.

Recent years have witnessed increasing interest in studying noncoding RNAs (ncRNAs) including long noncoding RNAs (lncRNAs), microRNAs (miRNAs) and circular RNAs (circRNAs), which are considered as important epigenetic regulators of many physiological processes. Huge efforts have been made to use these RNA molecules as predictive biomarkers for several diseases including metabolic disorders [5–9]. Nowadays, the landscape of miRNAs is by far the most characterized in relation to metabolic diseases whereas the role of circRNAs has not yet been precisely defined.

CircRNAs belong to the ever-growing world of ncRNA molecules. They are covalently closed single-stranded molecules generated from precursor mRNA back-splicing [10, 11] and can originate from different genomic regions. The lack of the typical terminal 5′ cap and 3′ polyadenylated tail renders circRNAs more stable and resistant to RNase R digestion compared to the linear RNA counterparts [12–14]. With respect to their biogenesis, detailed mechanisms have not been fully elucidated. Several possible models have been proposed including direct back-splicing with ALU and inverted repeats complementation, exon lariat, and RNA binding protein mediated models [15, 16].

Over the past few years, high throughput technologies have enabled a significant breakthrough in discovery of circRNAs. Today, thousands of circRNAs have been identified and annotated. Based on their genomic location, circRNAs can be classified into at least three types with distinct regulatory functions across multiple mammalian cells and species: (1) exonic circular RNAs (ecircRNAs); (2) circular intronic RNAs (ciRNAs); and (3) exon-intron circular RNAs (EIciRNAs) [11]. EcircRNAs appear to be the most abundant RNAs accounting for over 80% of the already known circRNAs. Moreover, the application of highly sophisticated bioinformatics tools has helped create several circRNA databases with searching and browsing functions [17].

CircRNAs are highly represented in the eukaryotic transcriptome, evolutionary conserved across species, and often show tissue or development stage-specific expression patterns [12, 18–21] suggesting their functional relevance [18, 19, 22, 23]. Interestingly, earlier studies indicated that the expression of a circular RNA does not correlate with the expression of its cognate linear mRNA [24]. In some cases, circRNAs can be more abundantly expressed than their associated linear mRNA isoform [25] while in other situations no circRNA can be detected despite high levels of mRNA expression [22, 23, 26]. The striking expression differences between circRNAs and their mRNA counterparts suggest that the production of circRNAs is a highly orchestrated process [23]. As to their potential functions, research is still limited and challenging. Studies have reported that some circRNAs may act as a sponge for miRNAs via competition with miRNA/mRNA binding or they may interact with RNA-binding proteins (RBPs) or regulate genes at the transcriptional and posttranscriptional levels [11, 27–30]. With these possible functions, specific circRNAs may control essential biological processes and contribute to the pathogenesis of diverse diseases including metabolic disorders [31–33]. However, the exact regulatory mechanisms by which these molecules may carry out these roles are not known. Thus, a more comprehensive understanding of how circRNAs function and what characteristics they should have to interact with other players to orchestrate gene expression in health and diseases states may lay the foundation for the development of RNAs-based diagnostic and therapeutic interventions for complex metabolic diseases. Below I will discuss the most important published studies of circRNAs in DM, NAFLD and obesity. CircRNAs that are most likely to be involved in some of these disorders as well as their putative functions are summarized in Table 10.1.

10.2 CircRNAs and Metabolic Diseases

10.2.1 DM

DM is a multiple-etiology metabolic disorder characterized by chronic hyperglycemia resulting from defects in secretion and/or insulin action [34]. Defects in insulin-mediated uptake of glucose can trigger pathogenic signals including mitochondrial dysfunction, oxidative stress, hypertension, inflammation and dyslipidemia. Additionally, diabetic patients with chronic hyperglycemia are more likely to suffer from many life-limiting and life-threatening complications, such as macrovascular-related stroke, heart disease, peripheral artery disease and/or microvascular-related retinopathy, neuropathy, nephropathy and cancer [35–37]. A major concern with these diabetic complications is that the number of DM cases and associated mortality are constantly increasing globally while the effectiveness of current treatments is limited, and this represents a heavy socioeconomic burden. Thus, identification of novel biomarkers that reflect or predict insulin-secretion dysfunction in individuals could transform the way we deal with diabetes, allowing for early prevention and guided therapy as a step toward precision medicine [38].

Table 10.1 Relevant circular RNAs associated with metabolic diseases

Circular RNA	Expression	Potential function and phenotype	Ref
Diabetes			
CDR1as/cirRS-7	↑	Improves insulin secretion by sponging miR7	[40]
Hsa_circ_0054633	↑	Associated with prediabetes and T2DM in peripheral blood cells Potential biomarker for T2DM	[42]
CircRNA-HIPK3	↑	Promotes retinal vascular disorders by blocking miR-30a-3p function Control of key b-cell functions by sequestering miR-124-3p/miR-138-3p	[44] [41]
CircRNA-0005015	↑	Involved in diabetes retinopathy by acting as miR-519d-3p sponge	[45]
CircANKRD36	↑	Correlated with inflammation in T2DM patient peripheral blood leukocytes Potential biomarker for screening chronic inflammation in T2DM patients	[104]
Hsa-circRNA11783–2	↓	Related to both coronary artery disease and T2DM in peripheral blood	[105]
NAFLD			
CircRNA-0046367	↑	Inhibits hepatic steatosis by preventing hepatotoxicity of lipid peroxidation	[68]
CircRNA-0046366	↑	Inhibits hepatic steatosis through miR-34a/PPARα signaling	[69]
CircScd1	↓	Affects steatosis of NAFLD via JAK2/STAT5 signaling pathways	[71]
Obesity			
CirRS-7	↓	Levels decreased in pancreatic islets of ob/ob and db/db mice	[41]

Ref reference number, *T2DM* type 2 diabetes mellitus

Over the last decade, efforts have been made to understand the disruption of mRNA-miRNA-lncRNA interaction networks under diabetic conditions [39]. More recently, scientists have shifted their research focus to circRNAs, hoping to develop these molecules as new biomarkers for early detection and management of diabetes. In this respect, the most well-known endogenous circRNA related to diabetes in the literature is CDR1as/ciRS-7 (a natural antisense transcript of CDR1) [19, 28]. Overexpression of this circRNA leads to improved insulin production and secretion in mouse β-cells [40]. By acting as a miR-7 sponge [20], CDR1as promotes islet β-cell proliferation and insulin secretion in diabetes via inhibiting miR-7 and enhancing Myrip and Pax6 expression [40]. These encouraging data suggest that the CDR1as/miR-7axis could serve as a potential therapeutic target for the treatment of diabetes. Similarly, another study reported that CDR1as and circHIPK3 silencing in wild-type animal models causes defective insulin secretion and lower islet cell proliferation [41]. By performing microarray and confirming the data by qRT-PCR, Zhao and colleagues measured the differential expression of circRNAs in the peripheral blood of pre-diabetes and T2DM patients compared to matched control

subjects. The most significantly upregulated circRNA was hsa_circ_0054633 (Table 10.1) implying its potential as diagnostic biomarker for prediabetes and type 2 diabetes mellitus (T2DM) in the clinical setting [42]. Circular RNAs have also been investigated in diabetic vascular complications, which are a major cause of mortality among patients with diabetes [43]. In this context, Shan et al. reported that circHIPK3 was significantly induced in the retinas of patients with diabetes [44]. The same study group showed that depletion of circHIPK3 in a mouse model for diabetic retinopathy alleviated the retinal disorder [44]. Mechanistically, circHIPK3 competitively binds different miR-30 isoforms to restore the expression of their target genes including VEGF, FDZ4 and WNT2 which are involved in cell viability, proliferation and migration. In a more recent study, Zhang and colleagues identified circ_0005015 as the most significantly upregulated circRNA in plasma, vitreous samples and fibrovascular membranes of diabetic retinopathy patients [45]. Furthermore, the authors demonstrated that siRNA-mediated silencing of circ_0005015 significantly reduced human retinal vascular endothelial cell proliferation, migration and tube formation. Additional analyses revealed that circ_0005015 acted as an endogenous miR-519d-3p sponge to sequester and inhibit miR-519d-3p, thus facilitating retinal endothelial angiogenic function [45]. Together, these findings suggest that circ_0005015 may be considered as an ideal candidate biomarker for monitoring diabetic retinopathy. CircRNA_000203 is an additional circular transcript linked to diabetes. Tang and colleagues found that circRNA_000203 was upregulated in the diabetic mouse myocardium and in angiotensin (Ang) II-induced mouse cardiac fibroblasts [46]. In fact, circRNA_000203 could specifically increase the expression of fibrosis-associated genes (Col1a2, Col3a1) and α-SMA in cardiac fibroblasts via inhibiting the interaction of miR-26b-5p with the target genes. Therefore, circRNA_000203 might serve as a potential target for prevention and treatment of cardiac fibrosis in diabetic cardiomyopathy [46].

All of the above-mentioned findings suggest that the circRNAs-miRNAs-mRNAs regulatory axis could be a useful therapeutic target for the pathogenesis of diabetes and its complications. However, much more remains to be learned about the biology of circRNAs in diabetes and their beneficial clinical application appears to be a future endeavor.

10.2.2 NAFLD

Non-alcoholic fatty liver disease is emerging as the most common cause of chronic liver disease worldwide. It is a multifaceted disorder that ranges from the simple accumulation of triglycerides in hepatocytes (hepatic steatosis) to steatosis with inflammation, non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, which may evolve towards cirrhosis and hepatocellular carcinoma [47–50]. The prevalence of NAFLD has been estimated to be between 25% and 45% of the general population [51, 52] and 70–90% among patients with obesity, DM or metabolic syndrome [53–55].

Although the pathophysiology of NAFLD has not been fully elucidated, recent investigations have brought forward evidence that this disorder may be caused by a plethora of factors including hepatic lipid accumulation, adipose tissue and mitochondrial dysfunction, a high fat diet, obesity, a chronic inflammatory state, insulin resistance, and genetic and epigenetic factors [56–58]. NAFLD is clinically important because fatty liver can progress to steatohepatitis in many patients and lead to liver cirrhosis and hepatocellular carcinoma. There is also growing evidence that in patients with NAFLD, hepatic steatosis is closely linked with obesity and the metabolic syndrome [59], which have been well-established as complex metabolic diseases with substantial heterogeneity. It is therefore important to identify biomarkers that may enable earlier prediction and diagnosis of NAFLD and to provide efficient treatment and better management.

An ongoing research effort is attempting to identify biological targets and signals closely associated with NAFLD. Some studies have indicated that miRNAs may have a potential role in this hepatic chronic disease [60]. Indeed, several processes relevant to the development and progression of NAFLD were found to be related to miRNAs [61, 62]. For instance, miR-34 is upregulated in NAFLD and has the potential to be a biomarker for diagnosis of this disorder [63, 64]. Several attempts have been made to translate miRNAs findings to clinical practice. In this sense, Regulus Therapeutics and AstraZeneca have started the development of RG-125, a GalNAc-conjugated anti-miR targeting microRNA-103/107 for the treatment of NASH in pre-diabetes and T2DM patients [65]. Hence, these encouraging findings, combined with the ongoing progress in the field of ncRNAs research, are expected to yield new insights into the pathogenesis of NAFLD.

The circRNA family has also become a key area of focus for research in NAFLD. There is now increasing evidence linking circRNAs to the pathogenesis of NAFLD even though studies in this respect have only just begun. Previous reports have established that the expression of PPARα and associated signaling pathways are inhibited by PPAR1 in patients with NAFLD [66, 67] but the underlying mechanism is not clear. Regarding circRNAs and hepatic steatosis, Guo and colleagues [68] found that circRNA_0046367 was significantly decreased in high-fat-induced hepatic steatosis [68]. Subsequently, the authors demonstrated that the decrease in circRNA_0046367 expression led to miR-34a/PPARα interaction and lipid peroxidative damage, while circRNA_0046367 normalization by intrahepatic overexpression prevented this interaction and therefore reduced steatosis [68]. In a different study, the same authors identified another circRNA, circRNA_0046366, whose expression was also decreased during free fatty acid-induced hepatocellular steatosis and its upregulation abolished the miR-34a-dependent inhibition of PPARα signaling, leading to a marked reduction in triglyceride levels and suppression of hepatocytes steatosis [69]. These findings suggest that the circRNA_0046367/miR-34a/PPARα and circRNA_0046366/miR-34a/PPARα axes play an important role in the pathogenesis of NAFLD [68, 69]. As circRNA_0046367 and circRNA_0046366 have the same target, it would be of interest to examine whether or not these two circRNAs act in synergy and if their transcripts display significant sequence similarities. In another study, the same group used the same model of NAFLD to show

that circRNA_021412 is also associated with hepatic steatosis through the circRNA_021412/miR-1972/LPIN1 signaling cascade [70] (Table 10.1). Finally, in a recent study, Li et al. reported that the expression of circScd1 was significantly lower in NAFLD tissues than control groups whereas its over-expression promoted steatosis of NAFLD via JAK2/STAT5 signaling [71].

Together, the above pioneering studies suggest that circRNAs are potentially involved in NAFLD and have the potential to serve as useful tools for the development of diagnostic and interventional pharmacology. However, as mentioned earlier, circRNA data are still lacking functional evidence and their underlaying mechanisms are still awaiting elucidation. Therefore, further carefully designed prospective studies to emphasize and validate the potential use of circRNAs as NAFLD biomarkers are warranted.

10.2.3 Obesity

Obesity is another chronic metabolic disorder affecting adults and children in developed and developing countries [72]. Genetic predisposition, epigenetics, environment, and lifestyle preferences such as diet and low physical activity play crucial roles in excess body fat development and obesity [73, 74]. Obesity is known to be the main risk factor for several disorders including T2DM, cardiovascular disease, hypertension, coronary heart disease, and certain types of cancers [75, 76]. Due to the considerable impact of obesity on human health, it is therefore essential to develop new strategies with potential for early diagnosis and effective treatment.

While the involvement of miRNAs in the physiological processes of obesity has been closely studied [8, 77, 78], the role of circRNAs remains poorly elucidated. To the best of our knowledge, no groundbreaking studies have ever examined the potential link between circRNAs and obesity in humans. However, examination of the potential impact of circRNAs on diverse metabolic processes and a review of examples in the literature, suggest that circRNAs may play a role in the pathogenesis of obesity. For instance, based on the above studies revealing a significant association between circRNA expression and diabetes and NAFLD, and the fact that both are complications of obesity, it is conceivable that circRNAs may also contribute to the development of obesity. In addition, the antisense non-coding RNA in the INK4 locus (ANRIL), a complex gene with many reported linear and circular isoforms (circANRIL), is generated by the 9p21 locus has polymorphisms that have been associated with increased risk of developing cardiometabolic disease, including type 2 (obesity-related) diabetes and manifestations of atherosclerosis such as coronary artery disease [79–81]. Furthermore, a previous study by Murray et al. reported that lower level of CpG methylation within the promoter of ANRIL at birth is associated with increased cardiovascular risk [82] and adiposity [83] in later childhood. Carrara and colleagues hypothesized in their recent review that ANRIL could be a genomic site of environmental epigenetic influence on obesity [84]. An additional example that would argue in favor of a possible implication of circRNAs

in obesity was shown by Li et al. when they attempted to identify the potential circRNAs associated with adipogenesis and lipid metabolism [85]. The authors analyzed the expression profile of these RNA molecules in subcutaneous adipose tissues of large White pig and Laiwu pig using RNA sequencing technology and bioinformatic methods. Among the differentially expressed circRNAs, they identified circRNA_11897 as the most significantly downregulated while circRNA_26852 was the most significantly upregulated. Subsequent analysis revealed that subcutaneous miR-27a and miR-27b-3p are targets for circRNA_11897 and subcutaneous miR-874 and miR-486 are targets for circRNA_26852 [85]. These target genes are enriched in pathways associated with adipocyte differentiation and lipid metabolism. Since miR-874 and miR-486 were shown to be targets of circRNA_26852, the authors hypothesized that circRNA_26852 may play a role in adipogenic differentiation and lipid metabolism through these miRNAs [85]. On the other hand, since miR-27a is known to promote lipolysis [86] and inhibit adipocyte differentiation by targeting PPARγ [87], it is reasonable to assume that circRNA_11897, which binds miR-27a and miR-27b-3p and consequently provokes upregulation of their target genes, may be implicated in the regulation adipogenic differentiation and lipid metabolism. The fact that several miRNAs have been shown to be involved in the processes of adipogenesis and obesity [8, 88] and lipid metabolism [89, 90], and considering the existing regulatory link and the dynamic interplay between different circRNAs and miRNAs, it is possible to assume that circRNAs may also be part of the complex machinery that orchestrates the regulation of genes associated with obesity. Obesity has been reported to induce a decline in the activity and the amount of PPARγ [91] and an upregulation of miR-130b and miR-138 levels. Considering that miR-130b is known to target 3′-UTR and certain sequences within the coding region of PPARγ [92], while miR-138 indirectly inhibits the expression of PPARγ [93], it is possible that the obesity-associated decline in PPARγ expression may be due to a decline in the expression of yet unknown circRNAs, that normally act as miRNA sponges to target miR-130b. There are reports in the literature that may support this scenario. In a previous study, Deng et al. observed that miR-548 can be regulated by the PPARγ gene, a heart-protective factor shown to be downregulated in acute myocardial infarction (AMI) [94]. Subsequently, when Deng et al. explored the expression profile of circRNAs comparing plasma expression of circRNAs in AMI patients with healthy volunteers, they identified circRNA_081881, which contained seven competitive binding sites for miR-548 as the most significantly downregulated circRNA in AMI. The authors concluded that circRNA_081881 may regulate PPARγ expression by functioning as a competing endogenous RNA (ceRNA) of miR-548 [94].

Collectively, these hypotheses and speculative scenarios are proposed for the purpose of serving as basic framework for further understanding of circRNAs in obesity and providing investigators with potential research directions that may be used for generating new hypotheses for further studies on circRNAs. Finally, as circRNAs research continues, it is expected that new information on the role of these molecules will arise in the field of metabolic diseases. It is hoped that this information will bring evidence for the potential role of circRNAs in metabolic diseases.

10.3 Conclusions and Future Perspectives

Even though circRNAs are increasingly being recognized to play critical regulatory roles in the development of metabolic diseases, the lack of their large exploration and characterization may delay their consideration for clinical settings. In this respect, many concerns are left for their potential future studies. The analytical approaches used in the identification and prediction of circRNAs are still part of a relatively new field of investigation, thus, their sensitivity and specificity require improvement. Furthermore, lack of prospective studies, poor study design and complicated statistical analyses could impede the translation of circRNA results to preclinical and clinical trials, thus, limiting the success of prospective biomarkers. The complex interplay of circRNAs with networks involving transcription factors, mRNA, miRNAs, RBPs and metabolic pathways makes it difficult to evaluate the functions of these RNA molecules under complex metabolic diseases. It should also be noted that circRNAs as transcriptional and posttranscriptional regulators themselves undergo extensive regulation from their biogenesis to the effects that they exert on their target molecules and pathways. Therefore, interpretation of such complex data could be enhanced by deploying systems biology approaches to refine our understanding of circRNAs dynamic and provide insights into their potential regulatory circuits in metabolic disorders. Another limitation that may generate huge incoherencies in circRNA results within a group of patients with metabolic diseases is drug use and other treatment modalities not taken in consideration. Using the example of miRNAs, previous studies reported that statins [95, 96], anticoagulation [97], and antiplatelet drugs [98] can affect quantification of these RNAs in blood samples and therefore should be taken in account. Regarding the patients with metabolic disease, thiazolidinedione drugs are frequently used for patients with impaired fasting glucose tolerance while abdominal obesity can be treated with a variety of lower calorie diets along with regular exercise [99]. Hence, drugs as well as confounding parameters should be also taken in account when examining circRNAs in patients with diabetes, NAFLD, obesity, and metabolic syndrome, as these may impact the disease through these RNA species. Metabolic diseases represent a cluster of disorders such as T2DM, insulin resistance, metabolic syndrome, NAFLD and hypercholesterolemia, which could be linked by numerous metabolic pathways. The interplay between these clinical situations is challenging. Thus, although each of these disorders has different physiological and clinical symptoms, it would be important to identify a signature or set of markers including circRNAs, shared by all disorders constituting metabolic diseases. This idea proses that, rather than relying on a single circRNA biomarker for disease diagnosis, one can use a group of disease-relevant biomarkers which will likely be more accurate and efficient in predicting a complex phenotype. Another type of circRNA that has not been well explored in metabolic diseases is circRNA found within exosomes (exo-circRNAs). The presence of abundant circRNAs within exosomes was firstly reported by Li and colleagues [100] and a web-accessible database (http://www.exoRBase.org), exoRBase, a resource containing all available long RNAs (circRNA, lncRNA and mRNA) derived from RNA-seq data of human blood exosomes, has been recently constructed [101].

With respect to metabolic diseases, many questions remain uncertain. For instance, what is the role of exo-circRNAs in metabolic disorders? What is their origin? Are they horizontally transferred via exosome vehicles to recipient cells as in the case of mRNAs [102]? In studies of cancer, Li et al. [100] observed that the abundance of tumor-derived exo-circRNAs in the serum of patients with colorectal cancer was correlated with tumor mass. They also found that the expression profile of exo-circRNAs in cancer serum was significantly different from that in normal serum. More importantly, a recent study revealed that treatment of lean mice with exosomes isolated from obese mice induced glucose intolerance and insulin resistance in mice [103]. Hence, future studies should aim for answering these questions in order to understand the origin, mode of secretion, target cells and organs of exo-circRNAs. This knowledge may help us to gain more insights into the function of circRNAs in the field of metabolic diseases. With respect to the epigenetic regulation of complex metabolic diseases by ncRNAs, there are only a few published data associating the dysregulation of circRNAs with genes involved diabetes and NAFLD, as mentioned above. Unfortunately, no data are yet available on the potential implication of circRNAs in obesity and metabolic syndrome. Likewise, no reports are available on the potential link between circRNAs and the chronic low-grade inflammation associated with diabetes, obesity and the metabolic syndrome apart from one report indicating an association between circANKRD36 and inflammation in patients with T2DM [104]. All of these pertinent questions represent important issues that must be solved in future investigative attempts to fully understand the role of circRNAs in the pathogenesis of metabolic diseases.

In summary, although the existing studies support a possible association between circRNA molecules and metabolic diseases, it is too early to consider and develop these molecules as sensors and biomarkers for metabolic disorders as claimed by existing reports. Further research in this area is worthwhile and new powerful strategies should be employed to uncover the full biological relevance of circRNAs and their potential therapeutic applications.

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Chapter 11
Deciphering Endothelial Dysfunction in the HIV-Infected Population

Genevieve Mezoh and Nigel J. Crowther

Abstract Cardiovascular disease (CVD), as a possible consequence of endothelial dysfunction, is prevalent among HIV-infected patients despite successful administration of antiretroviral drugs. This warrants the routine clinical assessment of endothelial function in HIV-positive patients to circumvent potential CVD events. Several different non-invasive strategies have been employed to assess endothelial function in clinical research studies yielding inconsistencies among these reports. This review summarises the different techniques used for assessing endothelial function, with a focus on proposed blood-based biomarkers, such as endothelial leukocyte adhesion molecule-1 (E-selectin), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWF), TNF-α, interleukin 6 (IL6) and soluble thrombomodulin (sTM). The identification of suitable blood-based biomarkers, especially those that can be measured using a point-of-care device, would be more applicable in under-resourced countries where the prevalence of HIV is high.

Keywords Endothelial dysfunction · HIV · Biomarkers · Inflammation · Cardiovascular disease

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11.1 Introduction

There are currently more than 36 million people in the world living with HIV, with sub-Saharan Africa accounting for up to 53% of this population [1].The introduction of highly active antiretroviral therapy (HAART) has increased the life expectancy of HIV-positive patients, and presently, cardiovascular disease (CVD) has emerged as an important cause of mortality among infected individuals. Despite effective antiretroviral therapy, HIV-positive patients have been observed to have a higher risk of myocardial infarction and cardiovascular death, than age-matched uninfected controls [2, 3]. An increased prevalence of traditional risk factors such as dyslipidaemia and smoking, among individuals infected with HIV, is thought to contribute to this increased cardiovascular morbidity. However, growing evidence suggests that HIV infection and treatment may lead to endothelial dysfunction, likely through inflammation and immune dysregulation [4, 5].

The endothelium is constantly in contact with foreign particles that circulate within the bloodstream [6], and is one of the first points of contact between tissues and circulating blood-bourne pathogens. Therefore, the endothelium is subjected to immune system defence strategies that can lead to endothelial damage [7]. This in turn can initiate repair mechanisms that lead to a cascade of events that may culminate in further vascular damage and plaque formation [8, 9]. This review seeks to explore the association between HIV infection and endothelial dysfunction, with an emphasis on the serum biomarkers that have been used to assess the level of endothelial dysfunction in such disease states.

11.2 Endothelial Dysfunction

The endothelium constitutes a single layer of cells that runs throughout the body internally lining the walls (intima) of the blood vessels. It is therefore the principal barrier separating blood from tissues. The endothelial cells secrete a number of substances that confer upon the endothelium its ability to regulate numerous blood vessel functions including vascular tone, cell adhesiveness, and coagulation [10]. An imbalance in the levels of these mediators can result in endothelial dysfunction [11]. This systemic pathological state of the endothelium is the earliest clinically detectable stage of cardiovascular disease [7].

Cytokines are soluble proteins secreted by a variety of cells, mainly leukocytes, as well as endothelial, stromal and mast cells, in response to foreign entities [12]. They therefore play a vital role in the regulation of innate and adaptive immunity, hematopoiesis and lymphocyte development [13]. Endothelial cells express transmembrane adhesion proteins such as endothelial leukocyte adhesion molecule-1 (E-selectin), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The expression of these adhesion molecules by the endothelium is increased in response to proinflammatory cytokines such as tumour

necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [14] and in response to turbulent blood flow. These factors increase the expression of endothelial adhesion molecules by activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [15]. This phenomenon, referred to as endothelial cell activation, enables the transmigration of monocytes across the endothelium, which is a primary factor in the initiation of atherosclerotic plaque formation [14, 16].

Endothelial dysfunction is defined as the decreased bioavailability of nitric oxide (NO) derived from the endothelium, and is caused by a number of factors including hypercholesterolaemia, smoking and oxidative stress [16]. The synthesis of NO in endothelial cells is catalysed by the enzyme endothelial nitric oxide synthetase (eNOS), which is activated by shear stress brought about by the continuous pulsatile flow of blood [17]. Endogenous NO has several functions which include inhibition of white cell and platelet activation, inhibition of vascular smooth muscle cell proliferation, smooth muscle relaxation and vasodilation [18]. Levels of NO are reduced by its interaction with superoxide, which is produced from oxygen by the action of NADPH oxidase [19]. Failure of the endothelium to respond to shear stress would therefore lead to a decrease in the endothelium-derived NO with a concomitant build-up of superoxide which further decreases levels of NO. Given the vital role NO plays in smooth muscle relaxation, a prerequisite for vasodilation, a decrease in NO therefore leads to vasoconstriction [19, 20]. A decrease in the bioavailability of NO also leads to greater endothelial activation which results in increased platelet aggregation, vascular smooth muscle cell proliferation, greater monocyte adhesion and transmigration and oxidative stress, which promote atherosclerosis [8].

Post-mortem studies show that the atherosclerotic process starts in individuals as early as childhood, and by the early third decade of life, 20% will have advanced lesions present [21, 22]. Core factors known to influence endothelial function include age [20], gender [23], family history of diabetes and CVD [24], physical activity [25], insulin resistance [26], hyperglycaemia [27], active and passive smoking [28], visceral fat [29], obesity [26], hypertension [30] and dyslipidaemia [31]. Infection with HIV has been proposed as a further risk factor for endothelial dysfunction [32, 33] (Fig. 11.1).

The endothelium is exposed to shear stress induced by turbulent blood flow, which is common in areas where the vasculature branches, and are sites at which atherosclerotic plaques are often formed [34]. Endothelial damage at such sites initiates a repair process, which involves the localization of circulating cells originating from the bone-marrow. These cells include circulating angiogenic cells (CACs) and endothelial progenitor cells (EPCs). Endothelial damage equally results in the release of circulating endothelial cells (CECs) and endothelial micro-particles (EMPs) into the blood circulation [35]. As a consequence, CACs are recruited to the site of endothelial damage for vascular repair [36]. Angiogenic cytokines are produced from the site of endothelial damage, and induce movement of EPCs from the bone marrow, to the damaged endothelium, where they are integrated into the sites of active angiogenesis [15, 37]. The release of EPCs from the bone marrow is modulated by the activation of matrix metalloproteinase (MMP)-9, in a mechanism

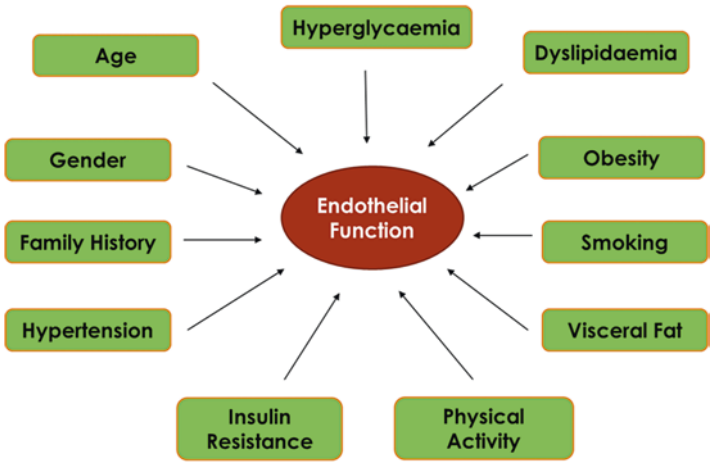

Fig. 11.1 Main factors that affect endothelial function. Several factors are known to have an impact on the endothelial cell state. This includes hyperglycaemia, dyslipidaemia, obesity, smoking, visceral fat, physical activity, insulin resistance, hypertension, family history of endothelial dysfunction, gender and age

coordinated by the bioavailability of NO [38, 39]. Therefore, reduction in the levels of NO, as observed during endothelial dysfunction, hinders recruitment of EPCs to the site of endothelial damage [38]. A recent study has further shown that in an animal model of atherosclerosis, treatment with EPCs reduces the level of atherosclerosis [40].

The formation of an atherosclerotic plaque can therefore be seen as a complex process, involving both endothelial activation and dysfunction. Reduced production and/or activity of endothelial-derived NO appear to be central to this process, but the molecular pathways involved are only partially understood. In addition, recruitment of EPCs to the site of endothelial damage seems to be essential to the attenuation of plaque formation. Many factors can initiate endothelial dysfunction and activation, and HIV infection may be one such agent.

11.3 HIV Infection and Endothelial Dysfunction

Infection with HIV has been associated with the development of CVD [2, 3], which may occur via the induction of endothelial dysfunction brought on either by the initiation of an inflammatory response or by the direct action of the HIV proteins Nef [42] and gp120 [42]. In vitro and in vivo studies conducted using human microvascular endothelial cells and C57BL/6 rats respectively, showed that adhesion of leukocytes to the endothelium was selectively activated by the synergistic action of HIV-1 Tat and TNF-α. In a study conducted by Dhawan and colleagues [44] using human umbilical vein endothelial cells, HIV-1 Tat was found to induce expression of E-selectin, ICAM-1 and VCAM-1. Research conducted by Wang et al. [41] using

human coronary arterial endothelial cells (HCAECs), and Indian rhesus macaque heart tissue, showed that HIV-1 Nef contributes to endothelial dysfunction via two distinct pathways; an NADPH oxidase-dependent mechanism resulting in the apoptosis of endothelial cells, and by the expression of monocyte chemoattractant protein-1 (MCP-1) through the NF-κB signalling pathway. In addition, gp120 with TNF-α pre-treatment was found to cause endothelial dysfunction by downregulating the expression of endothelial nitric oxide synthase and upregulating ICAM-1 expression in HCAECs [43]. Hence, research conducted thus far shows that the circulating viral proteins act individually via different mechanisms to induce endothelial dysfunction, with HIV Tat and gp120 acting in synergy with TNF-α, and HIV Nef being secreted in vesicles and entering the endothelial cells by cell-to-cell transfer [41] (Fig. 11.2).

Elevated plasma levels of endothelial cell derived markers such as endothelial leukocyte adhesion molecule-1 (E-selectin), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWF), and the inflammatory cytokines TNF-α, interleukin 6 (IL6) and interleukin 8 (IL8), have all been observed in HIV-infected individuals with elevated CVD risk factors (Table 11.1). In addition, an association between

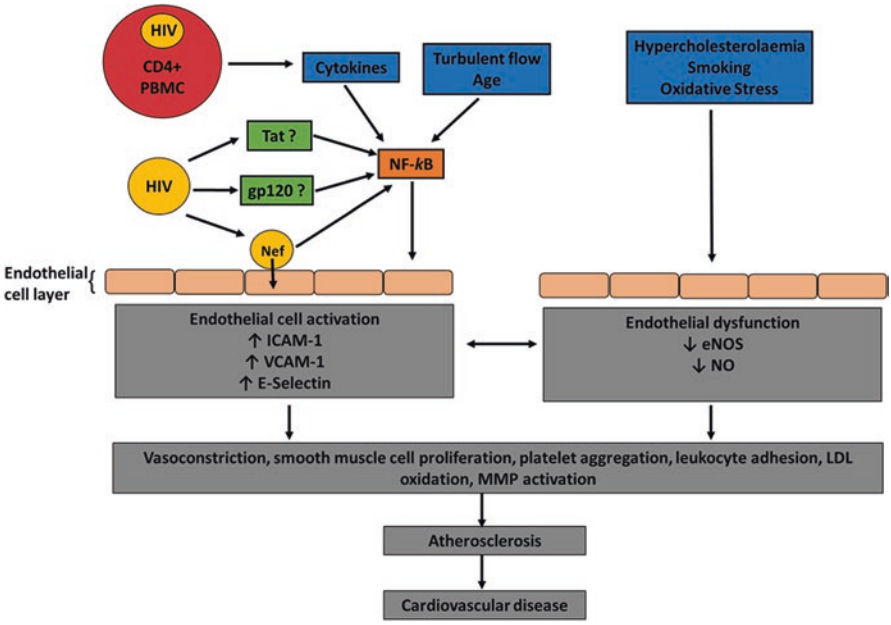

Fig. 11.2 Schematic illustration of the potential mechanisms behind CVD in the HIV-infected population. HIV-infected individuals are more predisposed to endothelial dysfunction. This occurs not only as a result of inducers such as hypocholesterolaemia, smoking, oxidative stress, turbulent blood flow and age, but also as a result of immune activation upon HIV infection and direct interaction between HIV viral proteins such as Nef, Tat and gp120 with endothelial cells. (Adapted from Liao [16])

Table 11.1 Studies comparing markers of endothelial dysfunction in HIV-infected and non-infected subjects

Authors	Study populations	Effectors and markers of endothelial function analysed	Results of comparison of cases versus controls	Associations
[72]	106 HIV-infected children, of which 86% were on HAART, against 55 normal control children aged 12–15 years	CRP IL6 MCP-1 sICAM-1 sVCAM-1 E-Selectin	Increase Increase Increase Increase Increase Increase	Correlation between all endothelial markers with higher waist:hip ratio, low CD4 counts and high viral load.
[148]	83 HIV-infected children on HAART against 83 uninfected healthy children aged 7–13 years	CIMT CRP	Increase Increase	Positive association between HIV infection with CIMT and elevated CVD risk factors. Negative association between CIMT with total cholesterol and HDL-C
[146]	A cross-sectional study of 39 HIV-infected ARV-naïve patients aged 29–45 years compared with three different groups; (i) 26 age-matched (27–37 years) and (ii) 26 elderly (64–81 years) uninfected healthy controls; and (iii) 26 non-survivor elderly subjects (74–87 years).	CRP TNF-α INF-γ IL-1β IL-6 IL-8 IL-17	Increase Increase Increase Below detection Increase Increase Below detection	No association between any markers of inflammation and HAART.
	A longitudinal study of 39 HIV-infected ARV-naïve patients at baseline, and after 48 and 96 weeks of ARV administration.	CRP TNF-α INF-γ IL-1β IL-6 IL-8 IL-17	No change Decrease Decrease Below detection Decrease Decrease Below detection	
[115]	494 HIV-infected individuals, of which 82.4% were on HAART, compared with 5386 healthy controls aged 45–76 years; and 287 HIV-infected individuals, of which 72.5% were on HAART, compared with 3231 participants aged 33–44 years.	CRP IL-6	Increase Increase	Positive association between endothelial markers and HIV infection. No association between levels of endothelial markers and HAART.

(continued)

Table 11.1 (continued)

Authors	Study populations	Effectors and markers of endothelial function analysed	Results of comparison of cases versus controls	Associations
[46]	300 ARV-naïve HIV-infected participants compared with 300 uninfected controls aged 36–52 years.	IL-6 CRP sICAM-1 sVCAM-1 Carotid radialis pulse wave velocity (crPWV)	Increase Increase Increase Increase No difference	Positive association between crPWV and age in the HIV-infected group. Positive association of HIV-infection with levels of ICAM-1 and VCAM-1.
[145]	66 HIV-positive subjects on HAART compared with 165 HIV-negative controls.	IL-6 CRP sICAM-1 sVCAM-1 crPWV	No difference No difference Increase Increase No difference	Positive association between HIV-infection with ICAM-1 and VCAM-1. Inverse association between VCAM-1 with CD4 count.
[99]	90 HIV-positive subjects on HAART versus 90 uninfected controls	vWF sTM E-selectin TNF-α IFN-α	Increase Increase Increase Increase Increase	Negative correlation between sTM and CD4+ cell count. Positive correlation of vWF with TNF-α and IFN-α Higher level of markers of endothelial dysfunction in HIV-positive subjects.
[149]	112 HIV-infected adults on long-term HAART, 35–45 years of age, compared with 84 HIV-uninfected controls aged 33–43 years.	IL-6 sICAM-1 sVCAM-1 E–selectin CIMT	No difference No difference Increase No difference No difference	Positive correlation between HIV infection with increased levels of VCAM–1 but not ICAM–1 or E–selectin. No association of IL-6 with markers of endothelial dysfunction.

markers of inflammation such as C-reactive protein (CRP) and TNF-α with CVD in the HIV-positive population has also been reported [45, 46].

Although traditional risk factors such as hypercholesterolemia, smoking, oxidative stress and age cause endothelial dysfunction, HIV-infected individuals have an additional increased risk of endothelial dysfunction due to the action of HIV (Fig. 11.2). Invasion of cells of the immune system by HIV initiates immune responses that involve an upregulation of cytokine production [47]. In addition, studies have shown immune reconstitution inflammatory syndrome (IRIS) to occur upon ARV administration, characterised by excessive proinflammatory cytokines

[48]. Cytokines, as well as age and turbulent blood flow are factors known to activate the NF-κB pathway resulting in endothelial cell activation, which subsequently leads to endothelial dysfunction [16]. The HIV viral protein, Nef, is suggested to cause endothelial dysfunction via activation of the NF-κB pathway within endothelial cells [41]. Endothelial activation and dysfunction put into motion a series of events which include vasoconstriction, smooth muscle cell proliferation, platelet aggregation, leukocyte adhesion, LDL oxidation and matrix metalloproteinase (MMP) activation, which initiates atherogenesis, and as consequence, the development of CVD.

11.4 Assessment of Endothelial Function

There are several methods by which endothelial function can be assessed, which can be either invasive or non-invasive (Table 11.2) [49]. Non-invasive strategies are more applicable in terms of cost, patient compliance and are easier to conduct compared to invasive strategies [49, 50], and therefore, only these methods will be described.

The quantification of cells involved in the process of endothelial repair, such as the endothelial micro-particles (EMPs) and endothelial progenitor cells (EPCs), by flow cytometry, has been used as a strategy to investigate endothelial dysfunction [51]. However, this technique is limited by the fact that the circulating EPCs are present in low numbers, and there is no specific marker currently available that can be used for the identification of EPCs [52]. To address this problem, a combination of markers has been utilised to measure EPC numbers [51, 53]. Researchers have also proposed the use of CECs and EMPs as prognostic markers in evaluating atherosclerotic vascular disease [54]. However, there are several drawbacks to the use of these flow cytometric methods for the assessment of endothelial dysfunction such as the requirement of highly specialised equipment, trained personnel, and low turnaround times.

Another non-invasive strategy is the measurement of flow-mediated dilation (FMD). This involves the ultrasound measurement of the diameter of the brachial arteries following a brief period of occlusion and reflow in which reactive hyperaemia is induced. Reduction of endothelial function as demonstrated by a decrease in flow-mediated vasodilation of the brachial artery has been observed in HIV-infected subjects [32, 55]. However, this procedure is not subject to automation, is time consuming and requires the availability of specialised equipment and skilled personnel. In addition, significant variation has been observed in day-to-day measurements, most likely due to changes in the biological circadian rhythms [50].

A more recent non-invasive approach is the plethysmographic recordings obtained from the fingertip using the EndoPAT (endothelial peripheral arterial tonometry) device [56, 57]. The finger arterial pulse wave amplitude is recorded at rest, following which the forearm is cuffed for 5 min to induce reactive hyperaemia, and plethysmographic readings taken in 30 s intervals for 4–5 mins following occlu-

Table 11.2 Methods used for the assessment of endothelial function

	Technique	Principle	Reference
Invasive strategies	Quantitative coronary angiography	Infusion of vasodilator (e.g. acetylcholine, bradykinin or serotonin) followed by measurement of changes in vascular diameter. Patients with good endothelial function show a vasodilatory response whereas either vasoconstriction or no change in vascular diameter is observed in those with endothelial dysfunction	[11, 139]
	Intracoronary doppler	Infusion of an endothelium-dependent vasodilator (such as acetylcholine) and direct dilator of vascular smooth muscle (such as papaverin) via a Doppler catheter followed by measurement of intracoronary blood flow velocity and cross-sectional arterial area	[140]
	Strain-gauge venous impedance plethysmography	Direct brachial artery administration of agonists of vasodilation in the forearm, followed by measurement of change in blood flow	[141, 142]
Non-invasive strategies	Brachial artery ultrasound (flow mediated vasodilation)	Brachial artery diameter measured using high-resolution ultrasound transducer before and after brief occlusion. Reflow after occlusion results in raised shear stress that causes endothelial dependent dilation in patients with healthy endothelial function. This function is impaired in patients with endothelial dysfunction	[23, 143]
	Peripheral arterial tonometry (EndoPAT)	Arterial blood volume measured in fingertip using plethysmograph before and after a brief period of occlusion. Reflow results in arterial volume changes which increases the measured signal	[56, 144]
	Immunoassays including enzyme-linked immunosorbent assays (ELISAs)	Formation of an antibody-antigen complex, with antigen concentration determined via the quantification of light emitted by a conjugated fluorescent protein (antibody orantigen)	[145, 146]
	Flow cytometry	Quantification of forward and scattered-light emitted by labelled cells	[51, 147]
	Immuno-magnetic separation	Antibody-antigen binding of CECs (similar to ELISA) coupled with fluorimetry for quantification of the selected autofluorescent CECs	[126, 132]

sion. Advantages of the EndoPAT over FMD include the fact that it is easier to perform, less variability in day-to-day readings and higher sensitivity [49]. However, this technique is more expensive compared to FMD as it requires the use of disposable finger probes. Therefore, as with FMD, this approach may not be easily adopted in resource-limited countries. Moreover, EndoPAT readings have been associated with several traditional and metabolic cardiovascular risk factors, thus, non-endothelial factors can influence the signal [58, 59].

Ultrasound measurement of the thickness of the two inner layers of the carotid artery (the intima and the media), referred to as carotid-intima media thickness (CIMT), has been proposed as a non-invasive strategy to assess endothelial dysfunction as it provides a quantitative measure of sub-clinical atherosclerosis. Several studies report a positive correlation to exist between CIMT and CVD risk [60, 61]. However, a lack of correlation between CIMT and markers of endothelial function has been observed [62, 63].

The use of circulating plasma markers for the assessment of endothelial function in HIV-infected patients would be a more practical and sustainable approach in developing countries. Given that this technique uses immunoassays to analyse the level of targeted analytes in blood samples, it can easily be applied to automated systems, thereby enabling the processing of hundreds of samples with a short turn-around time. However, this technique may lack specificity in that some of these markers are elevated under conditions not associated with endothelial dysfunction [64, 65], and do not necessarily originate from endothelial cells [66]. This problem may be partially overcome by measuring multiple blood markers. The following section of this review will discuss each of these blood markers of endothelial function in more detail.

11.5 Blood-Based Markers of Endothelial Function

11.5.1 Adhesion Molecules

The ICAM-1 and VCAM-1 molecules are trans-membrane proteins belonging to the immunoglobulin superfamily often found on the surface of leukocytes and endothelial cells [66, 67]. Though attached to the membrane, they also exist in a soluble form in biological fluids [66, 68]. These adhesion proteins are similar in structure and function. They play a major role in cell-cell adhesion by stabilizing cell-cell interactions [69], facilitate the migration of leukocytes across the endothelium, and are expressed upon cytokine stimulation [67]. Thus, cytokines such as TNF-α and IL-6 activate the signal transducers and activators of transcription (STATs) signalling pathway, and the STATs bind to NF-κB in the ICAM-1 promotor leading to upregulation of expression [70]. Reactive oxygen species (ROS) are required for the binding of STATs to NF-κB [71]. Therefore, increases in the levels of ROS as a result of endothelial dysfunction would result in further increased levels of ICAM-1. Increased expression of ICAM-1 on the endothelial cells results in the recruitment of inflammatory immune cells [72, 73].

In addition, studies show ICAM-1 to be the entry point of viruses including the human rhinoviruses [74, 75], and the malarial parasite Plasmodium falciparum [76, 77]. The ICAM-1 and VCAM-1 molecules are not simultaneously expressed in all diseases involving an inflammatory process [78]. Thus, a study conducted by Mason et al. showed the levels of sICAM-1 and sVCAM-1 to be significantly higher in

patients with rheumatoid arthritis compared to healthy controls, however, in patients with lupus erythematosus, only sVCAM-1 was elevated and not sICAM-1 [79]. Therefore, different inflammatory diseases may be associated with specific adhesion or inflammatory molecules.

Elevated levels of ICAM-1 and VCAM-1 in blood would imply an increase in their expression on the cell surface, and thus, an increase in the transmigration of leukocytes across the endothelial cells. This increase in movement across the endothelium results in the build-up of pressure at the surface of the endothelial cells [80]. Eventually, endothelial shedding will occur, resulting in the detachment of the endothelial trans-membrane proteins and their shedding into the blood circulation [15].

Blood flow through the blood vessels is generally laminar. However, obstruction to blood flow as a result of atherosclerotic plaques generates rhythmic patterns. In vitro studies show that laminar and oscillatory shear stress upregulates ICAM-1 expression [81, 82]. However, up-regulation of VCAM-1 is either to a much lesser extent compared to ICAM-1 or non-existent [80, 83], and prolonged exposure to shear stress appears to down-regulate both adhesion molecules [81, 84].

Endothelial leukocyte adhesion molecule-1, often referred to as E-selectin or ELAM-1, is similar to VCAM-1 and ICAM-1 in that it is also a trans-membrane adhesion molecule that facilitates cell-cell interaction, and the transmigration of leukocytes into tissues following cytokine activation. However, E-selectin differs from VCAM-1 and ICAM-1 in that it is solely expressed on endothelial cells. In addition, it promotes the adhesion of resting CD4+ memory cells to endothelial cells [81, 85]. This adhesion molecule could therefore be regarded as a plasma marker of choice for the assessment of endothelial function given that it is endothelium specific. However, continuous blood flow and influx of leukocytes to inflamed tissues results in shear stress, which has been shown to inhibit E-selectin expression in vitro [81, 86]. Levels of sICAM-1, sVCAM-1 and E-selectin have been found to be elevated in other conditions not associated to endothelial dysfunction such as multiple sclerosis [87] and cancer [88, 89]. Interestingly, sICAM-1 and sVCAM-1 levels have been shown to be influenced by other factors such as ethnicity [90] and high-density lipoprotein cholesterol (HDL-C) [91, 92]. The latter down regulates expression of the adhesion molecules and upregulates endothelial cell migration and proliferation [92].

11.5.2 von Willebrand Factor (vWF)

von Willebrand factor (vWF) is a glycoprotein present in blood plasma that plays a role in haemostasis [93]. It is synthesized by endothelial cells [94] as well as megakaryocytes [95]. It is thought to be an excellent blood-based marker for the assessment of endothelial function, with several studies demonstrating a strong inverse correlation between vWF and FMD [96, 97]. Thus, a study conducted by Felmeden et al. [96] in which 89 hypertensive subjects were compared against 43 healthy subjects, before and

after 6 months of cardiovascular risk management, revealed a significant ($r = -0.517$, $P < 0.001$) inverse correlation between vWF and FMD. Following 6 months of antihypertensive treatment and intensive cardiovascular risk factor management, vWF levels decreased with a simultaneous increase in FMD. Moreover, both vWF and FMD were found to correlate significantly with 10-year cardiovascular risk. In contrast, another study in which 32 obese children were compared against 20 healthy controls with regards to measurements of E-selectin, vWF, thrombomodulin, FMD and carotid intima-media thickness (CIMT), no association was found to exist between FMD and plasma endothelial markers [98]. Within the context of HIV infection, a study performed by Seigneur et al. involving 90 HIV-positive subjects versus controls revealed the HIV-positive cohort to have higher levels of vWF, sTM (soluble thrombomodulin), E-selectin, TNF-α and IFN-α compared to the HIV-negative cohort [99]. Levels of vWF correlated positively with TNF-α and IFN-α while sTM correlated negatively with CD4+ cell count.

11.5.3 Thrombomodulin

Similar to E-selectin, thrombomodulin (TM) is a trans-membrane protein specifically expressed on the surface of endothelial cells [100]. However, it differs in its function in that it acts as a protein C co-factor and possesses anti-coagulant activity [100]. Thrombomodulin has been found to exist in a soluble form within the blood circulation, thus, easily quantified by ELISA [101, 102]. Clinical studies revealed a positive association between levels of sTM and endothelial damage, thereby warranting its use as a plasma marker of endothelial damage [101, 103, 104]. However, a positive correlation between plasma levels of sTM with CECs, but not with E-selectin, was observed by Strijbos et al. [105]. On the other hand, a strong negative correlation has been reported to exist between levels of sTM with CD4+ cell count [99]. The authors suggested increased levels of sTM to be a true representative of endothelial dysfunction, as this marker was found to be elevated only in patients with more severe HIV infection, whereas vWF is elevated largely as a result of an inflammatory stimulus [99]. Moreover, results originating from the measurement of sTM to assess endothelial function could be more reliable, as plasma level of sTM is not generally influenced by age. However, women within the age range of menopause are observed to have higher levels of sTM compared to younger women [106].

11.5.4 Markers of Inflammation

C-reactive protein (CRP) is a pentameric protein found in plasma and largely synthesised by hepatocytes in response to acute inflammation [107, 108]. However, expression of CRP in other cells such as neurons, monocytes, lymphocytes and foam cells have been reported, though, most likely at insignificant plasma levels [109, 110]. The

C-reactive protein stimulates phagocytosis by activating the complement pathway, and binds to immunoglobulin receptors, thereby playing a role in innate immunity as a defence mechanism against infections [108]. Expression of CRP is principally induced by the inflammatory cytokine, interleukin-6 (IL-6), which can be enhanced by interleukin-1 (IL-1) [111]. C-reactive protein has gained acknowledgement as an independent marker of inflammation and can be used in the clinical evaluation of endothelial function and CVD risk [112, 113]. The C-reactive protein has been observed to be high in HIV-positive patients, despite long periods of HAART administration [114, 115]. C-reactive protein can decrease endothelial nitric oxide synthase mRNA [7], and upregulate the adhesion molecules, ICAM-1 and VCAM-1, and chemoattractant chemokines such as monocyte chemoattractant protein 1 (MCP-1) [50, 116]. In addition, in vivo and in vitro studies have shown that CRP upregulates angiotensin type 1 receptor in vascular smooth muscle cells, thereby increasing proliferation and migration of these cells, as well as increasing restenosis and the production of reactive oxygen species [117]. These mechanisms would ultimately lead to a decrease in NO levels, and as consequence, endothelial dysfunction.

Interleukin-6 is a soluble protein secreted by a variety of cells such as monocytes, macrophages, fibroblasts, vascular smooth muscle cells, stromal cells and endothelial cells [118, 119]. It acts not only as a pro-inflammatory cytokine, but also has anti-inflammatory effects, mediated by its ability to inhibit TNF-α and IL-1, and activate the IL-1 receptor antagonist (IL-1Ra) and IL-10 [118]. Interleukin-6 activates the NF-κB pathway, resulting in the expression of ICAM-1, VCAM-1 and E-selectin, and inhibits NO production by downregulating eNOS expression [120]. Interleukin-6 also plays a role in the development of atherosclerotic plaques. Thus, after migration of monocytes across the endothelium, these cells develop into foam cells as they absorb modified lipoproteins, and secrete IL-6 and TNF-α. Both of these cytokines further mobilize immune cells, leading to the progressive development of the atherosclerotic plaque [121].

Interleukin-8 (IL-8) is a cytokine produced not only by macrophages, but also by other types of cells, such as epithelial, smooth muscle and endothelial cells [119, 122]. Interleukin-8 also plays a role in atherogenesis, however, to a much lesser extent when compared to IL-6 [119].

Tumour necrosis factor alpha (TNF-α), is a trans-membrane cell signalling protein primarily expressed by activated macrophages in response to acute systemic inflammation [123]. During inflammation, TNF-α and IL-1 are released by the macrophages in the inflamed tissue, which in turn induces overexpression of the adhesion molecules, VCAM-1, ICAM-1 and E-selectin [124, 125]. In a study performed in HIV-infected subjects, TNF-α was found to correlate strongly with levels of vWF [99].

11.5.5 Circulating Cells

Studies conducted by da Silva et al. [51] demonstrated FMD and the systemic levels of EPCs to be lower, and EMPs to be higher, in HIV-infected ARV-naïve individuals when compared to healthy controls. A report from López et al. revealed the numbers

of EPCs to be significantly lower, and CACs to be significantly higher, in HIV-positive ARV-naïve subjects versus controls [126]. The authors hypothesized that direct infection of EPCs by HIV could account for the reduced levels of EPCs in HIV-positive patients, as these cells may possess the chemokine receptors (CCR5 and CXCR4) that are used by HIV to infiltrate host cells [127]. This is evident by the observation that administration of HAART to HIV-positive patients fully restores the level of EPCs [128]. This observation was confirmed by Costiniuk et al. [129], who observed reduced levels of EPCs in HIV-infected versus HIV-negative controls, in a study conducted with 30 ARV-naïve HIV-infected men against 36 HIV-uninfected men. However, no correlation was found to exist between EPC levels and CD4+ cell count or viral load [129]. In contrast, a cross-sectional and a longitudinal study conducted with 50 and 66 chronic HIV-infected subjects respectively, in which both cohorts were receiving ARVs, showed EPC levels in HIV-infected subjects to be significantly higher compared to negative controls [128]. Moreover, a correlation was found to exist between EPC numbers and CD4+ cell count, but no association could be seen between EPC numbers or change in EPC numbers over time with CIMT measurements.

Circulating angiogenic cell (CAC) levels and their migration to the site of endothelial damage have been shown to correlate with endothelial function [130, 131]. However, levels of CACs can be influenced by factors such as age [130] and dyslipidemia [132]. To date, no study has been conducted to assess levels of CACs in HIV-infected patients under conditions of continuous viral replication or suppressed viremia.

Studies have revealed a significant positive correlation between levels of CECs with vWF levels [132, 133], and a significant negative correlation ($r = -0.423$, $P = 0.002$) between levels of CECs and FMD measurements. Also, HIV-infected ARV-naïve patients are reported to possess higher EMP levels [51] and CEC levels [126] compared to healthy individuals. These data therefore suggest that HIV infection results in significant endothelial injury. However, to date, there are no studies to show if controlled viremia on administration of HAART reduces levels of EMPs and CECs.

11.5.6 Metabolites of Endothelial Nitric Oxide Synthetase

As previously discussed, the enzyme, eNOS, plays a vital role in maintaining the body's vasculature in a healthy state. Endothelial dysfunction is characterised by a decrease in eNOS with the concomitant decrease in NO and increase in ROS. An increase in ROS results in the increase oxidation of NO to NO_2^-, which is further oxidised to NO_3^-. This increase in NO_3^- and NO_2^-, upon oxidation of NO, can be measured in human biological fluids [134]. Given that NO has a short half-life in blood of about 0.1 s [135], the measurement of the NO metabolites, NO_3^- and NO_2^-, as biomarkers for the assessment of endothelial dysfunction, has been proposed by Lomelí and colleagues [134, 136]. A study conducted on 32 subjects with Marfan

syndrome, a connective tissue disorder, with 35 healthy controls, revealed an inverse correlation to exist between NO_3^- / NO_2^- and NO_2^- with FMD [136]. However, although a significant difference was observed between the two groups with regards to NO_3^- / NO_2^- ($p = 0.002$), NO_2^- ($p = 0.03$) and VCAM-1 ($p = 0.03$), no significant difference was observed between the groups with regards to markers of inflammation IL-6, IL-8 and TNF-α, and a marker of endothelial function, ICAM-1 [136]. In addition, no studies on these molecules have been performed in HIV-positive subjects.

11.6 Conclusions

Although a standard method for the assessment of endothelial function in HIV-positive or HIV-negative subjects is yet to be established, the use of plasma biomarkers, including vWF, VCAM-1 and ICAM-1, remains a promising option. Furthermore, these protein-based biomarkers can easily be adopted for use in point-of-care devices [137, 138]. One such example is the development of an integrated fluorescence correlation spectroscopy point-of-care device by Olson and colleagues [137] that can be used for the measurement of vWF. The advantages of using point-of-care devices include the fact that they are user-friendly, allow for the simultaneous measurement of multiple analytes, they give fast and precise results, are easily transported, do not require specialised equipment and have a low cost of production [138]. Therefore, these methodologies could be more easily sustained in developing countries. However, more research is warranted in this field to reach a consensus on the best biomarker, or a panel of biomarkers, with accurate diagnostic and predictive values, for the clinical assesment of endothelial dysfunction, to monitor the development of CVD in individuals infected with HIV.

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Chapter 12
The Role of Inflammation in the Development of GDM and the Use of Markers of Inflammation in GDM Screening

Lungile Khambule and Jaya Anna George

Abstract Gestational diabetes mellitus is a hyperglycaemic state first recognised in pregnancy. GDM affects both mother and child. Women with GDM and their newborns are at risk of developing type 2 diabetes in the future. The screening and diagnostic criteria for GDM are inconsistent and thus novel biomarkers of GDM are required to strengthen the screening and diagnostic processes in GDM. Chronic low-grade inflammation is linked to the majority of the well-established risk factors of GDM such as old age, obesity and PCOS. This review provides an overview of the present knowledge on the pathology of GDM, the screening criteria applied, the role of inflammation in the development of GDM and the use of markers of inflammation namely cytokines, oxidative stress markers, lipids, amino acids and iron markers in screening and diagnosis of GDM.

Keywords GDM · Inflammation · Biomarkers · Cytokines · Pregnancy · Metabolites

12.1 Introduction

Gestational diabetes mellitus is defined as high blood glucose first discovered in pregnancy [1]. The lack of consistency in the testing protocols and diagnostic criteria affects prevalence rates. GDM is said to be steadily increasing with the rising prevalence of obesity and type 2 diabetes (T2D). The global prevalence ranges between 1% and 14% with higher rates reported from Asian, Latin American and Middle Eastern populations [2, 3]. A meta-analysis from India

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noted prevalence estimates ranging from 0% to 41.9%, with International Association of the Diabetes and Pregnancy Study Groups (IADPSG) criteria picking up significantly more GDM (19.19% [CI: 15.5, 23.6]) compared to the World Health Association (WHO) criteria (10.13% [CI: 8.17, 12.50]) [3]. They also noted a higher prevalence in urban compared to rural areas. Data from Mexico showed that the prevalence of GDM was higher in the north in keeping with a higher prevalence of obesity in the north [4]. Little is known about GDM in Africa. A recent meta-analysis noted that the prevalence varied from 0% in Tanzania to as high as 13.9% in Nigeria [5]. There has been an increase in the reported prevalence of diabetes in Africa, which is expected to affect 41.5 million people by 2035, because of lifestyle changes [6].

Short term complications of GDM include infants who are large for their gestational age, preeclampsia, polyhydramnios, stillbirth, hypoglycaemia and hyperbilirubinemia, and respiratory distress syndrome [7]. The long term complications are: neonates born from mothers with GDM have increased risk of being overweight in childhood with impaired pancreatic beta-cell function as an adult, which may lead to T2D [8, 9]. Women with GDM are at increased risk of developing T2D, with high cardiovascular risk and early atherosclerosis after pregnancy [10]. A recent meta-analysis showed that treating GDM results in less preeclampsia, shoulder dystocia, and macrosomia but has no effect on neonatal hypoglycaemia or future poor metabolic outcomes [11].

The determination of risk factors for GDM is complicated by inconsistencies in diagnostic factors referred to above as well as by inconsistencies in measurement of risk factors across studies. Despite these issues, a number of risk factors have emerged consistently including advanced maternal age, overweight/obesity, excessive gestational weight gain, ethnicity, genetic polymorphisms, low or high birthweight, family or past history of GDM, and other insulin resistant states, such as polycystic ovarian syndrome (PCOS) [12–14]. Ethnicity and obesity are two strong independent risk factors for GDM [15]. In a cohort of 123,040 women without recognized pre-gravid diabetes, Asian and Filipino women had an increased risk of GDM at a lower Body Mass Index (BMI) cut point, particularly as compared with non-Hispanic white and African American women [16]. These risk factors are either directly or indirectly associated with impaired β-cell function and insulin sensitivity.

Considering that obesity is a major risk factor for GDM, it is possible that underlying biological functions that are affected by high body fat mass could be detected in patients who are at risk of GDM. Inflammation, particularly chronic low-grade inflammation is associated with obesity [17]. Although normal pregnancy is also associated with inflammation, mostly due to innate immune response to the foetal allograft [18], women with GDM have been shown to have even higher inflammatory markers when compared to women with a normal pregnancy [19]. Therefore, this review provides an overview of the present knowledge on the pathology of GDM, the screening criteria applied, the role of inflammation in the development of GDM and the use of markers of inflammation such as cytokines, lipids, amino acids,

oxidative stress and iron markers in screening and diagnosis of GDM. The review will only cover markers that are directly or indirectly linked to inflammation, upregulated or downregulated in GDM patients and linked to the features of GDM such as obesity and insulin resistance. Inflammatory markers, oxidative stress markers, lipids and amino acids that can be detected in blood will be discussed as these are more feasible and applicable for screening and diagnosing GDM.

12.2 Pathophysiology of GDM

As pregnancy advances the demand for insulin increases and, in most cases, the pregnant women can meet the demand. Placental secretion of diabetogenic hormones, such as growth hormone, corticotropin releasing hormone, placental lactogen, and progesterone, leads to increasing insulin resistance. The inability to overcome the insulin resistance of pregnancy despite β-cell hyperplasia leads to GDM.

β-cell dysfunction occurs when the β-cells lose the ability to adequately sense blood glucose levels or to release sufficient insulin in response to a glucose load. β-cell dysfunction is thought to result from prolonged excessive insulin production in response to a chronic fuel load. Reduced insulin-stimulated glucose uptake is damaging to β-cells, leading to a vicious cycle of hyperglycaemia, increased insulin resistance and worsening β-cell dysfunction [20].

The cause of the insulin resistance on GDM is multifactorial and arises because of hormones released from the fetal-placental unit and maternal fat accretion, excess lipolysis and release of free fatty acids (FFA) and of inflammatory mediators and adipokines [21]. The ongoing low-grade inflammation in adipose tissue impairs insulin signalling, which further stimulates production of proteins implicated in insulin resistance. Adipocytes synthesize substances with chemotactic and adhesive properties such as macrophage chemotactic protein-1 (MCP-1) and vascular and intracellular adhesion molecules (VCAM and ICAM), which enhance influx of lymphocytes and monocytes. These then interact with adipocytes to initiate a perpetuating cycle of macrophage recruitment, production of inflammatory cytokines and impairment of adipocyte function, with adverse effects including insulin resistance and endothelial dysfunction [21]. This is summarized in Fig. 12.1.

The placenta contributes to insulin resistance during pregnancy via its secretion of hormones and cytokines as well as via the transport of glucose, amino acids, and lipids across the placenta. Glucose transport occurs via GLUT1, by carrier-mediated sodium-independent diffusion and maternal hyperglycaemia contributes to foetal macrosomia [22]. In addition to the effects that are seen in the fetus because of transport of nutrients across the placenta, recent studies have reported that GDM is associated with placental DNA hypermethylation as well as release of miRNAs [23, 24].

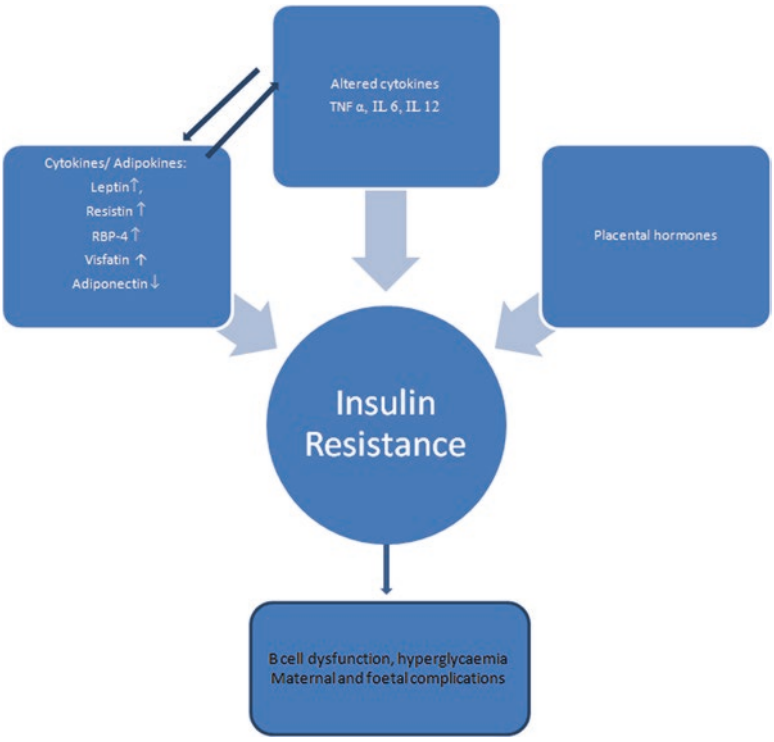

Fig. 12.1 Women who are obese have insulin resistance, down-regulation of adiponectin and up-regulation of leptin, resistin and retinol-binding protein-4 (RBP4), with features of chronic inflammation characterised by increased tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-12 (IL-12). Fetal and placental hormones may contribute to inflammation and increase insulin resistance

12.3 Screening and Diagnosis of GDM

While the evidence shows that treatment of GDM is of benefit to both mother and child [11], there is a lack of consensus in the approach to the screening and diagnosis for GDM. The initial criteria for the diagnosis of GDM were chosen to identify women at high risk of developing diabetes after pregnancy [25]. Data that has emerged since then has shown that the risk of adverse perinatal outcomes is associated in a continuous and graded fashion with maternal hyperglycaemia [1]. It is because of this that the International Association of Diabetes Study Groups (IADPSG) recommends universal or selective screening for high risk women to detect overt diabetes mellitus at the first antenatal visit. They recommend universal screening at 24–28 weeks using 75 g of glucose in a 2-hour oral glucose tolerance test (OGTT). On the other hand the NICE guidelines recommend either self-monitoring of blood glucose or a 75 g OGTT as soon as possible after booking for women who have had GDM in previous pregnancies and risk factor based screening at 24–28 weeks for others [26]. There is no agreement about which factors best

Table 12.1 Diagnostic criteria for gestational diabetes

Glucose (mM)	IADPSG	WHO	NICE	NDDG	ADA 2 steps	ADA 1 step
Test	75 g OGTT	75 g OGTT	75 g OGTT	100 g OGTT	50 g non-fasting OGTT	75 g OGTT
0 hours	5.1	7.0	5.6	5.8		≥5.1 mM
1 hour	10.0	–	–	10.6	≥10 mM	≥10 mM
2 hours	8.5	7.8	7.8	9.2		≥8.5 mM
3 hours				8.0		
Number of abnormal values needed for the diagnosis of GDM	≥1	≥1	≥1	≥2	If 1 hour ≥10 mM go on to 3 hr. 100g OGTT	≥1

Values are presented in mmol/L. *NDDG* National Diabetes Data Group, *OGTT* oral glucose tolerance test, *IADPSG* The International Association of Diabetes and Pregnancy Study Groups, *WHO* World Health Organization, *NICE* National Institute for Health Care Excellence, *NDDG* National Diabetes Data Group

define GDM risk. For example the NICE guidelines classify a family history of diabetes as a risk factor while the Australian Diabetes in Pregnancy Society (ADIPS) does not [15, 26]. Several groups recognize increased BMI as a risk factor but the definition of an increased BMI varies with NICE defining it as BMI ≥ 30 kg/m^2 and ADIPS defining it as ≥25 kg/m^2 [9, 10].

Another area of controversy is with the diagnostic criteria. Some of these are summarized in Table 12.1.

12.4 The Role of Inflammation in the Development of GDM

Inflammation is a complex mechanism that occurs in response to the invasion of foreign matter or in the case of injury. This process is characterized by redness, heat, swelling, pain and loss of function in the affected area which soon disappears after the infected area has been cleared. However, in a case of chronic inflammation, which is characterized by persistent secretion of cytokines and chemokines, it is more likely to cause more damage. The role of inflammation in the development of GDM is described below and the use of inflammatory markers, lipids, amino acids, oxidative stress markers and iron markers in the screening and diagnosis of GDM are discussed.

12.4.1 Cytokines

Cytokines are signalling proteins that bind onto their receptors which are present in various cells and they induce inflammation through the activation of the of the c-JUN N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) pathways. The

JNK activation signals for phosphorylation of the serine residues in the insulin receptor substrate-1 (IRS-1) which prevents insulin signalling that would normally occur through the tyrosine kinase cascade [27]. Like, the JNK pathway, NF-κB activation has been implicated in various chronic inflammatory diseases such as rheumatoid disease, atherosclerosis, asthma and chronic obstructive pulmonary disease [28]. The activation of the NF-κB by cytokines and chemokines leads to the production of pro-inflammatory cytokines and recruits leukocytes and monocytes to the site of injury or infection [28]. It is important to note that cytokines could function in both ways as they could collaborate to propagate inflammation or work antagonistically depending on the type of cytokine. In most cases, the anti-inflammatory cytokines inhibit the pro-inflammatory cytokines and vice versa.

Obesity, a known risk factor for GDM, is a chronic and heterogenous disease disproportionately affecting women, with up to 30% of women in general and over half of the pregnant women in some populations considered overweight or obese [29]. Adipose tissue which was originally believed to be a passive depot of energy is now considered an endocrine organ that secretes adipokines incuding adiponectin and leptin as well as numerous cytokines (TNF-α, IL-6 and IL-1) which have wide ranging metabolic effects [30, 31]. In addition to adipocytes, macrophages from adipose tissue also secrete pro-inflammatory adipokines [32]. There is a positive loop between pro-inflammatory cytokines, macrophages and monocytes in the presence of enlarged adipocytes (Fig. 12.2). Adipocytes are not the only source of cytokine secretion in pregnancy. The placenta contributes to inflammation and insulin resistance by also secreting pro-inflammatory cytokines, as mentioned above.

Adipokines contribute to insulin resistance in obese individuals and may be implicated in the insulin resistance seen in GDM, directly through regulation of insulin secretion and indirectly through inflammatory mediators and their effects on glucose metabolism [33].

The proportion of studies aimed at investigating the role of cytokines in GDM are far less than those involved in investigations of T2D and cardiovascular diseases (CVS) even though GDM shares similar characteristics with these diseases. The sections below discuss various cytokines including adipokines which are in keeping with the criteria mentioned in the introduction above.

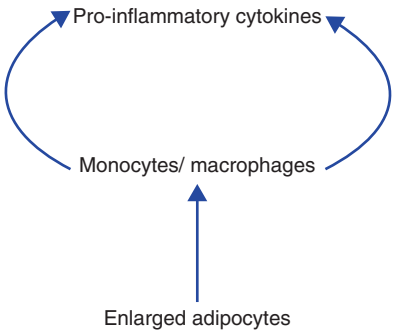

Fig. 12.2 Schematic diagram indicating the positive loop between pro-inflammatory cytokines macrophages and monocytes in the presence of enlarged adipocytes

12.4.1.1 Adiponectin

Adiponectin is an anti-inflammatory protein and is capable of amplifying insulin secretion by signalling for the expression of insulin gene and exocytosis of insulin granules [34]. It has been shown to induce insulin secretion and fatty acid oxidation in part by 5′ adenosine monophosphate-activated protein kinase (AMPK) mediation. AMPK is an enzyme that plays a role in muscle fatty acid oxidation and insulin sensitivity [35–37]. The AMPK activity is reduced by recruitment of pro-inflammatory cytokines and also feeds into the dysfunctional lipid metabolism and inflammatory pathways seen in individuals with elevated adipose tissue. Adiponectin is also known to supress pro-inflammatory cytokine production, seen in mice and in humans [38, 39]. Pro-inflammatory cytokines like TNF-α indirectly terminate the activation of AMPK to reduce fatty acid oxidation and insulin sensitivity [40] (Fig. 12.3). In GDM studies, adiponectin has been investigated more than all other adipokines [34]. The majority of these studies, including prospective ones, have led to agreement that adiponectin is inversely related to GDM [30, 34, 41–49] (Table 12.2). In the few studies that reported no differences in adiponectin levels between women with GDM and those without [50–52], the sample size was low and the lack of adjustment for co-factors such as BMI could have affected the results. López-Tinoco et al. showed that in their population, low adiponectin levels were seen in obese women who later developed GDM related to pregravid BMI [30]. In accordance with López-Tinocoet al, other studies showed an association of obesity with low adiponectin levels in non-pregnant individuals [53, 54]. In contrast, low adiponectin levels independent of pregravid BMI were observed in GDM cases in women with normal glucose tolerance [49]. However, gestational weight gain was not discussed despite its role in low adiponectin levels. Park et al. found that women with GDM gained more weight in pregnancy and had low adiponectin levels when compared to women who were not diagnosed with GDM [43]. These findings suggest that body weight gain does not favour secretion of adiponectin and therefore tilts the balance between pro-inflammatory and anti-inflammatory cytokines towards the pro-inflammatory side.

12.4.1.2 Tumor-Necrosis Factor-α (TNF-α)

Tumor-necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that is predominantly activated by macrophages and T lymphocytes. TNF-α can be expressed in placenta, adipose tissue, immune cells such as mast cells, B lymphocytes, natural killer (NK) cells, neutrophils and endothelial cells [55]. The binding of TNF-α onto either TNF-receptor type I (TNF-RI) or -receptor type II (TNF-RII) mainly activates the NF-κB pathways which leads to a further pro-inflammatory cytokine and immune cell secretion [56] (Fig. 12.3). Apart from inducing inflammation, TNF-α reduces insulin sensitivity by disrupting the translocation of glucose transporter GLUT-4 and insulin signal transduction [57, 58]. The expression of TNF-α in placenta is thought to contribute to the insulin resistance associated with pregnancy.

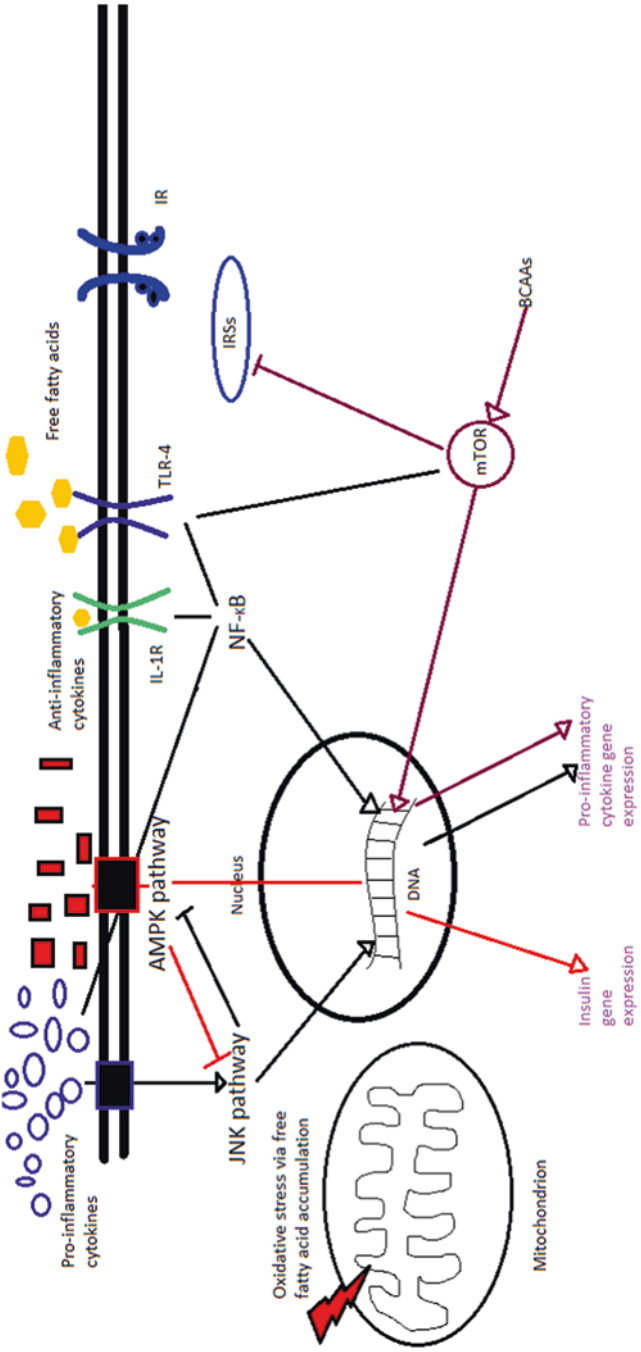

Fig. 12.3 Schematic diagram summarising the role of inflammation in target tissues. Elevated pro-inflammatory cytokine mediate JNK and NF-κB which inhibit the AMPK pathway and induce production of pro-inflammatory cytokines. JNK and NF-κB are also activated by TLR-4 and IL-1R in response to free fatty acids. NF-κB and BCAAs activate the mTOR pathway which in-turn inhibits the function of IRS and thus reduces insulin signalling. Anti-inflammatory markers induce the AMPK pathway and thus increase expression of insulin genes. Oxidative stress occurs in mitochondria due to elevated ROS

Table 12.2 Markers of inflammation and their effect in GDM

Marker	General function	Effect in GDM
Adiponectin	Anti-inflammatory cytokine	↓in women with GDM [30, 41–43, 45–49] ↔between GDM cases and controls [50–52]
TNF-α	Pro-inflammatory cytokine	↑ in women with GDM [30, 59–64] ↔between GDM cases and controls [65, 66]
IL-6	Pro-inflammatory cytokine	↑ in women with GDM [70–73] ↔between GDM cases and controls [30, 46]
AFABP	Pro-inflammatory cytokine	↑ in women with GDM [76, 79, 80]
Leptin	Pro-inflammatory cytokine	↑ in women with GDM [30, 41, 62, 92–94] ↔between GDM cases and controls [42, 46, 50, 52, 95]
RBP-4	Pro-inflammatory cytokine	↑ in women with GDM [96, 98, 99, 101, 102] ↓in women with GDM [100]
Resistin	Pro-inflammatory cytokine	↔between GDM cases and controls [46, 66, 103, 108]
Visfatin	Pro-inflammatory cytokine	↑ in women with GDM [110, 111] ↓ in women with GDM [43]
Free fatty acids	Biological signaling, energy storage, source of fuel, cell membrane formation	↑ in women with GDM [119, 120, 122]
BCAAs	Fuel source, protein synthesis, insulin regulation and biological signaling	↑ in women with GDM [119, 134–136, 174]
Alanine	Fuel source, protein synthesis, insulin regulation and biological signaling	↑in women with GDM [119, 135, 137, 138]
MDA	Product of oxidative stress	↑ in women with GDM [146–150]
Ox-LDL	Product of oxidative stress	↑ in women with GDM [146, 152]
Nitrotyrosine	Product of oxidative stress	↑ in women with GDM [66, 154]
AGEs	Product of oxidative stress	↑ in women with GDM [158]
GSH/GPx	Anti-oxidants	↓ in women with GDM [149, 160]
Ferritin	Iron storage	↑ in women with GDM [170, 171, 175]
Hepcidin	Iron regulator	↑ in women with GDM [172]

↑ = increased levels, ↓ = decreased levels, ↔ no differences in level, *GDM* gestational diabetes mellitus, *TNF-α* tumor-necrosis factor-α, *IL-6* interleukin-6, *AFABP* adipocyte fatty acid-binding protein, *RBP-4* retinol binding protein 4, *BCAAs* branched chain amino acids, *MDA* malondialdehyde, *Ox-LDL* oxidized low-density lipoproteins, *AGEs* advanced glycated end products, *GSH* glutathione (GSH), *GPx* glutathione peroxidase

However, in normal pregnancy, β-cells can compensate for the low insulin sensitivity by producing and secreting more insulin. TNF-α expression may come from different sources and, when combined, contribute to the development of GDM as seen in Fig. 12.3. For example, in addition to TNF-α expression in the placenta, it is also activated by the increase in adipose tissue, hyperglycaemia and chronic inflammation.

The majority of the studies have shown that TNF-α levels are elevated in women with GDM compared to healthy pregnant women [59–64] (Table 12.2). However, other studies did not show a significant difference in TNF-α levels between GDM cases and controls [65, 66]. There are only a few prospective studies on TNF-α and out of four prospective studies included in the current review, one study showed a non-significant difference in TNF-α levels between GDM cases and healthy controls [66]. Furthermore, a systematic review and meta-analysis on cytokines showed that TNF-α levels are higher in GDM cases than in controls [67]. Multivariate linear regression analysis reported that pregravid BMI was the most predictive indicator of TNF-α levels in women with GDM [49]. Similarly, Winkler et al. suggested that the elevated TNF-α levels seen in women with GDM is strongly linked to the increase in body fat mass [64]. Moreover, TNF-α correlated inversely to insulin sensitivity and correlated with c-peptide concentrations in a BMI matched population [49, 68]. These studies suggest that there is a correlation of body weight with TNF-α in pregnancy, although there are other factors in play such as insulin resistance that contribute to the development of GDM.

12.4.1.3 Interleukin-6

Interleukin-6 (IL-6) operates similarly to TNF-α to induce inflammatory responses in non-pregnant individuals with obesity and /or T2D [19, 69]. Many of the studies performed in GDM are cross sectional but the majority of the studies have indicated a positive correlation of IL6 with GDM [70–73] (Table 12.2). Serum IL-6 levels were significantly high in GDM females as compared controls and IL-6 levels correlated with pregravid BMI, fasting blood sugar (FBS) levels and postprandial sugar levels [70]. Similarly, women with GDM had significantly higher IL-6 and resistin levels compared to pregnant women with normal glucose tolerance [71]. In Pima Indians, a population with high rates of obesity and T2D, IL-6 levels correlated positively with adiposity and negatively with insulin action in women with GDM [72]. Furthermore, Morisset et al. suggested that IL-6 is a strong biomarker for GDM independent of BMI in women with GDM [73]. This is consistent with the possibility that adipose tissue plays a role in the development of GDM by inducing inflammatory responses and activating pro-inflammatory cytokines. In contrast, IL6 showed no significant difference between women with late-onset GDM and controls although TNF-α, leptin and adiponectin levels were significantly different between the groups [30]. A recent study showed that high molecular weight and fasting glucose are more robust risk factors for GDM compared to adiponectin, omentin-1 and

IL-6 when using the IADPSG criteria [74]. It is possible that the inconsistency in results of the studies mentioned above is caused by the differences in methodology, sample size and adjusted variables.

12.4.1.4 Adipocyte Fatty Acid-Binding Protein

Adipocyte fatty acid-binding protein (AFABP) functions as a pro-inflammatory cytokine and is highly expressed in macrophages and endothelial cells [75]. Previous studies of non-pregnant individuals have shown a link between the AFABP, obesity and associated complications such as metabolic syndrome [76–78]. In GDM, AFABP is a promising marker as it has been seen to be elevated in GDM cases compared to non-GDM women independent of their insulin sensitivity state [76, 79] (Table 12.2). Furthermore, this binding protein has been linked to adiposity markers in fetuses of women with GDM [79] which may connect GDM with macrosomia. One prospective study performed in an Indian population indicated that elevated AFABP levels in the first trimester of pregnancy may be useful in identifying women who are at risk of GDM [80]. Further studies, particularly, prospective ones are still required to investigate the significance of AFABP in GDM.

12.4.1.5 Cytokines/Adipokines with Contradictory Results

There are other promising cytokines that are either not extensively studied or have shown contradictory results in GDM. These cytokines have been implicated in inflammation in animal models and in non-pregnant individuals. Therefore, these could play a role in the development of GDM. Table 12.2 summarizes the relevant studies performed in GDM.

Leptin is a cytokine active in adipocytes but also found in various body organs such as the brain, placenta, gastro-intestinal tract (GIT), skeletal muscle and immune cells [81]. The binding of leptin to leptin receptors induces the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway which mainly regulates energy homeostasis, including insulin sensitivity regulation and appetite [82–85]. Leptin can pass through the blood brain barrier (BBB) and acts on the hypothalamus to promote satiety and regulate energy expenditure [82]. Apart from its actions in the central nervous system, leptin can act on peripheral organs such as adipose tissue, muscles, pancreas and liver to improve insulin sensitivity, reduce lipolysis, reduce amino acid absorption in intestines, increase glucose uptake in muscles and reduce gluconeogenesis in the liver [82, 86]. This effect appears to be attenuated in obesity [87]. An abnormal increase in leptin is observed in obese individuals and is indicative of leptin resistance that may be caused in part by a negative feedback loop between leptin and suppressor of cytokine signalling (SOCS)-3 transcription factor which in turn inhibits the action of leptin [88, 89].

During acute infection, leptin activates dendritic cells, monocytes, macrophages, neutrophils and natural killer cells [90, 91]. Moreover, leptin stimulates the production of other pro-inflammatory cytokines namely, IL-6 and TNF-α [90]. In the state of prolonged activation, leptin can contribute to the manifestation of chronic inflammation as seen in obesity and T2D. However, the function of leptin in pregnancy is not clearly understood. A systematic review in pregnant women has indicated that first and second trimester leptin levels are higher in women who later develop GDM compared to those who do not, although there has been significant study heterogeneity [30, 41, 62, 67, 92–94]. Other studies have indicated that leptin is not significantly different among women with GDM and those without [42, 50, 52, 95]. Similar to studies performed in non-pregnant individuals, leptin increases with the increase in body weight in pregnancy [30, 93].

Retinol binding protein 4 (RBP-4) is a member of the lipocalin family of proteins and is predominantly synthesized in the liver and partly in adipocytes [34]. As mentioned above, this pro-inflammatory cytokine is suggested to play a role in the development of GDM. However, more studies, particularly prospective ones, are required to gain more conclusive information. In multi-marker diagnostic models, a model that consisted of BMI, RBP-4, n-acetylaspartic acid and C16:1 (cis-7) could identify women with GDM better than other models including those using other cytokines and lipids [96]. RBP-4 correlated with fetal birth weight of neonates born from women with GDM [97]. The plasma RBP-4 concentration was significantly higher in women with GDM and correlated positively with transthyretin, fasting plasma glucose, insulin and triglyceride concentrations, as well as blood pressure, abdominal fat area, and homeostasis model assessment of insulin resistance (HOMA-IR) [98]. In one prospective study there was evidence of a positive association of early pregnancy elevated RBP-4 concentrations with increased GDM risk, particularly among women with advanced age and obesity [99]. In contrast, Krzyzanowska and associates reported low levels of RBP-4 in women with GDM and RBP-4 was not significantly associated with glucose or HOMA-IR [100]. Although RBP-4 levels were higher in women with GDM than in controls, there was no significant correlation between RBP-4 and age, BMI, insulin and HOMA-IR [101, 102]. A prospective study indicated that RBP-4 was not significantly higher altered in pathological pregnancies including GDM [103].

Resistin is a hormone that belongs to a family of proteins generically known as resistin-like molecules (RELM), which are characterized by the consistent presence of a segment rich in cysteine at the C-terminal end [104]. This hormone contributes to inflammation by inducing the production of monocytes and macrophages and correlates with other pro-inflammatory markers such as TNF-α and IL-6 [75, 104]. Despite its association with adipose hypertrophy [19, 105, 106], previous studies have shown that resistin is not associated with insulin resistance and its indices [71, 105]. Furthermore, other studies reviewed in a meta-analysis have shown that resistin levels are not significantly different between women with GDM and women without GDM [107]. Four prospective studies have indicated that resistin levels cannot predict women who are at risk of GDM [46, 66, 103, 108].

Visfatin is a cytokine which is mostly expressed in visceral adipose tissue (VAT) and is thought to improve insulin sensitivity by mimicking insulin [19]. However, this effect could be explained by its ability to up-regulate cytokine secretion in monocytes [109]. Despite its conflicting effects, visfatin appears to be elevated in women with GDM. In case control studies, visfatin levels were found to be higher in women with GDM despite non-significant correlation with insulin, BMI, glucose [110, 111]. However, Park et al. reported low visfatin levels in GDM cases when compared to controls [43].

12.4.2 Lipids and Their Metabolites

Fatty acids are the building blocks of lipids and are stored in adipose tissue. Increase in adipose tissue means that the storage of free fatty acids and lipids is limited and thus lipids leak from adipose tissue and into the bloodstream which ultimately elevates circulatory free fatty acids, cholesterol, lipoproteins and phospholipids. Normal pregnancy is associated with an increase in free fatty acids in the first and second trimester which normalizes in the third trimester of pregnancy [112]. This could be a result of hyperphagia and lipid synthesis associated with normal pregnancy in order to ensure sufficient energy for the growing foetus. In general, postprandial insulin secretion aims to supress lipolysis to sustain energy levels and the reverse occurs during fasting state [113, 114]. Low insulin sensitivity associated with GDM indicates that the suppressing role of insulin in the postprandial state is compromised and therefore lipolysis is not regulated which further explains the increase in free fatty acids in women with GDM compared to controls. Furthermore, elevated free fatty acids are connected to fetal complications such as macrosomia because of elevated fatty acid transportation to the fetus in GDM cases [115].

Elevated circulatory free fatty acids have been shown to induce the innate immune response by binding to toll-like receptors (TLRs). TLR activation induces the secretion of pro-inflammatory cytokines [116]. In addition, free fatty acids have been shown to play a role in β-cell inflammation by inducing IL-1β, IL-6, and IL-8 secretion in human islets and IL-1β secretion in mouse islets via the IL-1 receptor [117] (Fig. 12.3).

A case control study reported that pregravid BMI is a significant contributor to the increase in serum fatty acids at 28 weeks of pregnancy and serum fatty acids were higher in GDM cases when compared to controls [118]. In addition, serum triglycerides and pregravid BMI significantly correlated with fetal birth weight [115]. The Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study showed that pregnant women with high fasting plasma glucose had higher triglyceride levels and lower insulin sensitivity in the third trimester of pregnancy [119]. Similarly, free fatty acids were associated with insulin resistance in early pregnancy [120]. Furthermore, women with GDM and high pregravid BMI had altered mRNA expression levels of genes involved in fatty acid uptake and metabolism [121]. A systematic review of 60 studies indicated that triglyceride levels were significantly

elevated in women with GDM compared with those without GDM and HDL-C levels were significantly lower in women with GDM compared with those without GDM in the second and third trimester [122]. In summary, lipids and free fatty acids levels are associated with GDM and GDM-associated risk factors, thus promising potential biomarkers of GDM (Table 12.2).

12.4.3 Amino Acids and Their Metabolites

The demand for amino acids is higher in pregnancy because of their crucial role in protein synthesis, gluconeogenesis and ketogenesis. In addition to protein synthesis and fuel generation, some amino acids are regulators of biological processes. For example, branched chain amino acids (BCAAs) play a role in regulating the immune system and insulin sensitivity. Alanine can inhibit cell apoptosis. Arginine can be broken down into nitric acid and used to regulate inflammation. Tyrosine, produced from phenylalanine, is a precursor for the synthesis of catecholamines which acts as a messenger during the immune response [123].

Elevated circulatory BCAAs have been linked to insulin related diseases such as the metabolic syndrome and T2D [124–126]. The proposed mechanism used by the BCAAs, particularly leucine, involves the amino acids induction of the mammalian target of rapamycin (mTOR) pathway [127–130]. The mTOR pathway is a serine-threonine kinase that behaves as a nutrient sensor. The function of the mTOR signalling pathway is to relay signals related to nutrition, cell growth and cell survival. BCAAs, particularly leucine, activate the mTOR pathway through the activation of the class III phosphoinositide 3-kinase (PI3K) and consequently mTOR phosphorylation (Fig. 12.3). The activation of mTOR leads to the phosphorylation of two main downstream effectors, namely, ribosomal substrate 6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [131]. The activation of mTOR signalling pathway has been linked with secretion of pro-inflammatory cytokines in cancerous cells through the activation of IL-1A [132] and in peripheral blood mononuclear cells (PBMCs) [133].

Investigations have shown a link between elevated circulatory amino acids with GDM and T2D [43, 119, 134–136]. The majority of these studies indicate that BCAAs are elevated in women with GDM. Alanine has also been identified as a predictor of GDM in metabolomic studies [119, 135, 137, 138] (Table 12.2).

12.4.4 Oxidative Stress Markers

Oxidative stress is a state when there is an imbalance between reactive oxidative species (ROS) and anti-oxidants with the ROS being higher than the anti-oxidants. ROS are charged species and can bind with other compounds in the body changing their natural structural morphology and possibly their function. It is believed that

elevated ROS play a role in inducing insulin resistance by interacting with the insulin receptor and the JNK signalling pathway [139]. In non-pregnant individuals, oxidative stress activates the NF-κB and JNK pathways which further feeds into inflammation [140].

Elevated free fatty acids have been shown to cause oxidative stress through mitochondria uncoupling which give rise to ROS [139, 141]. The combination of ROS and lipids could lead to the formation of oxidized lipids [120]. Oxidized lipids can attenuate lipid metabolism, lipid transport and placental development [113]. Another mechanism of how enlarged adipocytes can cause oxidative stress is by overconsumption of oxygen which generates free radicals in the mitochondrial respiratory chain [54].

Oxidative stress is a feature in normal pregnancy and is partly generated in the placenta but is heightened in pathological pregnancies such as GDM [142, 143]. It is possible that an increase in adipose tissue common in women who develop GDM exacerbate oxidative stress by further elevated free fatty acids. Evidently, GDM is associated with elevated ROS and defects in antioxidant defences [75].

Oxidative stress markers such as malondialdehyde (MDA), oxidized low density lipoproteins (Ox-LDL), nitrotyrosine, advanced glycated end products (AGEs) and anti-oxidants, glutathione (GSH)/glutathione peroxidase (GPx) are promising biomarkers for GDM [144] (Table 12.2). However, there have been only a few studies on oxidative stress in GDM.

MDA is formed by lipid peroxidation of polyunsaturated fatty acids [144] and has been shown to be associated with intrauterine growth restriction (IUGR) [145]. In a Turkish population, elevated MDA and ox-LDL levels were evident in all pregnancies but higher in women with GDM [146]. Similarly, MDA levels were found to be higher in GDM cases when compared to healthy controls and MDA levels correlated positively to leptin and resistin and were negatively correlated with HOMA-IR [147]. In two longitudinal studies, MDA levels were found to be significantly higher in obese women later diagnosed with GDM [148, 149]. In a GDM and preeclampsia study, MDA was the only oxidative stress marker elevated in GDM cases when compared to controls among other oxidative stress markers such as protein oxidation markers (AOPPs), myeloperoxidase (MPO) and lipid hydroperoxide (LHP) [150].

Oxidized low density lipoproteins are one of many oxidized lipid markers and are linked to diseases associated with dyslipidaemia, such as atherosclerosis [151]. In GDM, Ox-LDL has been shown to be elevated when compared to healthy controls [146, 152]. The study further proposed that Ox-LDL is linked to macrosomia [146].

Nitrotyrosine is formed through the binding of the highly reactive ROS species peroxynitrite (ONOO-) with serine, tyrosine and threonine hydroxyl groups [153]. Lyall and colleagues have indicated that nitrotyrosine oxidative stress occurs in the placenta of women with diabetes in pregnancy (GDM and overt diabetes), which is in agreement with previous studies showing that the placenta induces oxidative stress [154, 155]. Georgiou and associates have shown that nitrotyrosine is higher in

GDM cases at the first trimester (11 weeks) but declines after 28 weeks of pregnancy [66].

AGES are formed by glycation and oxidative reactions and can alter signalling pathways inside cells, the conformation of molecules and the expression of genes. In addition, this attenuation could ultimately cause inflammation by inducing pro-inflammatory markers through NF-κB dependent pathways [144, 156, 157]. As expected, AGEs have been shown to be associated with hyperglycaemia-related illnesses such as retinopathy and nephropathy and thus is a potential marker of GDM [156]. In GDM, AGEs were found to be higher in women with GDM [158]. However, it is more likely to be used for diagnostic processes than early screening because of its dependency on hyperglycaemia which is only present later in pregnancy in most cases of GDM.

GSH is an antioxidant which reduces oxidative stress by donating electrons to unstable ROS resulting in the formation of ox-GSH [144, 159]. Alternately, GSH functions as a substrate for the antioxidant GPx. GPx requires GSH to neutralize reactive peroxides. It is unclear at what concentration GSH and GPx are present in women with GDM. Peuchant and colleagues have shown that GPx levels are significantly lower in GDM cases and in pregnant women with T2D [160]. A longitudinal study by Arribas et al. showed that GPx was significantly lower in early pregnancy when compared to healthy controls but increased later in pregnancy [149].

12.4.5 Iron Markers

Iron is a transitional element used in various biological systems such as oxygen transportation, cellular proliferation, protein synthesis, antimicrobial effects and energy production [161]. However, due to the reactive nature of iron, too much iron could produce ROS through a process known as Fenton reactions [162]. Elevated iron could also alter insulin signalling in the liver and adipocytes and increase non-esterified fatty acid oxidation in muscles [163, 164]. Elevated serum ferritin levels have been observed in numerous chronic inflammatory diseases such as lupus and diabetes [165–168] including GDM [169–171]. The high circulatory iron concentration activates hepcidin thus increasing hepcidin levels as seen in women with GDM and women with impaired glucose tolerance (IGT) [172]. In contrast, Akhlaghi and colleagues have reported low serum iron concentrations in women with GDM as compared to healthy controls [173].

The mechanisms involved in the increase of iron levels are not clearly understood although chronic inflammation could be an important piece of the puzzle [169]. One other way that iron levels in pregnancy are elevated is through iron supplement consumption. Although the majority of the studies mentioned above support the case that circulating iron levels are higher in GDM cases, more studies aimed at investigating the mechanisms involved in iron increase in pregnancy are required (Table 12.2).

12.5 Conclusions

It is likely that the development of GDM is caused by different factors which synergistically collaborate to suppress the production of insulin, in part via secretion of pro-inflammatory cytokines and mediation of pro-inflammatory signalling pathways. The balance between the pro-inflammatory cytokines and anti-inflammatory cytokines is what distinguishes the disease state from the healthy state. The disease state favours pro-inflammatory cytokine secretion. Furthermore, the activation of pro-inflammatory signalling pathways through fatty acids, amino acids, ROS and markers of oxidative stress and iron contributes to the development of GDM.

In summary, there is strong possibility that adiponectin, TNF-α, IL-6, AFABP-4, lipids, BCAAs, alanine, oxidative stress markers and iron markers are biomarkers of GDM and could be used for screening in early pregnancy or late pregnancy. However, more prospective studies are required to confirm this. The conflicting reports in RBP-4, resistin, visfatin and leptin levels may indicate that these markers are not involved in the development of GDM. However, it is important to consider the heterogeneity between studies which could have led to the conflict in results. Future studies should assess the diagnostic ability of the markers reviewed in the current review as a combined set considering the substantial knowledge that these markers are interlinked and are all implicated in the development of GDM.

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Chapter 13
PFKP Signaling at a Glance: An Emerging Mediator of Cancer Cell Metabolism

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Abstract Phosphofructokinase-1 (PFK-1), a rate-determining enzyme of glycolysis, is an allosteric enzyme that regulates the oxidation of glucose in cellular respiration. Glycolysis phosphofructokinase platelet (PFKP) is the platelet isoform and works as an important mediator of cell metabolism. Considering that PFKP is a crucial player in many steps of cancer initiation and metastasis, we reviewed the specificities and complexities of PFKP and its biological roles in human diseases, especially malignant tumors. The possible use of PFKP as a diagnostic marker or a drug target in the prevention or treatment of cancer is also discussed.

Keywords PFKP · Cancer · Gene regulations · Biologic roles · Diagnostic marker · Drug target

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13.1 Introduction

Cancer is a disease characterized by uncontrolled cell growth. A cancerous cell generates energy mainly through the process of aerobic glycolysis, better known as the Warburg effect [1]. The Warburg effect describes how cancer cells alter their method of metabolism to an increase in glycolysis followed by lactic acid fermentation even in the presence of excess oxygen [2].Cancer acquires and metabolizes nutrients through aerobic glycolysis synthesizing macromolecular precursors to supportcell proliferation. Phosphofructokinase-1 (PFK-1), a rate-controlling enzyme in glycolysis, catalyzes the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP). There are three isoforms of PFK-1, including PFKP (platelet), PFKM (muscle) and PFKL (liver). PFKL is the main isoform in liver and kidney, whereas PFKM and PFKP are the isoforms found in muscle and platelet, respectively. All isoforms are also present in other tissues [3]. Recently, PFKP is noted for its high prevalence in different types of cancer such as glioblastoma and breast cancer [4, 5].

13.2 Recent Advances in PFKP Biology

The molecular structure of PFKP (Fig. 13.1) in its active state or ATP-bound state is a tetrameric enzyme [6]. Upon hydrolysis of ATP, tetrameric PFKP changes its conformation and catalyzes the essential glycolytic reaction of converting F6P to F1,6BP.

13.2.1 Molecular Structure and Regulation of PFKP

The PFKP gene is located on the short arm of chromosome 10 in the human genome [7]. PFKP has 8 transcript variants and isoform 1 is the longest of these with a length of 784 amino acids. The regulation of the PFKP gene expression is closely intertwined with glycolysis. One specific example of PFKP gene regulation is found to occur in brown adipose tissue via correlation of the protein levels of hypoxia inducible factor-1α (HIF-1α) with activation of glycolytic genes [8]. Reduced expression of PFKP was observed in HIF-1α knockdown mature brown adipocytes, indicating that PFKP is one of HIF-1α targets [8]. One recent advancement revealed Krüppel-like factor 4 (KLF4)-mediated regulation of PFKP. KLF4 is a transcription factor that upregulates glycolytic metabolism by binding directly to the promoter region of the PFKP gene. Several KLF-binding regions exist in the G-C rich sequence of the proximal region of the PFKP promoter [5]. However, expression of the other two PFK-1 isoforms is not regulated by KLF4 activity due to lack of KLF-binding regions in the promoter sequences of PFKM and PFKL [5]. In contrast,

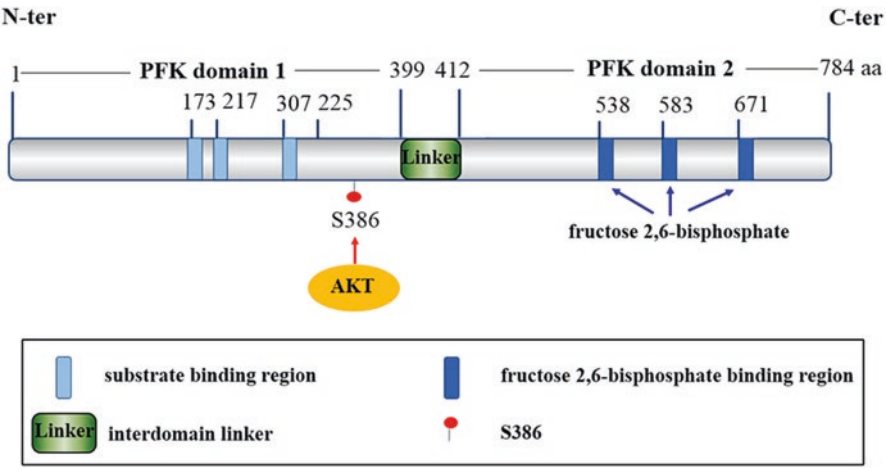

Fig. 13.1 Molecular structure and major function domains of the PFKP protein. Structurally, PFKP is comprised of two major domains, an N-terminal catalytic PFK domain 1 which ranges from amino acids 1 to 399 and C-terminal regulatory PFK domain 2 which ranges from amino acids 412 to 784. Three substrate binding regions (amino acids 173–175, 217–219 and 307–310) are located in PFK domain 1. The allosteric activator fructose 2,6-bisphosphate (F2,6BP) binds on PFK domain 2at amino acids 538–542, 583–585 and 671–674. S386 can be phosphorylated by AKT to increase PFKP stability

ZBTB7A, a member of the POZ/BTB and Krüppel (POK) family, directly binds to the PFKP gene promoter to represses its transcription [9].

There are several known post-translational modifications of PFKP such as acetylation, glycosylation and phosphorylation. The PFKP molecule has been presumed to have 18 phosphorylation sites and 12 acetylation sites (https://www.phosphosite.org/). Recently, S386 was identified as the key phosphorylation site tightly related with PFKP protein stability (Fig. 13.1). AKT binds to and phosphorylates PFKP at S386 and, as a result, inhibits the binding of TRIM21 E3 ligase to PFKP, which prevents degradation of PFKP protein, leading to an up-regulation of aerobic glycolysis [4].

Several domains and putative binding sites have been revealed based on the structure of PFKP protein. One important functional domain is the N-terminal catalytic PFK domain 1, which ranges in from amino acids 1 to 399. Another highly notable region of PFKP is the C-terminal regulatory PFK domain 2. This domain is situated from amino acids 412 to 784 (Fig. 13.1). An inter-domain linker connects PFK domain 1 with PFK domain 2. As an allosteric activator, fructose 2,6-bisphosphate (F2,6BP) can bind to the PFK domain 2 at amino acids 538–542, 583–585 and 671–674 to yield active PFKP (Fig. 13.1). The enzyme that produces F2,6BP is 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), which is a bifunctional enzyme with two distinct catalytic possibilities in a single polypeptide unit [10]. One catalytic possibility of PFKFB is F2,6BP synthesis, which leads to glycolysis. The other possibility is degradation of F2,6BP, resulting in gluconeo-

genesis. Due to this reciprocal regulation, the activation of glycolysis in association with this substrate activator results in a decrease in gluconeogenesis [11]. Regulation through PFKFB is indirectly intertwined with PFKP because the product of PFKFB fuels the activation of PFKP, which results in glycolysis [12]. PFKP is only active in its tetrameric state. From studies on the mechanisms of PFKP on ATP and substrate binding, it has been observed that no enzymatic activity takes place in the cases of the monomer or dimer forms [6]. In the tetrameric state, the two subunits of the dimer assume an antiparallel orientation with the active site located between the subunits [6].

13.3 Recent Advances of PFKP Function in the Development and Human Diseases

13.3.1 PFKP and Oocyte Development

PFKP acts as the key enzyme in glycolysis, participating in the development of the oocyte. Cumulus and mural granulosa cells reflect the characteristics of the oocyte [13]. The development of the oocyte occurs due to glycolysis in cumulus cells, which surround and nurture the oocyte. Mammalian oocytes are dependent on their cumulus cell counterparts because oocytes cannot form glycolytic intermediates [14]. Instead, oocytes release paracrine factors, which promote cumulus cell glycolysis by inducing the mRNA expression of PFKP [14]. Moreover, oocytes control glycolysis in granulosa cells by altering the expression of PFKP and other glycolytic enzymes, including enolase 1 (ENO1), pyruvate kinase (PKM2), triosephosphate isomerise (TPI), aldolase A (ALDOA), and L-lactate dehydrogenase 1 (LDH1) [15].

13.3.2 PFKP and Sertoli Cells

Sertoli cells are somatic cells of the testis that are essential for testis formation and spermatogenesis. These cells play a part in the progression of germ cells to spermatozoa [16]. GATA binding protein 4 (GATA4) is a sertoli cell marker that is involved in various cellular activities of the embryonic stage such as genital ridge initiation, sex determination and differentiation [17]. Deletion of GATA4 in adult mice has been observed to increase permeability of the blood-testis barrier (BTB) and disrupt spermatogenesis. Conditional deletion of the GATA4 gene in vivo is also associated with altered expression of several genes regulating lactate production, including PFKP [17]. Since sertoli cells give lactate to germ cells, any alteration in lactate production effects the development of germ cells [18]. Furthermore, knockdown of

GATA4 in MLTC-1 cells decreases production of sex steroid precursors as well as other metabolic pathways involved in glycolysis, leading to lower intracellular levels of ATP and increased extracellular levels of glucose [19]. More specifically, the expression levels of PFKP, hexokinase 1 (HK1), glucose phosphate isomerase 1 (GPI1), and phosphoglycerate mutase 1 (PGAM1) are down-regulated when GATA4 expression is repressed [19].

13.3.3 PFKP and Thyroid Hormone (TH)

TH secreted by the thyroid gland is important for normal growth, metabolism and development. Its biological effects are mainly mediated by 3,3,5-triiodothyronine (T3) which acts as a ligand to bind to the thyroid hormone receptors TRβ1, TRβ2 and TRα1 [20]. To better understand the broad scale effects of TH on gene expression in normal human cells, the expression of more than 15,000 genes in fibroblasts of normal cells was measured. Through quantitative fluorescent cDNA microarray analysis, PFKP was identified as a positively up-regulated target of TH [20]. HIF-1α is a direct target of the TH-thyroid hormone receptor (TR) axis. The PFKP as the target gene of HIF-1α was indirectly up-regulated by the TH-TR axis. The results of these studies point towards the possibility of glucose metabolism in human fibroblasts being regulated in part by TH. Moreover, the cytosolic phosphoinositide-3-kinase (PI3K)/AKT signaling pathway has been activated by TH-TR. Activated AKT can enhance the PFKP stability to increase cell glycolysis [4, 21]. These findings provide information on a link between TH and glycolysis in human fibroblasts.

13.3.4 PFKP and Rheumatoid Arthritis (RA)

RA is a systemic inflammatory autoimmune disease affecting many joints including the hands and feet. A study that explores the pathogenesis of RA compared protein levels between patients with and without RA illustrating that anaerobic catabolizing enzymes, such as PFKP and lactate dehydrogenase A (LDHA), were up-regulated in RA patients [22]. Moreover, the study evaluated the role of the HIF-1α pathway in catabolism. The results illustrated that aerobic oxidation enzymes and fatty acid oxidation were all down-regulated and the enzymes involved in anaerobic catabolism were increased in fibroblast-like synoviocytes (FLS) after HIF-1α knockdown [22]. These findings regarding the HIF-1α pathway indicate that reduced aerobic oxidation due to enzymes such as PFKP may be a newly recognized factor contributing to RA progression.

13.3.5 PFKP and Obesity

The relationship between PFKP polymorphism and obesity is controversial. A study recently found that the presence of the PFKP rs6602024 polymorphism allele A in women was associated with an increased risk of obesity [23]. However, a different study that focused on Danes could not find definitive data on a causal relationship between PFKP and obesity [24]. Moreover, no apparent relationship was found between PFKP rs6602024 and development of obesity in Danes. Another study on the relationship of PFKP and obesity found that several single nucleotide polymorphisms (SNPs) in PFKP genes were strongly correlated with increased BMI, hip circumference and body weight [25]. However, when attempts to replicate the findings of the study were performed in the GenNet study, the data on PFKP was found to be inconclusive [25]. Thus, further research needs to be done on the relationship between PFKP polymorphisms and obesity. Interestingly, the relationship of obesity and PFKP has been found to overlap with body weight in newborns [26]. Newborns can be characterized based on weight at birth as small for gestational age (SGA), large for gestation age or appropriate for gestational age. A study that observed newborns with small and appropriate sizes for gestation age found genetic variation in both cases [26]. Moreover, genetic variations were found in the PFKP potassium voltage-gated channel subfamily J member 11 (KCNJ11), brain-derived neurotrophic factor (BDNF), phosphotriesterase related (PTER) and protein transport protein Sec16B (SEC16B) genes in newborns with SGA, which was similar to genetic findings for type II diabetes and/or obesity [26].

13.3.6 PFKP and Heart Disease

Anti-human protein S antibody (anti-hPS Ab), an autoantibody, can be detected in patients with autoimmune diseases and this results in hypercoagulability in these individuals [27]. In a recent study, anti-hPS Ab was found to specifically cross-interact with PFKP and inhibit its activity in human coronary artery endothelial cells (HCAECs) [27]. Tissue factor (TF) was up-regulated when PFKP was silenced in these types of cells. Furthermore, activationof PFKP by F6P can block the effects of anti-hPS Abs on TF up-regulation in HCAECs. Transforming growth factor β (TGFβ1) pathways and peroxisome proliferator-activated receptor gamma (PPARγ) are essential for cardiovascular homeostasis. TGFβ1 stimulates vascular smooth muscle cell (VSMC) proliferation, while PPARγ acts as an inhibitor of TGFβ1 [28]. The inhibitory effects of PPARγon VSMC proliferation and TGFβ1-induced mitochondrial activation occur through regulation of PFKP and protein phosphatase 1 regulatory subunit 3G (PPP1R3G), a protein involved in the regulation of hepatic glycogenesis in a manner coupled to the fasting-feeding cycle [28].

13.4 PFKP and Cancer

The progression and prevalence of cancer is highly related with glycolysis. Being one of the rate-limiting enzymes in glycolysis, the aberrant expression of PFKP has been reported in different types of cancer,including but not limited to breast cancer, clear cell renal cell carcinoma and lung cancer, glioblastoma [4, 5, 29, 30]. High rates of glycolysis and subsequent lactic acid fermentation occur in cancer cells even in the presence of abundant oxygen. Hypoxia, as the specific microenvironment in cancer, positively regulates the expression levels of PFKP [31]. In addition to a tight relationship with tumor growth, PFKP also participates in cancer metastasis through interaction with oncogenes.

13.4.1 Elevated PFKP Expression Levels in Cancer

Currently, 44 somatic mutations of PFKP have been identified and these are known to have distinct effects in terms of enzymatic activity and allosteric regulation [6]. Three of these somatic mutations of PFKP (R48C, N426S, and D564N) have been well studied and this has shown that the R48C mutant reduces citrate inhibition, the N426S mutant is not effectively inhibited by ATP, and the D564N mutant has decreased maximum velocity and affinity for its substrate, F6P [6]. Deregulation of PFKP via mutations can lead to an arsenal of diseases including glycogen storage disease type VII, also known as Tarui disease, and mice that lack PFK may develop decreased fat stores [6]. Furthermore, deregulation through mutations in PFKP residues can alter the tetrameric state of the enzyme which again contributes to altered glycolytic flux [6]. Researching the functional consequences of unique somatic mutations of PFKP and its isozymes will allow for better understanding of how PFKP interacts in human cancer.

Amplification is the predominant gene alteration of PFKP in cancer, which is positively associated with PFKP transcription levels (Fig. 13.2). Compared to normal functioning cells, cancer cells are recognized for having abnormally high levels of glycolytic flux, which may explain why elevated PFKP is found in cancers as the rate-determining enzyme in glycolysis. The epigenetic regulation in the PFKP promoter region has also been studied recently. Approximately 15–30% of breast cancer cases are associated with the human epidermal growth factor receptor 2 (HER2) and result in a poor prognosis [32]. In HER2 positive breast cancer cells, HER receptors dimerize when activated and activate downstream multiple signaling pathways that result in cell cycle progression/survival and inhibition of apoptosis [32]. PFKP, as one of the important checkpointsof the glycolytic pathway, has been found with a hypermethylation on G-C rich region in HER2 positive breast cancer cells [32].

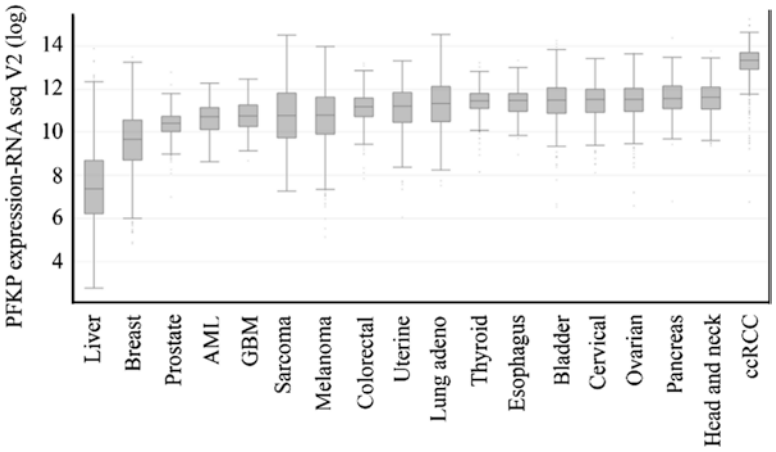

Fig. 13.2 Alteration of the gene and transcription of PFKP in human cancers. Alterations of PFKP expression were analyzed by cBioPortal in various types of cancer. The TCGA database chosen for this analysis must meet with a large number of patient samples (>100)

13.4.2 PFKP Contributes to Metabolic Pathways in Favor of Tumor Growth and Survival

Recently, epidermal growth factor receptor (EGFR) has been found to regulate cancer cell glycolysis through PFKP [33]. EGFR is a transmembrane tyrosine receptor for ligands that bind to members of the EGF family. Once the EGFR is activated, acetylation of PFKP and subsequent translocation of PFKP to the plasma membrane occurs. At the plasma membrane, PFKP is phosphorylated by the EGFR [33]. Phosphorylated PFKP is involved in an indirect action that results in PI3K-dependent AKT activation [33]. The activation of PI3K results in phosphorylation of phosphofructokinase-2 (PFK-2) which leads to the production of F2,6BP which, in turn, activates PFKP and leads to increased levels of glycolysis promoting the Warburg effect. Through PI3K activation due to EGFR, AKT becomes activated and has been shown to stabilize PFKP in glioblastoma (GBM) tumorigenesis [4] (Fig. 13.3).

In a state of cancer tumorigenesis, cancers such as breast cancer require proliferation in nourished and resource-limited environments. Both of these environments require the rate-limiting step of aerobic glycolysis, PFK-1 or PFKP. A study elucidating the mechanistic link between snail (SNAI1) and PFKP found that the transcripts, protein abundance and kinase activity of PFKP were consistently up-regulated by SNAI1 deletion [34]. The mechanism that SNAI1 uses to regulate glycolytic activity is through the inhibition of PFKP in breast cancer [34]. Furthermore, the study found that SNAI1 represses PFKP on a transcriptional level because post-translational modifications were not affected by SNAI1 [34]. SNAI1 abundance in breast cancer cells is undergoing further study due to its key function in cancer invasion and metastasis. These results indicate that SNAI1 is a key

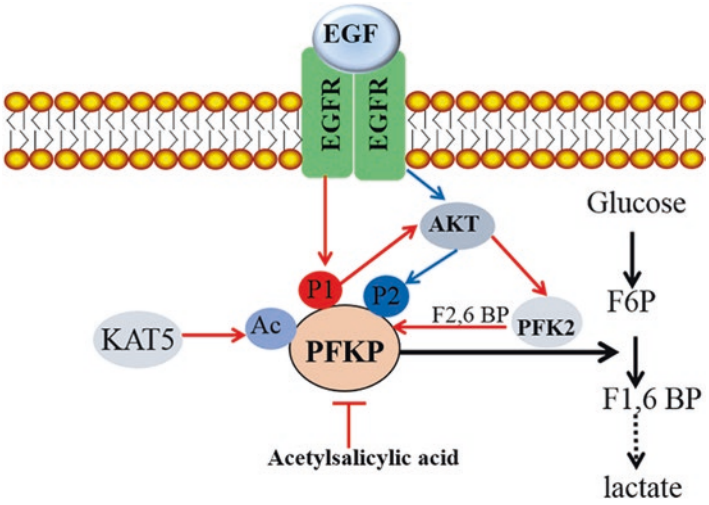

Fig. 13.3 Upregulation of PFKP by the EGFR to promote glycolysis. The EGFR enhances cancer cell glycolysis by up-regulation of PFKP through two distinct mechanisms: increasing its activity or stability. Upon EGFR activation, acetylation of PFKP by KAT5 and subsequent phosphorylation of PFKP at Y64 (P1) by EGFR activates AKT, which in turn activates PFKP by increasing PFK-2 activity to produce more F2,6BP. In addition, AKT activated by EGFR directly phosphorylates PFKP on S386 (P2), increasing PFKP stability by blocking its ubiquitination and degradation in the cytoplasm

transcriptional repressor of epithelial-mesenchymal transition (EMT) that allows for glucose metabolism to be altered in favor of the pentose phosphate pathway [34]. Due to the suppression of PFKP, the glucose flux switches from glycolysis to the pentose phosphate pathway which creates NADPH and other precursors for nucleotide synthesis [35]. Biosynthetic materials such as NADPH and pentose sugars as well as ribose-5-phosphate generated from glycolysis can then be used as precursors for the metabolites necessary for cancer metastasis [35].

Another area of study relating to activated PFKP involves muscle-invasive bladder cancer (MIBC). MIBC is a type of bladder cancer that spreads into the detrusor muscle of the bladder. Research showed that PFK-1 family genes were up-regulated in many cases of MIBC [36]. Although mutated or altered PFK-1 genes predict a history of non-invasive bladder cancer, the PFK-1 status does not indicate survival or disease relapse [36]. These observations suggest that PFKP may play an important role in cancer cell survival of patients with MIBC. Moreover, PFK-1 inhibitors significantly decreased the level of glycolysis and inhibited the growth and invasion of bladder cancer cells [36].Interestingly, PFKP is one of the many genes identified as possible lung cancer oncogenes due to its role in galactose, fructose and mannose metabolism, and the pentose phosphate pathway [37]. In clear cell renal cell carcinoma (ccRCC), elevated mRNA levels of PFKP were found, and suppression of PFKP results in attenuated cell proliferation and accumulation of apoptotic proteins [38], implicating the involvement of PFKP in growth and survival of ccRCC.

Zinc finger and BTB domain-containing protein 7A (ZBTB7A), also known as POKEMON, is a protein in humans that is part of the POK transcription repressor family. Although the mechanism of action of ZBTB7A is not completely understood, it acts as a negative regulator of glycolysis through directly binding to the promoter region of the PFKP gene and repressing its transcription levels in ccRCC [39]. Deletion of ZBTB7A leads to an increase in PFKP mRNA and protein levels, enhancing the Warburg effect in ccRCC [39].

Glycolytic fluctuations always occur in hepatocellular carcinoma (HCC). Tat activating regulatory DNA-binding protein (TARDBP), a novel glycolytic regulator in HCC cells, down-regulates glycolysis through inhibition of the expression of PFKP by miR-520. By inhibiting PFKP expression, the TARDBP can lead to the inhibition of HCC cell growth [40]. In contrast, miR-186 represses proliferation of MKN45 and SGC7901 cells by negatively regulating HIF-1α, which results in significant downstream effects. These downstream effects involve programmed death-ligand 1 (PD-L1) and certain glycolytic enzymes such as PFKP [41].

13.4.3 PFKP Participates in Tumor Metastasis

Tumor metastasis is dependent on glucose uptake from the extracellular environment and its subsequent breakdown which results in lactate production through glycolysis [42]. PFKP participates in tumor metastasis by tightly regulating lactate production, which has the potential to stimulate cancer cell migration andmetastasis [43, 44]. Recently, some studies showed that WNT5A signaling can reduce migration/invasion and lactate production with significantly decreased PFKP expressionin breast cancer [42]. The WNT5A ligand is a glycoprotein that binds to a specific cellular receptor to initiate an intracellular signaling cascade resulting in various cellular processes, including cell proliferation, differentiation and migration. PFKP is positively regulated by β-catenin to control aerobic glycolysis and lactate production in breast cancer migration. The inhibition of aerobic glycolysis by WNT5A signaling occurs via a β-catenin-PFKP axis, resulting in reduced lactate production in breast cancer cells [42]. Blocking β-catenin by XAV939, a potent tankyrase (TNKS) inhibitor that antagonizes β-catenin-dependent WNT signaling significantly decreases PFKP expression in MDA-MB-468 and MDA-MB-231 breast cancer cells [42]. PFKP down-regulation by WNT5A signaling correlates with the aforementioned function of WNT5A to reduce lactate, and knockdown of PFKP by siRNAs significantly decreases lactate production and impairs migration in breast cancer cells [42]. These results suggest that PFKP expression levels can promote cell migration and invasion by modulating lactate production in breast cancer.

The transcription factor KLF4 acts as anoncogene in cancer cell migration and invasion. In breast cancer specifically, it has been found that KLF4 up-regulates glycolytic metabolism through direct binding to the promoter site of PFKP [5]. Once PFKP expression is activated by KLF4, glycolysis begins to occur at a high rate, promoting breast cancer metastasis. The findings confirm that PFKP is an

essential marker in tumor metastasis because the expression of the other phosphofructokinase isoforms, PFKL and PFKM, were unaffected by KLF4 due to the lack of KLF-binding regions in their promoters [5]. Overall, the study suggests that PFKP plays a major role in glycolytic metabolism related with cancer metastasis regulated by transcriptional initiation of KLF-4.

13.4.4 PFKP Is Marker for Poor Prognosis in Cancer

The prognosis of patients with non-small cell lung cancer (NSCLC) is determined by polymorphisms of certain genes associated with glycolysis. In a research study, 44 various SNPs were evaluated to determine a correlation with that of the prognosis of NSCLC [45]. Four of the studied SNPs, one being PFKP, were concluded to be highly significant in terms of survival outcomes [45]. Muscle-invasive bladder cancer is associated with PFKP expression [36]. Currently, the correlation between this specific cancer and the PFK family only illustrates a history of non-invasive bladder cancer, although there is no association with survival or disease relapse [36]. PFKP expression predicts the overall survival in breast cancer patients [42]. In an investigation of breast cancer survival using Kaplan-Meier survival analysis, the findings shows that high PFKP expression correlate with decreased patient survival while the other two isoforms of PFK-1 did not [42].

13.5 PFKP as a Novel Anticancer Target

As a major isoform of PFK-1 in cancer glycolysis, PFKP has become an emerging anticancer target. Until now, two possible strategies have been developed to target PFKP for cancer therapy: (1) direct inhibition of PFKP; and (2) interfering with its ability to associate with the cytoskeleton.

Recently, several small molecular inhibitors of PFK-1 have been reported. Acetylsalicylic acid (ASA; aspirin) is an anti-inflammatory drug that has anticancer properties [46]. One of the possible pathways that ASA suppresses in cancer is the alteration of tumor glucose utilization [46]. Through alteration of PFK-1 quaternary structure, ASA has been found to successfully inhibit cellular glycolysis in a dose dependent manner [46]. Thus, ASA is a powerful research component in the search for therapeutic measures to regulate glycolysis and eventually, cancer. An effective competitive inhibitor of PFK-1 is 2,5-anhydro-D-glucitol-1,6-diphosphate. In rat liver, PFK-1 phosphorylates 2,5-anhydromanitol to 2,5-anhydro-D-glucitol-1,6-diphosphate [47]. Similar to inhibition of PFK-1 by high concentrations of F2,6BP, high concentrations of 2,5-anhydro-D-glucitol-1,6-diphosphate leads to PFK-1 inhibition [47].

Alteration the effects of PFK-1in glycolysis by disrupting its interaction with the cytoskeleton may open a new avenue to block PFKP-mediated cancer metabolism.

One important cytoskeleton inhibitor is known as vinblastine, which is an anti-mitotic agent acting by inhibition of tubulin assembly [48]. Since vinblastine and PFK-1compete for tubulin binding, the inhibitory effect of vinblastine is hindered by the presence of PFK-1 [48]. Another known cytoskeleton inhibitor is paclitaxel. Research conducted on melanoma cells has shown that paclitaxel induces detachment of PFK-1 from the cytoskeleton [49].Due to this detachment of the PFK-1enzyme, there is a decrease in the production of F1,6BP, which further emphasizes the result of paclitaxel. Other cytoskeleton inhibitors include lidocaine and bupivacaine. Both of these inhibitors are local anesthetics that result in the removal of PFK-1 from the cytoskeleton. The result of this action is a decrease in ATP provisions in cytoskeleton-membrane areas. Overall, the addition of these local anesthetics on melanoma cells has been found to induce decreased cell viability.

Clotrimazole (CTZ), an antifungal azole derivative, has been proposed to act as an inhibitor of glycolysis [50]. More specifically CTZ inhibits glycolysis by acting on PFK-1 [51]. Of the two strategies mentioned earlier, CTZ utilizes the second strategy in which case the inhibitor interferes with the ability of PFK-1 to associate with the cytoskeleton. On a molecular level, the tetrameric form of PFK-1 dissociates from the cytoskeleton [50]. Research has concluded that the detachment of glycolytic enzymes from the cytoskeleton occurs before cell death. Thus, cell death does not result in the process of enzyme detachment. Instead, CTZ is able to induce separation [50].

As mentioned earlier, the pathway towards PFK-1 regulation involves F2,6BP which is produced from F6P. The substrate F2,6BP has been found to activate PFKP which allows for F1,6BP production. Controlling F2,6BP concentrations requires further regulation from PFKFB3. In order to reduce PFK-1 activation, PFKFB inhibitors have been studied further. PFK-158 is a novel PFKFB3 inhibitor. This inhibitor is in phase I clinical trials with hopes to treat advanced cancers [52]. Another important inhibitor associated with glycolytic disruption is itaconate. Unlike the inhibitors discussed earlier, itaconate is an analogue of phosphoenolpyruvate that does not inhibit PFK-1. Instead, itaconate is an inhibitor of F2,6BP [53]. Thus, the role of itaconate is to prevent the pathway of glycolysis through a mechanism that involves the inhibition of the F2,6BP that activates PFK-1. Research conducted on itaconate found that it reduces visceral fat in rat liver [53]. The proposed general mechanism for this is driven by the idea that all ingested carbohydrates go into glycogen synthesis and excess glucose moves towards fatty acid synthesis. However, in liver cells, itaconate prevents fat synthesis by inhibiting glycolysis.

13.6 Current Challenges and Future Prospects

Cancer cells maintain a high glycolytic status even during states of increased citrate levels and sufficient ATP concentrations in order to maintain the anabolic metabolic pathways which support cell proliferation and growth [5]. In order to maintain high

levels of glycolysis, certain rate-determining glycolytic enzymes such as PFKP need to be highly up-regulated. PFKP is involved in the "committed step" of glycolysis in which it catalyzes the conversion of F6P and ATP to F1,6BP and ADP. The reaction takes place when PFK is a complex tetrameric enzyme. In cancer cells, the characteristic activity or rate of PFKP in glycolysis is enhanced. A major disadvantage for the PFK-1 class of inhibitors is the inability to discriminate between each isozyme of PFK-1. Thus, these inhibitors cannot be used to specifically inhibit a certain isozyme of PFK-1, such as PFKP. Three isoforms of PFK-1 evolved from a common prokaryotic ancestor via gene duplication and mutation events. The N and C terminal major domains are similar in all three isoforms [54]. However, PFKP rather than other two isoforms is highly related with cancer growth and metastasis. Unlike PFKM and PFKL, PFKP is prominent in breast carcinoma, ascites tumors, B- and T-cell leukemias, and other types of cancer in which total PFK-1 expression is up-regulated [4]. Furthermore, PFKP is the primarily expressed PFK-1 isoform compared to PFKM and PFKL as well as the most closely correlated isoform with total PFK-1activity in GBM cells [4]. A mechanism to specifically target the PFKP isoform would consist of regulation of the PFKP S386 phosphorylation site [4]. Perhaps another means of targeting PFKP would be to stray from the traditional methods of studying mammalian tetrameric PFK-1 which use the native protein or recombinant protein formed from yeast or bacteria since this does not discriminate between the three major PFK-1 isoforms [6]. Instead, the production of recombinant PFKP by using a baculovirus expression system would overcome this current limitation [6].

Use of glycolytic inhibitors to target PFK-1 poses certain challenges that must also be recognized. Since various tissues of the body use glucose as the main energy source, disruption in the pathway of glycolysis may cause potential toxicities to these specific tissues [55]. The currently available glycolytic inhibitors are not potent as blockers of PFK-1 function and high concentrations are required for efficacy [55]. Thus, these glycolytic inhibitors, especially fortargeting PFKP, need to be revaluated with increased potency levels before being considered as effective therapeutic measures.

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Chapter 14
ATAD3A on the Path to Cancer

Yong Teng, Liwei Lang, and Chloe Shay

Abstract The ATPase family AAA-domain containing protein 3A (ATAD3A), a nuclear-encoded mitochondrial enzyme, is involved in diverse cellular processes, including mitochondrial dynamics, cell death and cholesterol metabolism. Overexpression and/or mutation of the ATAD3A gene have been observed in different types of cancer, associated with cancer development and progression. The dysregulated ATAD3A acts as a broker of a mitochondria-endoplasmic reticulum connection in cancer cells, and inhibition of this enzyme leads to tumor repression and enhanced sensitivity to chemotherapy and radiation. As such, ATAD3A is a promising drug target in cancer treatment.

Keywords ATAD3A · Mitochondria · Oncogene · Drug target · Cancer treatment

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14.1 Introduction

The AAA ATPase is a large and functionally diverse superfamily of NTPases that are characterized by a conserved AAA+ module [1, 2]. More than 53 members have been identified in this superfamily, and these proteins mainly localize to mitochondria and play diverse roles in cellular processes, including protein degradation, membrane fusion, peroxisome biogenesis, cytochrome assembly, regulation of enzymatic activity microtubule severing, helicase activity and gene expression [3]. ATPase family AAA domain-containing protein 3 (ATAD3), an ATPase localized in the mitochondrial inner membrane (MIM), was discovered as a novel molecular target of c-Myc [4, 5]. In primates and humans, ATAD3A, ATAD3B and ATAD3C are three tandemly repeated genes of ATAD3, essential for the development several kinds of organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculu*s [6–8]. ATAD3A functions as a critical regulator of lipid and fatty acid metabolism, contributing to metabolic diseases (e.g. cancer) involving disturbances in energy production and anabolism tightly related with mitochondria [6, 9, 10]. Here we summarize the biologic role of ATAD3A in the regulation of mitochondria-related metabolism, autophagy and apoptosis, communication between the endoplasmic reticulum (ER) and mitochondria in cancer development and treatments. Specific challenges in current research and future prospects are also discussed.

14.2 The Structural Characteristics and Modifications of ATAD3A

Compared with other types of organisms, primates including humans have three tandemly-repeated genes of ATAD3, ATATD3A, ATAD3B and ATAD3C, which are located in chromosome 1 at locus p36.33. ATAD3A is believed to be the ancestral gene, which was duplicated and mutated to generate the other two paralogs, ATAD3B and ATAD3C [11]. ATAD3A has three predicated transcript variants. Isoform 2 of ATAD3A acts as the main one being 586 amino acids (aa) in length, shorter than isoform 1, which is 634 aas and longer than isoform 3 at 507 aas [12]. The genetic structure and protein sequence of the ATAD3B is similar to ATAD3A [11]. Compared with its ancestor, ATAD3C is mutated on the translation initiation site. There is 87% identity in the similar region of the ATAD3C transcript and ATAD3A transcript 2 [11].

The putative promoter of ATAD3A contains numerous regulatory elements linked to cell growth, including CCAAT/enhancer binding protein (C/EBP), core binding factor (CBF), iron regulatory protein (IRP), cAMP response element binding protein (CREB), erythroid transcription factor (GATA-1), OCTAM-binding protein (Oct-1) and transcription factor II D (TFIID) [11]. The expression of ATAD3A is ubiquitous and has been detected in all tissues and cell lines tested, with

higher expression in the brain/cerebellum, heart, liver and kidney [12]. ATAD3B is expressed in human astrocytoma cell lines, in embryonic tissues and in the pituitary gland and heart [13, 14]. However, there is no available study regardingt ATAD3C expression.

ATAD3A can be divided into two parts, the N and C terminals. The N-terminal portion constitutes the specific domain of ATAD3A. It contains a flexible region rich in proline residues (aas 18–27), transmembrane domain 1 (TM1, aas 225–242), transmembrane domain 2 (TM2, aas 247–264), and two coiled-coil regions (CC1, aas 85–115; CC2, aas 180–220), offering an interaction zone which could be important for the oligomerization of ATAD3A monomers and/or for interaction with partners [15–17] (Fig. 14.1). The C-terminal part of the protein constitutes the ATPase domain, which is involved in the binding and hydrolysis of ATP, a second region of homology (SRH) motif, sensor I, sensor II, arginine (Arg) finger, and Walker A (WA) and Walker B (WB) sites [11, 16]. The N-terminal domain (aas 1–245) is positioned in the MIM and interacts with the mitochondrial outer membrane (MOM). The first 50 aas of the N-terminus are located close the mitochondrial surface. The topology of ATAD3 was analyzed and confirmed by trypsin digestion, which showed that the C-terminal domain is localized in matrix compartment [15, 18].

ATAD3A has been reported as a specific binding target of S100B, one member of the S100 family of EF-hand calcium binding proteins. S100B could assist the newly synthesized ATAD3A protein to fold and locate in the mitochondria [19]. ATAD3A contains many putative phosphorylation sites, including those for PKC, PKA, GSK3, CDC2, CKI, CKII, DNAPK, RSK, Cdk5, PKG and p38MAPK and INSR [11]. The most probable kinase for ATAD3A is protein kinase C at the possible phosphorylation sites Thr335, Thr338, Thr359 [18] (Fig. 14.1). The regulation of ATAD3A after phosphorylation and stabilization by PKC has been confirmed [20]. The expression of ATAD3A was found to be decreased by the pan-PKC inhibitor Calphostin C, and increased by ectopic expression of PKC isozymes [20].

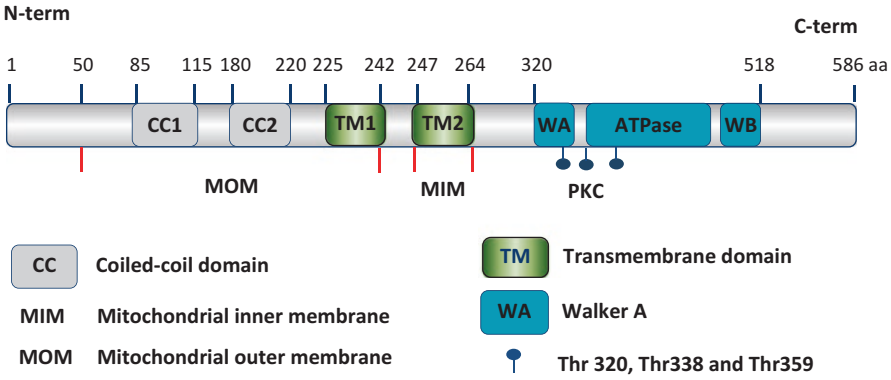

Fig. 14.1 The molecular ATAD3A structure with the main domains. The main domains and PKC phosphorylation sites of ATAD3A (isoform 2 for example) are exhibited

14.3 ATAD3A Functions as a Mitochondria Protein

It has been showed that ATAD3 plays an important role in mitochondrial dynamics that is mediated by fission and fusion of these organelles [15]. Knockdown of ATAD3A by RNAi in HeLa and lung cancer cells showed increased mitochondrial fragmentation and a decreased co-localization of mitochondria and the ER [15, 20]. The interactions of ATAD3A with mitochondrial fission (Drp1) and fusion (mitofusins, OPA1) proteins have been identified [6, 15, 18]. Mitochondrial fragmentation was also observed following over-expression of a deficient Walker A or mutant ATAD3A [15, 21]. Knockdown of Drp1 by siRNA in U373 cells inhibited mitochondrial fragmentation induced by mutant ATAD3A over-expression. As such, ATAD3A is required for the maintenance of mitochondrial integrity in mammalian cells. Mitochondrial DNA (mtDNA) molecules are usually clustered and attached on the inner mitochondrial membrane in nucleoprotein complexes called nucleoids [22]. The nucleoproteins mediating the interaction with MIM are important in mtDNA organization and distribution. ATAD3 appeared in HeLa cell mitochondrial nucleopro the possible binding to the D-loop of mtDNA [23, 24]. Gene silencing of ATAD3 by RNAi altered the structure of mitochondrial nucleoids and induced the dissociation of mitochondrial DNA fragments [25]. The nucleoid size is inversely correlated with ATAD3A/ATAD3B expression, although nucleoid number is tightly related with ATAD3 expression. Fibroblasts from human individuals with an ATAD3 deletion (chimeric ATAD3A/ATAD3B fusion gene) display mtDNA abnormalities [10].

14.4 The Role of ATAD3A in Lipogenesis

Mitochondria are important organelles for lipid metabolism. Knockdown of ATAD3 in *Caenorhabditis elegans* affects the intestinal fat tissue and the gonads at a time when these cells initiate mitochondrial biogenesis and lipogenesis [6]. ATAD3 is also a limiting factor for the processes of adipogenesis and lipogenesis in mouse adipocyte model 3T3-L1 cells [9]. Downregulation of ATAD3 inhibited adipogenesis, lipogenesis and impeded over-expression of many mitochondrial proteins. These phenotypes were rescued after ATAD3 re-expression. Lipogenesis was increased by over-expression of ATAD3 and inhibited by a dominant-negative mutant. Moreover, downregulation of lipogenesis by knockdown of ATAD3 was not related with insulin signaling, but linked to another mitochondrial protein, Drp1 [9]. ATAD3 has also been found to enhance hormonal-induced steroidogenesis in MA-10 mouse tumor Leydig cells [26]. Interestingly, ATAD3A has been identified in the mitochondrial-ER contact site formation, where it was found to transfer and metabolize cholesterol from the ER into mitochondria for steroidogenesis [15, 26, 27] (Fig. 14.2). Cholesterol can bind the translocator protein (TSPO) monomer, and with hormone stimulation, polymerized TSPO associates with the protein complex

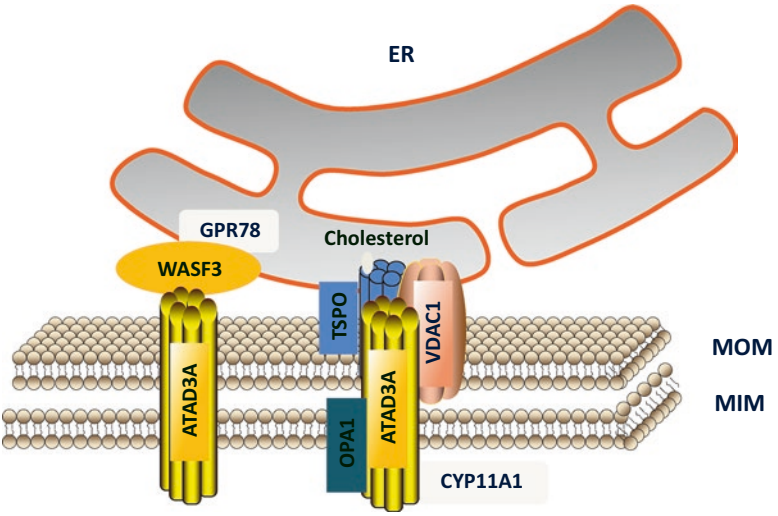

Fig. 14.2 ATAD3A interacts with WASF3 and other proteins for cholesterol import at the ER-mitochondria contacts. ATAD3A/WASF3/GPR78 protein complex and ATAD3A/TSPO/VDAC1 are the two main protein complexes are currently found at the contact sites of the mitochondria and ER

and voltage-dependent anion channel (VDAC1). Cholesterol bound to TSPO is translocated to the MIM through the formation of a contact site by VDAC1 and ATAD3A. Then, cholesterol is metabolized by CYP11A1 in the protein complex [27] (Fig. 14.2). The N-terminus of ATAD3A containing 50 aas in the MOM is associated with ER. Deletion of the ATAD3A N-terminus resulted in the reduction of hormone-stimulated progesterone biosynthesis [26]. The important role of ATAD3A in cholesterol metabolism has been linked to human disease recently. Deletion in the ATAD3 locus that generates the chimeric ATAD3A/ATAD3B fusion gene causes fatal congenital pontocerebellar hypoplasia in humans [10]. Fibroblasts from these individuals display altered cholesterol metabolism and mtDNA abnormalities. Moreover, cholesterol homeostasis disturbed by drugs can cause mitochondrial DNA disorganization in control cells [10].

14.5 The Role of ATAD3A in Development

ATAD3A is essential for the development of multicellular organism. Silenced ATAD3A by RNAi can cause severe defects in the worm *Caenorhabditis elegans*, including early larval arrest, gonadal dysfunction and embryonic lethality [6]. The indispensable role of ATAD3A in the development *Drosophila melanogaster* has also been confirmed. There is a high degree of similarity (70%) between the *Drosophila melanogaster* ATAD3A (dATAD3A) ortholog and human ATAD3A

[15]. Homozygous mutants of dATAD3A showed growth arrest during larval development [15]. Functional genomic studies identified the dATAD3A ortholog as a major gene positively regulated by the target of rapamycin (TOR) signaling pathway involved in cell growth and division [28]. ATAD3 deficient mouse embryos died around embryonic day 7.5 (E7.5) due to growth retardation and defective development of the trophoblast lineage immediately after implantation into the uterus [8]. This was caused by disrupting mitochondrial biogenesis and ATP production in mouse embryo. Fatal congenital pontocerebellar hypoplasia has been observed in humans with the deletion in ATAD3 locus that generates chimeric ATAD3A/ATAD3B fusion genes. In addition, rearrangements of the ATAD3 family genes can lead to late-onset encephalopathy [10].

14.6 The Role of ATAD3A in Human Diseases

14.6.1 ATAD3A in Cancer

Over-expressed ATAD3A has been found in different kinds of cancer, including head and neck cancer, gliomas, uterine cervical cancer, lung adenocarcinomas, prostate cancer and breast cancer [13, 18, 20, 29–32]. The gene alterations of ATAD3A, including those caused by mutation and amplification, are rare in different types of cancer. Elevated ATAD3A seems not to be directly related to gene alteration but rather to transcription and modification. ATAD3A was identified as one of the tumor antigens with strong overexpression in head and neck cancer [32]. It was also identified as one of cell surface antigens in acute myeloid leukemia (AML) [33]. Elevated ATAD3A expression increases glioma cell proliferation, and is tightly related with the growth and invasive potential of prostate cancer cells [13, 30]. ATAD3A was detected with a high expression rate in prostate cancer tissue compared with normal to non-tumor prostate epithelium (NTPE) or benign hypertrophic prostate epithelia (BHPE). Moreover, the ATAD3A expression levels were associated with disease status, tumor grade, serum prostate-specific antigen (PSA) level, lymphovascular infiltration as well as expression of the androgen receptor (AR) in prostate cancer [30]. ATAD3A overexpression was also detected in uterine cervical cancer patients [29]. A significant correlation has been found between ATAD3A expression and the presence of hrHPV (high-risk human papillomavirus), disease stage, lymph node involvement, and patient survival in uterine cervical cancer [29]. Additionally, silencing of the hrHPV E6/E7 expression decreased ATAD3A expression and uterine cervical cancer cell survival, which may be associated with p53 and pRB [34]. Mechanistic studies showed that HPV infection may stabilize ATAD3A expression to inhibit cell autophagy and apoptosis as well as increase chemotherapy drug resistance in uterine cervical cancer [29]. Elevated ATAD3A has been found in lung adenocarcinoma (LADC) cell lines and patient samples, and metastatic lymph nodes [18]. Moreover, LADC patients with over-expressed

ATAD3A have a high risk of recurrence and short time of survival [18]. Our previous study revealed that ATAD3A increased breast cancer metastasis, accompanied with GPR78 through the metastasis promoter WASF3 [31]. WASF3 is a member of the Wiskott-Aldridge family of proteins that are involved in actin polymerization tightly related with cell movement and invasion [31, 35]. ATAD3A interacts with WASF3/GPR78 at the ER-mitochondrial contact site (Fig. 14.2). Knockdown of ATAD3A showed reduction of cancer cell anchorage-independent growth and invasion in breast cancer MDA-MB-231 and colon cancer SW620 cells [31]. Silencing ATAD3A also suppressed breast tumor growth and metastasis in an orthotopic mouse model. Compared with the tumors derived by knockdown control breast cancer cells, the tumors generated by ATAD3A knockdown breast cancer cells were markedly smaller and showed significantly reduced expression of CD31, the marker for the tumor vasculature. Reduced metastases were also observed in lungs of the mice receiving ATAD3A knockdown breast cancer cells [31].

ATAD3A was identified as the most sensitive gene related with growth in the inhibition of the TOR pathway by rapamycin in *Drosophila melanogaster* [28]. The potential of a hyperactive mammalian TOR-ATAD3A axis needs to be confirmed and elucidated in cancer cells. Recently, a cell adhesion molecule, tumor suppressor Fat 1 protein (FAT1) was found to control cell growth and mitochondria function. ATAD3A is one of the interactors between FAT1 and mitochondria [36]. The mechanisms involved in ATAD3A-mediated cancer cell proliferation and survival are still unclear.

The mostly common types of cancer treatment are surgery, chemotherapy and radiotherapy. The resistance to chemotherapy and radiotherapy is a major cause of recurrence and mortality in cancer patients [37]. As a mitochondrial protein, ATAD3A has been identified as an anti-apoptotic factor in prostate cancer. Silencing ATAD3A expression in LNCaP cells markedly decreased the resistance to cisplatin treatment [30]. ATAD3A and ATAD3B are located on chromosome 1 (1p36-33) and usually lose their heterozygosity in the large distal regions of chromosome 1 in human oligodendrogliomas. Compared with the oligodendroglimas, astrocytomas are chemoresistant with an intact ATAD3A/ATAD3B gene on chromosome 1 [13]. It has been found that human glioma cell lines with over-expressed ATAD3A showed chemo-resistance to doxorubicin and temozolomide (TMZ) [20]. Moreover, high ATAD3A-expressing T98G cells exhibited higher resistance to radiotherapy compared with low ATAD3A-expressing U87MG cells. Knockdown of ATAD3A in T98 cells impaired the colony-formation ability. On the other hand, ectopic ove-expression of ATAD3A increased the radiation resistance [20]. Knockdown of ATAD3A using siRNA in uterine cervical cancer cells increases cell autophagy and apoptosis, and decreases resistance to anticancer drugs [29]. In lung cancer, knockdown of ATAD3A increased mitochondrial fragmentation and cisplatin sensitivity [18]. Serum starvation increases ATAD3A expression in lung cancer cells in a dose- and time-dependent manner [18]. The increase of ATAD3A expression is not caused by transcription but occurs more likely at the translational level. The cisplatin resistance is also associated with the increase in ATAD3A expression in response to serum starvation [18].

14.6.2 ATAD3A in Other Diseases

Dysfunctional central nervous system and neurological disorders are common clinical features of mitochondrial disorders. As a vital mitochondrial protein, ATAD3A gene alterations, including deletions and mutations, has been identified recently as a cause to these central nervous system dysfunctions [10, 21, 38]. 1p36 deletion syndrome is a congenital genetic disorder characterized by intellectual disability, delayed growth, hypotonia, seizures and other features [39]. The recent studies showed the potential role of ATAD3A located in 1p36 with this deletion syndrome. This has been observed in the fatal congenital pontocerebellar hypoplasia in humans with the biallelic deletion in ATAD3 locus that generates chimeric ATAD3A/ATAD3B fusion genes. The rearrangements in the ATAD3 genes, affecting the ATAD3B/ATAD3C genes on one allele and ATAD3A/ATAD3B genes on the other, manifest as later-onset encephalopathy with cerebellar atrophy, ataxia and dystonia [10]. The impaired mtDNA organization and cholesterol metabolism related with deletion of ATAD3A offers a pathogenetic explanation for these disorders. De novo mutations in ATAD3A c.1582C-T (p.Arg528Trp) has been found to result in global delayed development, hypotonia, spasticity, optic atrophy, axonal neuropathy and hypertrophic cardiomyopathy [38]. Tissue-specific overexpression of dATAD3AR534W, the Drosophila mutation homologous to the human c.1582C-T (p.Arg528Trp) variant, leads to a dramatic decrease in mitochondrial content, aberrant mitochondrial morphology and increased autophagy. Homozygous null dATAD3A larvae showed a significant decrease of mitochondria. ATAD3A variations represent an additional link between mitochondrial dynamics and recognizable neurological syndromes. Another dominantly inherited heterozygous variant c.1064G -A (p.G355D) in ATAD3A has been identified in two patients presented with hereditary spastic paraplegia (HSP) and dyskinetic cerebral palsy [21]. The dominant-negative patient mutation affects the Walker A motif, which is responsible for ATP binding in the AAA module of ATAD3A. The recombinant mutant ATAD3A protein has markedly reduced ATPase activity. Overexpression of the mutant ATAD3A fragments the mitochondrial network and induces lysosome mass [21].

14.7 Targeting ATAD3A for Novel Anticancer Therapy

ATAD3A overexpression has been confirmed to relate with cancer growth, metastasis and resistance to chemotherapy and radiotherapy. As a potential oncogene, ATAD3A represents a good candidate to develop novel therapy to combat cancer. However, there are no specific inhibitors of ATAD3A available. Two chemicals have been identified to decrease the ATAD3A expression. Calphostin C, the inhibitor of PKC, destabilizes ATAD3A through blocking its phosphorylation by PKC [18]. The other chemical drug is resveratrol, which provides a number of anti-aging health

benefits including improved metabolism, cardioprotection, and cancer prevention [40]. Resveratrol can reduce ATAD3A expression with increased cellular autophagy and apoptosis [29].

14.8 Conclusions and Future Prospects

ATAD3A, as a vital mitochondrial AAA-ATPase family member, shows essential roles in the lipid metabolism and ER-mitochondria communication. Mutation and deletion of ATAD3A is related with neurological disorders, while overexpression of ATAD3A tightly linked with cancer growth and metastasis. Cancer is mainly believed to be a disease with disrupt energy production and nutrient metabolism [41]. Elevated ATAD3A increases cancer cell anabolism especially for steroid synthesis and resistance to chemotherapy and radiotherapy at least in part through inhibition of autophagy and mitophagy. Several molecular mechanisms have been demonstrated recently to regulate ATAD3A in the control of mitochondrial functions, including the mTOR-ATAD3A functional axis and the ATAD3A-FAT1 interaction. Moreover, ATAD3A is involved in cancer metastasis through interacting with WASF3 and GRP78 at ER-mitochondria contacts. These findings support the critical role of ATAD3A in cancer development and progression. However, more regulations between ATAD3A and oncoproteins or tumor suppressors need to be elucidated.

Ion radiation affects various aspects of mitochondrial physiology, including mitochondrial respiration, ATP production and mitochondrial dynamics [42, 43]. Thus, investigating whether or not ATAD3A increases resistance to radiotherapy through modulating mitochondrial functions is an emerging research direction. Elevated ATAD3A has been shown to protect cancer cells from cytotoxic chemotherapy. Undoubtedly, novel therapies targeting ATAD3A will be investigating in the clinic as a potential single or combined anticancer agents in the future.

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Chapter 15
Impact of Exercise on Inflammatory Mediators of Metabolic and Vascular Insulin Resistance in Type 2 Diabetes

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Abstract The development of obesity is cornerstone in the etiology of metabolic and vascular insulin resistance and consequently exacerbates glycemic control. Exercise is an efficacious first-line therapy for type 2 diabetes that improves insulin action through, in part, reducing hormone mediated inflammation. Together, improving the coordination of skeletal muscle metabolism with vascular delivery of glucose will be required for optimizing type 2 diabetes and cardiovascular disease treatment.

Keywords Glycemic control · Insulin resistance · Insulin secretion · Exercise · Energy balance

15.1 Introduction

According to the World Health Organization [1], an estimated 380 million adults worldwide have type 2 diabetes (T2D). Type 2 diabetes is mainly characterized by insulin resistance and subsequent loss of β-cell functionality. Excess consumption of calories relative to low physical activity/exercise energy expenditure is considered a major contributor to the development of T2D as adiposity alters a variety of circulatory factors (leptin, free fatty acids, ET-1, IL-6, EVs, etc.) that drive reduced insulin action. Moreover, obesity is considered a chief component explaining why individuals with T2D are at a two- to four-fold increased risk for the development

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of cardiovascular disease (CVD) [2]. In fact, there are several overlapping etiological factors between T2D and CVD that support the idea that these two diseases share a "common soil" [3]. Since the proposal of the common soil hypothesis, insulin resistance in skeletal muscle has been highlighted as a factor that not only promotes reduced insulin-stimulated glucose uptake but also endothelial dysfunction [4, 5]. The primary explanation for how these metabolic and vascular abnormalities in insulin action share a common root remains to be fully elucidated, but inflammation is a leading candidate since it precedes T2D [6] and is associated with CVD development [7]. Interestingly, vascular dysfunction, specifically at the capillary and arteriolar endothelium, has been hypothesized to play a central role in the initiation of metabolic insulin resistance and T2D [8]. Thus, efforts should be placed on designing therapies that target reducing inflammation as a means to reduce T2D and CVD prevalence and severity. Exercise training is established to reduce insulin resistance [9, 10] as well as improve glucose tolerance [11, 12] and endothelial function in obese individuals [13–15]. However, the mechanism by which exercise decreases T2D and CVD risk remains largely unclear. Herein, we highlight novel metabolic and vascular mediators that interact with adipose tissue and contribute to T2D and CVD development. We also discuss how exercise, along with proximal dietary consideration, consequently affects these markers. Focus is placed on endurance exercise given that it is recommended for individuals most days of the week and increased aerobic fitness is established to decrease mortality [16].

15.2 Exercise Medicine for Diabetes Care

It comes as no surprise that the American Diabetes Association and the American College of Sports Medicine advocate for comprehensive lifestyle programs that increase physical activity while recommending a low-fat diet [17]. Indeed, current lifestyle recommendations for individuals at risk for T2D is to target weight loss of approximately 5–10% and increase physical activity to either 150 min/week or more of moderate intensity or 75 min/week of vigorous intensity aerobic exercise. Lifestyle interventions consisting of increased physical activity (i.e. aerobic and/or resistance exercise) and low-fat diet promote approximately 5 kg weight loss for 2 years or beyond and lower T2D risk by approximately 45% [18]. Interestingly, there appears to be a dose-response relationship with weight loss (i.e. energy deficit) and glycemic control, whereby a 2–10% reduction in body weight between 1–4 years is paralleled by decreases in HbA1c of 0.2–1.0% [18]. This improvement in glycemic control is clinically meaningful as lifestyle prescriptions of >5% weight loss via reductions in total fat under 30% of total calorie intake (with <10% coming from saturated fat), elevation in fiber consumption (i.e. 15 g/1000 kcal) and increases in physical activity (30 min/day) lowered the cumulative incidence of diabetes by 58% in people with prediabetes compared with controls [19]. In the landmark U.S. Diabetes Prevention Program (DPP) Study, an intervention based on 150 min/week and weight loss of approximately 7% reduced new diabetes cases by 58% in

people with prediabetes [20]. Further, subjects who lost the most weight and met physical activity/diet targets had >90% risk reductions of diabetes. These reports are consistent with emerging work demonstrating that higher intensities of aerobic exercise may benefit glucose control [21, 22] and increase exercise adherence more than moderate intensity exercise [23]. However, work from our group suggests that once exercise intensity is matched for energy expenditure differences in insulin action are not observed [24, 25]. These later observations highlight that the amount of energy expenditure may be critical for insulin sensitivity benefit. To this end, current work highlights that higher volumes appear related to greater glucose regulation [26–30] and cardio-metabolic health [31] as well as may be an important component of designing proper "doses" of exercise to improve HbA1c levels [32, 33]. How exercise contributes to this improved glycemic control above and beyond increases in insulin sensitivity remain to be determined, but much work in recent years have begun to focus on inflammation.

15.3 Adipokines

Hormones secreted from adipose tissue are collectively referred to as adipokines. These adipose derived hormones include both anti-inflammatory (e.g. adiponectin) and pro-inflammatory (e.g. leptin, FFA, etc.) chemicals that have direct impact in skeletal muscle and vascular glucose regulation [34].

15.3.1 Adiponectin

Adiponectin is an anti-inflammatory hormone that is mainly produced by adipocytes. Adiponectin decreases lipogenesis and increases fatty acid oxidation via activation of AMPK and PPARα pathways in the adipocyte, hepatocyte and myocyte [35, 36]. This adipokine also decreases gluconeogenesis [37] and upregulates insulin receptor substrates specifically in the liver to support glucose homeostasis [38]. In skeletal muscle, adiponectin increases glucose uptake and insulin sensitivity [36]. Further, adiponectin inhibits reactive oxygen species (ROS) production in endothelial cells and restores endothelial nitrous oxide synthase (eNOS) coupling [39], thereby favoring large conduit and microcirculatory blood flow. Together, adiponectin exerts favorable metabolic and vascular effects that protect against systemic inflammation impairing insulin action.

The high molecular weight (HMW) isoform of adiponectin is the most biologically active in regards to effects on insulin sensitivity [40]. In obesity and T2D, lower levels of HMW adiponectin are observed [41]. The lowering of HMW adiponectin is thought to be due to increases in extracellular redox [42], which converts HMW adiponectin to the low molecular weight isoform of adiponectin [43]. Further, during excess energy balance, adipocytes undergo hypertrophy and promote the

release of TNF-α [44]. This is problematic because TNF-α inhibits secretion of adiponectin and lowers circulating HMW levels [45]. As levels of HMW adiponectin lower, not only are less favorable metabolic effects observed, but β-cell dysfunction ensues [46]. Thus, adiponectin appears to be a potential insulin sensitizing target.

15.3.1.1 Effects of Exercise Training on Adiponectin

Exercise training is a beneficial treatment to increase secretion and circulating levels of adiponectin. After 6 weeks of high intensity exercise, HMW adiponectin increased and correlated with improvements in fat oxidation and insulin sensitivity in adults with T2D [47]. Similarly, 12 weeks of high intensity exercise increased circulating total adiponectin as well as adiponectin mRNA expression from adipocytes in obese men [48]. However, it is important to note that not all research supports exercise as a method to improve adiponectin levels. Hulver et al. [49] reported no change in total adiponectin levels despite insulin sensitivity increasing following 24 weeks of exercise. Further, no weight loss was observed within the study, suggesting that changes to adiponectin may occur only in the presence of weight loss. Conversely, others demonstrate that plasma adiponectin levels improve in overweight males after 10 weeks of exercise, independent of weight loss [50]. In addition, as little as 7 days of exercise significantly increased HMW adiponectin in older adults, and this increase in HMW adiponectin was directly related to improved insulin sensitivity and glucose tolerance [51]. It should be noted that most exercise studies to date have measured total adiponectin as opposed to HMW adiponectin. This is important as HMW adiponectin alterations are thought to be exercise dose-dependent [52], suggesting that interventions in which no change occurred could be the result of an inadequate exercise dose.

15.3.2 Leptin

Leptin is produced by adipose tissue and aids in the regulation of appetite, body weight and glycemia [53]. Contrary to adiponectin, leptin is a pro-inflammatory adipokine that stimulates oxidative and arterial stress [54] via the production of adipose-derived ROS, TNF-α and IL-6 [55]. Leptin secretion is thought to be proportional to adipocyte size, as individuals with obesity have higher circulating levels [53, 56]. However, individuals with T2D tend to have higher leptin levels independent of body fat [57] and it has been suggested that obese individuals, with or without T2D, become resistant to the action of leptin. In turn, hyperleptinemia ensues and has deleterious effects on glucose homeostasis via the hypothalamus [58] and peripheral insulin actions in the vasculature, skeletal muscle, liver as well as adipose

tissue [59, 60]. In addition, leptin may also inhibit β-cell insulin secretion through activation of ATP-sensitive calcium channels [61] as well as down-regulate proinsulin gene expression [62]. Further, data suggest that elevated leptin levels promote increases in inflammatory mediators in adipose (e.g. TNF-α, IL-6) and the vasculature (ET-1) [63] and contribute to arterial thrombosis [64] as well as endothelial dysfunction [63]. Therefore, leptin appears to be an important link between energy imbalance and T2D and CVD [65].

15.3.2.1 Effects of Exercise Training on Leptin

As leptin levels correlate directly with body mass [53, 56], it is not surprising that weight loss is proposed to decrease leptin secretion from adipocytes. This idea is supported by studies in which 12–16 weeks of exercise, with or without a low calorie diet, reduced circulating leptin as a result of weight loss [66]. However, Hickey et al. [67] reported that 12 weeks of moderate exercise lowered leptin levels in sedentary women, despite no alterations in fat mass. In addition, short-term exercise (7 days) effectively lowed leptin concentrations in obese adults with insulin resistance and this paralleled glucose tolerance [51]. These data suggest that exercise effectively lowers plasma leptin in the absence of weight loss for metabolic benefit. However, a limitation of these studies was that energy balance was not tightly controlled following exercise, such that adults may have been in an energy deficit. Subsequently, it remains unclear if exercise acts in part to reduce leptin and other inflammatory mediators through creating an energy deficit that fosters metabolic and/or vascular adaptation. Further work into the role of energy availability on the coordination of metabolic and vascular glucose regulation is required.

15.3.3 Free Fatty Acids

Adipose tissue is the primary supplier of circulating fatty acids. Typically in the fed state, free fatty acids (FFAs) decrease in response to the action of insulin on reducing hormone sensitive lipase activity. However, individuals with T2D are often adipose insulin resistant and continue to release FFAs into the general circulation. Moreover, FFAs can be released into the circulation in response to increased adipose tissue size, despite no changes in the rate of lipolysis [68, 69]. A major concern for elevated FFAs is that insulin-stimulated glucose uptake is inhibited in people, regardless of diseases status [70–72] through an inflammatory NFk-B mediated pathway [73]. In particular, FFAs have been purported to reduce skeletal muscle insulin-stimulated glucose uptake through impairments in glycolysis, accumulation of lipid species (e.g. diacylglycerols and/or ceramides) or incomplete lipid molecules (e.g. acylcarnitines) [74]. In regards to the endothelium, increased FFAs

concentration impairs NO bioavailability in the vascular wall [75]. This is thought to be due to FFA-induced action of PKCβ, an isoform in endothelial cells that inhibits the PI3K pathway and protein kinase b (Akt) and reduces NOS phosphorylation [76]. Research in individuals with T2D shows that inhibition of PKCβ reduces inflammatory activation and improved NO bioavailability as well as brachial artery flow-mediated dilation [77]. Therefore, FFAs are believed to play a crucial role in mediating insulin resistance and endothelial function.

15.3.3.1 Effects of Exercise Training on FFAs

We have shown that changes in circulating FFAs following moderate intensity training are directly related to improved peripheral insulin sensitivity [10] and short-term interval or continuous exercise increases adipose insulin sensitivity in adults with prediabetes [24]. However, few studies have directly examined the effect of FFAs on insulin resistance before and after exercise training. Solomon et al. [78] compared a eucaloric diet plus exercise to a hypocaloric diet plus exercise (i.e. diet-imposed 500 kcal/day deficit plus exercise induced energy deficit) in older, obese, sedentary adults. Despite individuals in the hypocaloric diet group losing approximately 4 kg more weight compared with people in the euclaoric group, clamp-derived peripheral insulin sensitivity following lipid infusion improved similarly in both groups. This was directly related to reduced FFA turnover and oxidation, suggesting that energy-induced energy deficit improves insulin action due to lower FFA availability in skeletal muscle. While no study to date has investigated the effect of lipid infusion on vascular insulin sensitivity or endothelial function following training, a cross-sectional study demonstrated that aerobic fitness was directly correlated with preservation of insulin-stimulated microcirculatory function in healthy young adults [79]. Moreover, recent work examined the effect of high fat diet on endothelial function following interval versus continuous training for 12 weeks in healthy inactive young adults, and reported that interval exercise increased brachial artery conduit artery function more so than continuous training alone [80]. Together, fitness mediated mechanisms are likely important for combating FFA-induced muscle and vascular insulin resistance.

15.4 Vascular Mediators

The vasculature releases various molecules that play critical roles in not only blood flow, but also endothelial function and arterial compliance. As a result, focus on how exercise modulates the coordination of nutrient utilization with delivery is critical for cardio-metabolic health.

15.4.1 Cellular Adhesion Molecules

Intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) are involved in the regulation of leukocyte migration and adhesion to the endothelium [81]. These cellular adhesion molecules (CAMs) are released from cell membranes through shedding and/or proteolytic cleavage. Expression of ICAM-1 and VCAM-1 on the surface of arterial endothelial cells is typically low. However, these CAMs are elevated in obesity [82], T2D and CVD [83, 84]. Interestingly, elevated CAM levels are also observed in individuals with endothelial dysfunction prior to T2D [85], supporting the notion that loss of vascular tissue integrity contributes to microvascular dysfunction and T2D risk.

ICAM-1 and VCAM-1 are expressed by endothelial cells and leukocytes in response to inflammatory cytokines, ROS and endothelial stress [86, 87]. Elevated CAMs promote inflammation in the vascular system via the accumulation of macrophages within the intima of the endothelium [88, 89]. These processes are thought to accelerate endothelial dysfunction and aid in T2D and CVD progression [90]. ICAM-1 and VCAM-1 are considered biomarkers for microvascular dysfunction [91, 92] as well as significant predictors of T2D incidence [93]. Collectively, these data highlight ICAM-1 and VCAM-1 as potential targets for improving vascular delivery of insulin for action on skeletal muscle glucose uptake.

15.4.1.1 Effects of Exercise Training on CAMs

While training improves endothelial function and overall cardio-metabolic health, data regarding effects of exercise on ICAM-1 and VCAM-1 are equivocal. For example, Ryan et al. [94] reported that while aerobic exercise, with or without weight loss, had no effect on circulating ICAM or VCAM, those individuals with the greatest reduction in circulating ICAM had the largest increase in insulin sensitivity. Further, Zoppini [95] observed that 24 weeks of progressive exercise training at a moderate intensity resulted in significant decreases of ICAM-1 in adults with T2D, independent of weight loss. In contrast, others have not observed changes in ICAM-1 or VCAM-1 following 12–24 weeks of aerobic exercise in obese adults with normal glucose tolerance or T2D [96, 97]. It should be noted that the most beneficial effects were observed when both exercise and diet were altered. In fact, when aerobic exercise and a low fat, high fiber diet were implemented concurrently, decreases in ICAM-1 and VCAM-1 were seen in obese adults with and without T2D [98, 99]. Thus, while these findings highlight that exercise training can improve overall cardio-metabolic health through modification of CAMs in sedentary adults at risk for T2D and CVD. More research is required to better understand the dose and intensity of exercise, with or without dietary alterations, on the relationship between CAMs and insulin sensitivity.

15.4.2 Endothelin-1

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by the endothelium [100]. Under normal physiological conditions, insulin promotes nitric oxide production by activating endothelium-derived nitric oxide synthase (eNOS) through a PI3K-dependent pathway. However, in insulin resistance, the PI3K pathway is impaired and the MAPK pathway is activated and ET-1 production is increased [101]. This increased production of ET-1 and exacerbated activation of the MAPK pathway is purported to drive the pathogenesis of endothelial dysfunction [102] and atherosclerosis [103]. Increased ET-1 levels are observed in obesity [104], T2D [105, 106] and CVD [107, 108]. Interestingly though, ET-1 is significantly elevated in obese and overweight adults, independent of other cardiovascular risk factors [104]. This is thought to be attributed to increased sympathetic nervous system activity [109] as well as oxidative stress [110], both of which are exaggerated in obesity [111, 112]. Further, elevated receptor-mediated ET-1 vasoconstriction has been directly linked to impairments in acetylcholine-stimulated endothelium-dependent vasodilation and promotes endothelial dysfunction as well as reduced glucose delivery to skeletal muscle for subsequent utilization [104].

15.4.2.1 Effects of Exercise Training on ET-1

There are few human data examining the effect of training on ET-1 as it relates to endothelial function, and no data relating ET-1 on vascular insulin sensitivity. Nonetheless, exercise reduced ET-1 in healthy sedentary adults after 8 weeks, suggesting potential vascular benefit [113]. Moreover, 12 weeks of exercise and a low-calorie diet lowered ET-1 concentrations in adults with impaired glucose tolerance [114], although it is unknown if these two therapies were additive or if one is superior regarding this vascular outcome. In either case, 12 weeks of exercise training reduced plasma ET-1 levels as well as mediated vasculature tone in obese adults [109]. These findings were also observed independent of weight loss, suggesting that fitness improved vascular function through ET-1.

15.4.3 Nitric Oxide

Nitric oxide (NO) is a signaling molecule that has a cellular, metabolic and vascular role [115]. eNOS is responsible for a majority of the NO produced in the endothelium [116]. This enzyme synthesizes NO in a pulsatile manner in response to increased intracellular calcium levels [117]. However, shear stress can also activate eNOS, independent of calcium [118]. NO produces vasodilation and reduces vascular smooth muscle cell migration, platelet aggregation and thrombosis, as well as monocyte and macrophage adhesion and inflammation [119]. Due to these actions, NO is considered to be a protective molecule in the vasculature.

In obesity and T2D, eNOS activity is impaired [120]. This impairment is thought to be in large part due to oxidative stress and inflammation. Oxidative stress and pro-inflammatory factors, such as TNF-α, promote the endothelium to express adhesion molecules (e.g. CAMs) that accelerate the inflammatory process [88]. This, in turn, interferes with the activation and expression of eNOS for reduced large conduit and microcirculatory blood flow [76]. Indeed, the inhibition of eNOS disturbs microvascular recruitment and blunts muscle glucose uptake in response to insulin [121] via reduced NO bioavailability [122]. Decreased NO bioavailability is a key factor of endothelial dysfunction and seems to play an important role in the development of insulin resistance [123].

15.4.3.1 Effects of Exercise Training on NO

Exercise training programs lasting 8–12 weeks have shown positive effects on NO bioavailability [124] and overall endothelial function [125]. These benefits have been observed in a wide array of disease states, including hypertension [126], T2D [125] and coronary artery disease [127]. In fact, healthy adults also show improved NO production [113] and responses to acetylcholine mediated conduit artery function following exercise training [124]. The increased NO bioavailability after training can be attributed to several effects. Exercise induces laminar shear stress within the vascular system [128]. This elevation in arterial shear rate is known to increase NO generation and bioavailability within endothelial cells through an increase in eNOS expression and phosphorylation [129]. In addition, the cumulative effects will reduce NO degradation and subsequently increase NO half-life [130], which is consistent with recent work showing a single bout of exercise enhances insulin-stimulated microvascular function, in part, through NO [131]. Overall, habitual exercise positively effects NO production and bioavailability and should be considered a therapeutic option to enhance vascular insulin sensitivity for glucose delivery and control.

15.4.4 Extracellular Vesicles

Extracellular vesicles (EVs) are cell bodies that carry and transfer proteins, lipids and nucleic acids to promote communication between organs [132]. Interestingly, EVs are elevated in people with prediabetes [133], T2D and CVD [134, 135] as well as hypertension [136] and have been suggested to serve as a biomarker of cardiometabolic health. It is physiologically important to acknowledge that EVs carry markers of their parent cell, thereby allowing for specific subpopulation identification (e.g. endothelium or leukocyte-derived) [132, 137] that can influence cross-talk between tissues and cells [137]. In fact, increased endothelial EVs in the circulation are considered to reflect vascular injury, while elevated leukocyte and platelet EVs represent pro-inflammation and coagulation, respectively. Thus, EVs are likely to

impact vascular health in a cell specific manner, potentially through an inflammatory mediated mechanism [138].

One possible mechanism by which EVs contribute to T2D and CVD relates to insulin resistance. Recent work demonstrates that macrophage-derived EVs (M0 THP-1) impair GLUT-4 translocation by decreasing p-Akt in human adipocytes [139]. This blunted GLUT-4 translocation was paralleled by activation of NfK-B, suggesting that inflammation may play a role. In addition, other studies have reported that adipocyte-derived EVs (CD14+) interfere with insulin signaling in both the liver [140] and skeletal muscle [141] via the transfer of adipokine content, thereby inducing insulin resistance. Another factor that may mediate disease relates to vascular physiology and blood flow. Werner et al. [142] reported that elevated endothelial EVs (CD31+/annexin V+) are correlated with reduced endothelium-dependent vasorelaxation. This is consistent with others reporting that elevations in these same endothelial EVs are related to reduced flow-mediated dilation as well as increased pulse wave velocity and carotid intima-media thickness [143, 144]. A possible reason EVs decrease vascular function may be related to in vivo inflammation. In vitro experiments demonstrate T-cell EVs promote TNF-α and IL-1β by monocytes [145], as well as promote the interaction and adhesion of leucocytes to endothelial cells [146]. These later findings are consistent with work by Mastronardi [147] demonstrating that injection of EVs from blood of patients with sepsis into mice promotes increased expression of iNOS, COX-2, and NFk-B in the heart and lung, thereby supporting a direct role of EVs at producing inflammation. Given the literature linking inflammation and oxidative stress to the pathogenesis of insulin resistance and endothelial dysfunction [148], EVs appear to be a potential target for improving cardio-metabolic health.

15.4.4.1 Effects of Exercise Training on EVs

There are few long-term exercise training studies that have investigated the relationship of EVs and T2D and/or CVD risk, and none have investigated if training increases skeletal muscle or vascular insulin sensitivity through an EV-mediated mechanism. Nonetheless, work by our group [149] recently reported that EVs correlate with aerobic fitness (i.e. VO_2 peak) and other cardiometabolic health factors in obese adults, highlighting that fitness may modulate EVs. Interestingly, these findings are consistent with other work reporting that 12–24 weeks of aerobic exercise with weight loss significantly lowered endothelial EVs (CD31+/CD41a; CD62E+) in pre-hypertensive men and women [150], as well as in African American women [151, 152]. Taken together, it would seem that exercise training may confer T2D and CVD risk reduction through reductions in circulating EVs. However, it is worth recognizing that not all exercise training studies on EVs show a reduction. Dimassi et al. [153] reported that 8 weeks of aerobic exercise increased circulating EVs in women with obesity, and that the micro-RNA (124a and 150) from these EVs correlated with adiponectin, TNF-α and IL-6 levels. Moreover, Kretzschmar et al. [151] demonstrated that endothelial EVs (CD31+/CD42b) [154] were

unchanged in post-menopausal, compared to a decrease in pre-menopausal, women following exercise training. It is not clear why post-menopausal women did not respond to exercise, but it may relate to the decrease in estrogen providing cardioprotective effects [155] or the notion that some individuals are "exercise resistant" to specific outcomes [156]. In either case, it appears that EVs are a novel target mechanism for modulating training responses and warrant further attention to elucidate the mechanism [157]. In fact, several recent studies suggest that acute bouts of exercise exert effects on EVs [158]. This highlights the point that EVs are responsive and may play roles in the adaptation process for health and disease.

15.5 Myokines

Skeletal muscle secretes hormones known as myokines. These skeletal muscle-derived hormones have been reviewed extensively elsewhere [159]. However, the focus here is on recent work describing how IL-6, myonectin, and vascular endothelial growth factor-A (VEGF) impact T2D and CVD risk.

15.5.1 IL-6

Interluekin-6 (IL-6) is both a pro-inflammatory adipokine as well as an anti-inflammatory myokine [160]. In this review, we focus on IL-6 and its effects as a myokine. Although skeletal muscle cells are capable of producing IL-6 in response to various stimuli, such as elevations in ROS and inflammatory cytokines [161], secretion predominantly occurs during skeletal muscle contraction [162]. IL-6 enhances glucose and lipid metabolism as well as insulin signaling [163] via activation of AMPK [164]. Furthermore, this myokine is believed to be an energy sensor as IL-6 release is enhanced when glycogen levels within the muscle are low [164] to stimulate hepatic glucose production [160].

As highlighted earlier, IL-6 is pro-inflammatory when released as an adipokine [7]. Therefore, it is difficult to quantify circulating IL-6 levels in disease states without performing invasive procedures. As an anti-inflammatory myokine, IL-6 is believed to inhibit TNF-α production and reverse insulin resistance [165]. Further supporting this idea, blocking IL-6 in patients with rheumatoid arthritis led to elevated cholesterol and plasma glucose levels [166], suggesting that lacking IL-6 may lead to insulin resistance and an atherogenic profile. However, another study in mice showed that IL-6 infusion reduced insulin-stimulated glucose uptake while subsequently raising FFA levels [165]. While the role of IL-6 in insulin sensitivity remains controversial, IL-6 production may represent a compensatory mechanism in individuals at risk for developing insulin resistance to stimulate alternative glucose homeostasis mechanisms [165].

15.5.1.1 Effects of Exercise Training on IL-6

As previously mentioned, IL-6 is secreted by skeletal muscle fibers in response to muscular contraction. IL-6 is increased during and immediately following exercise [162, 164]. The magnitude to which IL-6 is elevated is dependent upon the exercise intensity [167]. Following acute exercise, elevated levels of IL-6 promote an increase in IL-1ra and IL-10, two anti-inflammatory cytokines that are believed to dampen and protect against chronic low-grade inflammation [162, 164]. In addition, acute increases in circulating levels of IL-6 result in improved fat oxidation and insulin-stimulated glucose uptake [162, 164]. Chronic exercise training on the other hand appears to reduce plasma IL-6 but up-regulate expression of its receptor [168]. This suggests that IL-6 sensitivity is increased following chronic exercise. However, it is difficult to differentiate the effects of the muscle versus adipose tissue on circulating IL-6 [169, 170]. Furthermore, it cannot be determined if the benefits observed from exercise are a result of the last acute bout or chronic training. Nevertheless, data support the idea that exercise positively affects the IL-6 profile and is a contributor to improved insulin sensitivity and improved glucose tolerance following exercise [163, 171].

15.5.2 Myonectin

Myonectin, also known as CTRP15, is expressed and secreted predominantly from skeletal muscle [172]. This recently discovered myokine is thought to facilitate cross-talk between skeletal muscle and other metabolic tissues, namely adipose, to promote whole-body metabolism [173]. Myonectin lowers circulating levels of FFAs by increasing uptake in the liver and adipose tissue [172]. Interestingly, periods of fasting reduce myonectin levels, whereas re-feeding increases myonectin mRNA and serum concentrations, suggesting that it is responsive to acute energetic changes [173]. Earlier studies in murine models showed that obesity reduced expression and circulating levels of myonectin [172]. However, more recent work suggests that myokine levels are elevated in individuals with obesity, glucose intolerance as well as T2D [174]. These data have been corroborated by a recent study by Toloza and colleagues [175] who observed that plasma myonectin levels were positively associated with measures of insulin resistance in adults with T2D. Further investigation is warranted regarding myonectin and insulin resistance, although current research highlights the possible compensatory role of myonectin [175].

15.5.2.1 Effects of Exercise Training on Myonectin

Although most studies to date have been conducted in rodents [172], a recent report demonstrated that 10 weeks of aerobic exercise resulted in significant decreases in myonectin in healthy younger and older women [176]. Further, these decreases in myonectin correlated with increased VO_2max and adiponectin as well as decreased insulin resistance. More investigation is required to better understand if and how alterations in myonectin contribute to metabolic and/or vascular insulin sensitivity.

15.5.3 VEGF

Insulin mediated capillary recruitment and endothelium-dependent vasodilation is impaired in adults with T2D [177]. Vascular flow thereby augments delivery of insulin and glucose to skeletal muscle, resulting in insulin-stimulated glucose uptake. Vasculature endothelial growth factor-A (VEGF) is produced in skeletal muscle and contributes to angiogenesis for enhanced nutrient delivery. In rodents, it has been reported that muscle-specific genetic deletion of VEGF induces approximately a 60% reduction in capillary density which in turn contributes to nearly a 45% decrease in insulin-stimulated skeletal muscle glucose uptake [178]. Not surprisingly, VEGF in skeletal muscle is considered an important target for insulin and/or exercise mediated vascular effects in T2D [179] and therapies targeting VEGF are likely to benefit optimization of nutrient delivery capacity.

15.5.3.1 Effect of Exercise Training on VEGF

Skeletal muscle mediates local adaptation to increase capillarization for improved oxygen delivery and utilization [180]. VEGF is an angiogenic factor that increases in response to muscle contraction [181], although plasma concentrations are generally unaltered [182]. To that end, Wagner et al. [183] demonstrated that 12 weeks of exercise training increased VEGF in skeletal muscle of adults with T2D and this was directly related to peripheral insulin sensitivity. This is consistent with others showing that exercise stimulates PGC-1α to favor not only mitochondrial biogenesis but also capillarization [184]. However, not all exercise studies support the finding of increased VEGF and capillarization post-training. A recent study found that low volume interval training did not increase VEGF or capillarization when compared with continuous endurance training in people with T2D [185]. It was speculated that the dose of interval exercise was too low (20 min/session, 3 days/week) and that the longer duration seen with continuous exercise (40 min/session, 3 days/week) was important for shear stress induced adaptation despite increases in eNOS expression. Taken together, further work is required to understand how training doses impact adaptation responses for optimization of glycemia.

15.6 Conclusions

Insulin resistance in metabolic and vascular tissues is paramount to the regulation of circulating glucose and increases risk of T2D and CVD. Imbalances between exercise energy expenditure and energy intake are principle drivers of this “cross-talk” between organs that promote systemic and local inflammation for decreased insulin signaling (Fig. 15.1). As a result, further work is needed to examine the optimal exercise dose that reduces inflammation and increases insulin sensitivity and glucose tolerance in obese men and women, with and without diabetes. Additional

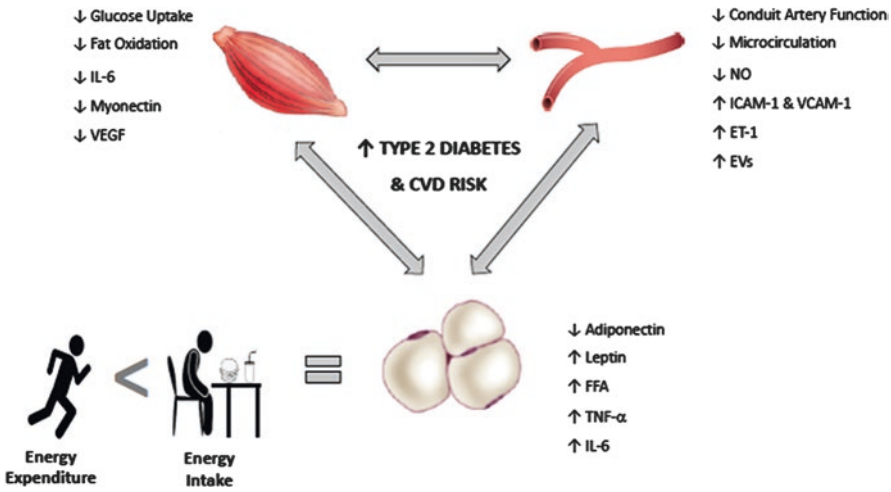

Fig. 15.1 Role of adipose on skeletal muscle and vascular metabolic physiology. Imbalance between energy expenditure from physical activity/exercise and energy intake leads to increases in adiposity. In turn, accretion of fat mass via energy surplus promotes inflammation. This inflammation has local and systemic effects that drive insulin resistance in skeletal muscle and the vasculature that worsens blood glucose control. IL-6; interleukin-6, VEGF; vasculature endothelial growth factor-A, TNF-α; tumor necrosis factor-alpa, FFA; free fatty acid, NO; nitric oxide, ICAM; intracellular adhesion molecule, VCAM; vascular cellular adhesion molecule, EV; extracellular vesicle, ET-1; endothelin-1

work is also warranted to understand how exercise combined with diet and/or pharmacology impacts the coordinated adaptation of metabolic and vascular tissue. Indeed, insulin resistance is multi-faceted and understanding the exact mechanism(s) that promotes metabolic and vascular abnormalities is critical for the prevention and/or reversal of chronic disease. By identifying novel biomarkers and underlying key targets of T2D, healthcare providers will eventually be able to provide precise treatment to individuals for lifelong health and well-being.

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