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Proteomics, Metabolomics, Interactomics and Systems Biology

Paul C. Guest *Editor*

Reviews on New Drug Targets in Age-Related Disorders

Part II

Springer

Advances in Experimental Medicine and Biology

Proteomics, Metabolomics, Interactomics and Systems Biology

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Editor

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Preface

Ageing is an inevitable part of life and may soon become a social, economic and health problem around the world. There is an ever-increasing proportion of individuals in the advanced age category, who have a higher risk of developing age-related disorders, such as type II diabetes mellitus, Alzheimer's disease, cancer, cardiovascular disorders and sarcopenia or frailty. New therapeutic approaches are needed to decrease or slow the effects of these diseases. The application of -omic technologies such as genomics, transcriptomics, proteomics and metabolomics has significantly advanced our understanding of disease in multiple medical areas as these allow the analysis of multiple molecular networks simultaneously to provide a more integrated view of health and disease pathways, and can even be used to monitor new therapeutic approaches. It is hoped that emerging data from these analyses will lead to the identification and development of novel drug targets to increase the human healthspan. In turn, this will lead to new therapeutic strategies and drug development projects by the pharmaceutical industry.

This book presents a series of reviews describing studies, which have resulted in identification of potential new drug targets for age-related disorders. Much of this information has come from -omic comparisons of healthy and disease states or from testing the effects of potential new therapeutic approaches. The authors in this series come from the six habitable continents from countries such as Australia, Brazil, Canada, France, Germany, India, Iran, Iraq, South Africa, South Korea, Thailand, United Kingdom, United States of America, Ukraine, Uruguay and Vietnam. This highlights the growing interest in this topic throughout the world.

Chapter 1 presents an overview of new therapeutics and biomarkers directed towards increasing the human healthspan. Chapter 2 describes new methods targeting metabolism in the treatment of cancer. Chapter 3 reviews monoclonal antibody-based treatments in non-small cell lung cancer. Chapter 4 describes the pathogenic metabolic signature in mitochondria that occurs in mitochondria. Chapter 5 covers the targeted treatment of fibromyalgia using coenzyme Q10 supplementation. Chapter 6 reviews the role of gut microbiota in the prevention and progression of multiple sclerosis. Chapter 7 summarizes the therapeutic applications of long non-coding RNAs in head and neck cancer. Chapter 8 describes studies which have found genotoxic effects of selective serotonin reuptake inhibitors in the treatment of depression. Chapter 9 covers the methods of circulating tumour cell isolation and detection, and the therapeutic strategies targeting these cells in different cancer types.

Chapter 10 describes the telomerase inhibitory effects of the natural compound curcumin in relation to its anti-cancer activity. Chapter 11 summarizes numerous experimental and analytical data that support the health and longevity benefits of aspirin treatment by affecting pro-longevity pathways. Chapter 12 describes methods involved in the targeting of stem cells as a treatment in diseases marked by chronic inflammation. Chapter 13 talks about new therapeutic approaches in Alzheimer's disease using nano-drug delivery systems. Chapter 14 describes the effects of age on stress outcomes in a large study of critical care nurses. Chapter 15 describes the use of viral vectors for delivery of gene-based therapeutics in neurodegenerative disorders. Chapter 16 presents the targeting of adipose stem cells to increase longevity and the healthspan. Finally, Chapter 17 describes the use of proteomic methodologies in the identification of new drug targets in psychiatric and neurodegenerative disorders.

The book will be of high interest to researchers in the areas of ageing and chronic disease, as well as to clinical scientists, physicians and the major drug companies since it gives insights into the latest ideas and technologies enabling progress in the area of healthy ageing. It will provide important information on disease mechanisms related to the ageing process, as each chapter will be presented in the context of specific chronic diseases or different therapeutic strategies.

Sao Paulo, Brazil Paul C. Guest

Contents

1 New Therapeutic Approaches and Biomarkers for Increased Healthspan

Paul C. Guest

Abstract

Healthcare costs have increased in developing countries over the last few decades, mostly due to the escalation in average life expectancy and the concomitant increase in age-related disorders. To address this issue, widespread research is now being undertaken across the globe with the aim of finding a way of increasing healthy aging. A number of potential interventions have already shown promise, including lifestyle changes and the use of natural products or pharmaceuticals that may delay the onset of diseases associated with the aging process. In parallel, a number of potential biomarkers have already been identified that can be used for assessing risk of developing age-associated disorders and for monitoring response to therapeutic interventions. This review describes the most recent advances towards the goal of achieving healthier aging with fewer disabilities that may lead to enhanced quality of life and reduced healthcare costs around the world.

Keywords

Life expectancy · Age-related diseases · Diabetes · Cancer · Frailty · Neurodegenerative conditions · Intervention · Lifestyle · Pharmaceuticals · Biomarkers

1.1 Introduction

After maturity is reached, a progressive decline in physiological functions occurs of all higher organisms over time. This aging process is a major risk factor for both healthspan and lifespan as it contributes to increased susceptibility to diseases, such as cardiovascular disorders, diabetes, cancer, frailty, and neurodegenerative conditions. It follows that delaying the aging process would lead to longer healthier lives. The first stage towards this goal is the identification of the main environmental and genetic factors associated with aging and morbidity [1, 2]. The next phase would be the identification of biomarkers associated with aging and disease processes which, in turn, would lead to identification of drug targets, leading to novel therapeutic avenues of intervention [3].

Currently, many countries of the world are now experiencing a change towards an increasing proportion of aged individuals, due to increased life

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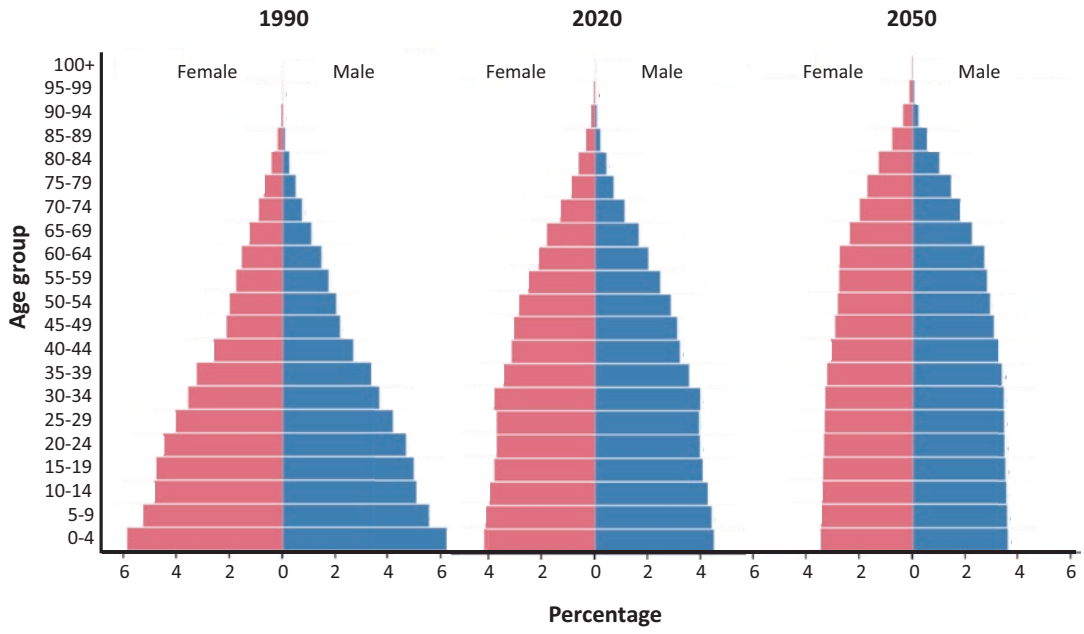

Fig. 1.1 Pyramid plots of various age groups from 0 to 100+ years-olds in the World from 1990 to 2020, with projections to the year 2050

expectancies from lifestyle adoptions and constant advancements in medicine. This can be seen easily by the change in shape of pyramid plots of various age groups from 0 to 100+ year-olds throughout the world from the years 1990 to 2020 and in projections to the year 2050 (Fig. 1.1) [4]. This shows a decreased proportion of individuals in the lower age groups to increased levels near the top of the pyramid. The highest proportions of centenarians (100+ year-olds), on a per capita basis, are localized to the continents of North America and Europe, with Africa being startlingly low in comparison (Fig. 1.2a). On a country basis, the highest proportion of centenarians appears in Japan (Fig. 1.2b), which appears to be the fastest growing country in the world with respect to this population group. In fact, from 1990 to 2020, the population of Japanese centenarians increased by an incredible 32-fold from 2397 to 78,636 individuals [4]. In all cases, the proportion of centenarians who are female exceeded that for males, consistent with the observation that females have an increased average lifespan compared to males [5–8].

At the time of writing (February 08, 2020), the longest-lived human was a French woman called Jeanne Calment who died on August 4, 1997, at the grand old age of 122 years and 164 days [9]. From the age of 109, Jeanne Calment was known to follow a strict daily routine, consisting of an early rise (6:45 am), a long prayer, seated gymnastics (hand and leg flexions and extensions while wearing a stereo headset), followed by a breakfast of coffee with milk and rusks. Besides having a robust appetite, she also consumed approximately 2 kg of chocolate per week with daily cigarettes and Port wine. The oldest person alive today is Kane Tanaka, who is 118 years and 42 days old as of February 18, 2021 [10]. Kane Tanaka still has a strong appetite, consumes sweets, three cans of coffee each day, along with sodas and various nutritional drinks, and attributes her very long life to faith in God.

Despite this information, the mystery of how these two women and other supercentenarians (110+ years-old) managed to live such long lives is not precisely known. Nevertheless, a number of studies have now been performed at the clinical and preclinical levels which have begun to shed new light on this subject. This review describes some of the latest breakthroughs in this exciting field.

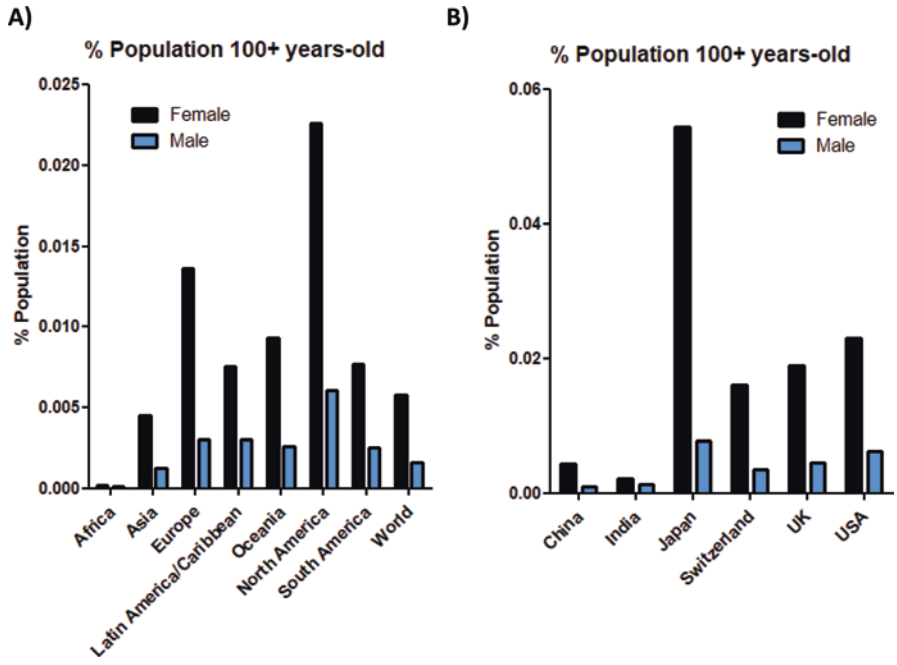

Fig. 1.2 Proportions of centenarians (100+ years-olds) per capita in (**a**) continents and (**b**) selected countries of the world

1.2 Body Composition

1.2.1 Adiposity

Overweight and obesity are risk factors for early mortality, and the prevalence of these conditions is on the rise [11]. In 2016, the World Health Organization (WHO) estimated that more than 1.9 billion adults were overweight [body mass index (BMI) = 25–30 kg/m^2] and 650 million were classified as obese (BMI > 30 kg/m^2) [11]. A meta-analysis of 239 prospective studies of 10,625,411 participants in Asia, Australia and New Zealand, Europe and North America found that all-cause mortality was lowest at normal body mass indices (18.5–25 kg/m^2), slightly higher below this range, and increased progressively in the overweight (25–30 kg/m^2) and obesity (>30 kg/m^2) classes [12]. However, these findings appear to conflict with those of another study which examined the association between BMI as a continuous variable and all-cause mortality in 4565 Geisinger Rural Aging Study participants aged 74.0 ± 4.7 years over an average 11-year follow-up period [13]. This study found a U-shaped association between mortality and BMI with a lower risk found for individuals who were either under-weight, overweight, or obese, compared to those with a normal-range BMI of 18.5–25 kg/m^2. Possible explanations for the findings of the second study include the possibility that excess fat could provide metabolic reserves during illness [14], lower fracture risk during falls [15], or surgeons may be more diligent or take more care with overweight or obese individuals during surgery [16]. However, in the case of both studies, body composition measurements, such as abdominal adiposity, waist:hip ratio, and muscle mass may be more appropriate measures than BMI [17, 18]. Another possible explanation is that the second study excluded deaths that occurred during the first two to five years of follow-up, and this could have removed data which might have affected the overall results.

The proposed U- or J-shaped survival curve in relation to BMI has been termed the obesity paradox. However, most of the studies which have

supported the existence of this paradox only took BMI into account as the measure of obesity. BMI does not consider other potentially useful parameters such as body composition and cardiovascular fitness. Although BMI has been used widely as a predictor of health risk, waist circumference might be a better indicator given its closer link with harmful visceral fat [19]. Aging is associated with changes in body composition including an increase in adiposity and reduced muscle mass. Higher fat in the abdominal region is associated with higher risk of age-related diseases [20–23]. A 5-year follow-up study of 58,609 individuals in the age range of 65–74 years at baseline found that a large waist circumference was associated with increased mortality across all BMI categories, compared to those with a small waist in the 'healthy' (20–25 kg/m^2) BMI category [24]. Thus, BMI may incorrectly classify individuals in terms of healthy or unhealthy bodyweights as it does not allow an assessment of adiposity. A study of 13,601 subjects aged 20–79.9 years from the Third National Health and Nutrition Examination Survey used bioelectrical impedance analysis to estimate percentage of body fat compared to BMI readings [25]. The bioelectrical impedance analysis showed that excess body fat was present in 50% of males and 62% of females using the WHO reference standard for obesity (>25%, males; >35%, females). However, 21% of the males and 31% of the females were classified as obese when BMI was used as the measurement. Thus, more than half of males and females with excess body fat were misclassified using the BMI system.

1.2.2 Muscle Mass

The composition of the body inevitably changes with aging, with a gradual loss of lean mass and a shift to increased fat mass [26]. A young person of 22-years-old consists of an average of 48% muscle and 19% fat, whereas the muscle mass is reduced to 25% and the fat increased to 35% in a 78-year-old person (Fig. 1.3) [27]. A review from 2012 found that the median loss in muscle mass over the entire lifespan was 0.37%/year in females and 0.47%/year for males [28]. The same study showed that this was increased to 0.64–0.70%/year and 0.80–0.98%/year in 75+ year-old females and males, respectively. Furthermore, this atrophy can be worsened during periods of inactivity, as can occur with extended hospital bed rest [29–31]. This leads to a decline in strength and an increase in the risk for disabilities and age-related disease [32].

There is also a loss of weight in older adults which is most often due to a reduction of lean mass and this is associated with increased risk of mortality [33–35]. A computed tomography and dual x-ray absorptiometry analysis of 1803 men and women initially aged 70–79 years found that greater loss of thigh muscle relative to overall

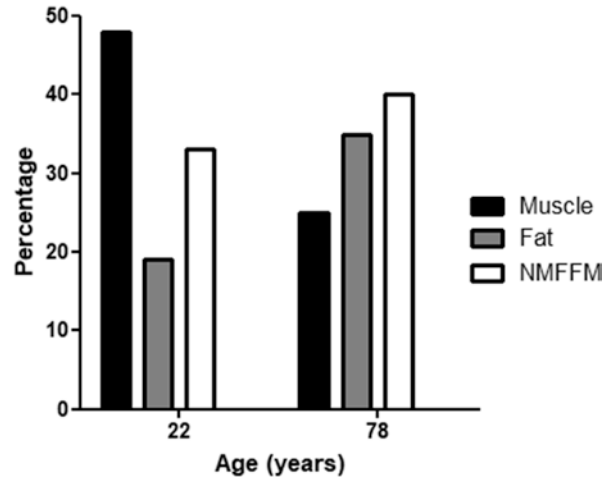

Fig. 1.3 Change in body composition with aging. *NMFFM* *non-muscle fat-free mass*

weight change had a higher mortality risk compared to persons with preservation of this muscle [36]. Another follow-up study analysed muscle quality of 511 people aged 50 years or older, using the ratio between knee-extension isokinetic strength and thigh muscle cross-sectional area [37]. The results showed that body composition, but not BMI, was associated with muscle quality decline. Therefore, the authors suggested that efforts to alter body composition in favour of decreased adiposity and increased lean mass would be sensible therapeutic approach. A more recent study published in 2019 employed magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DXA) to measure changes in thigh muscle in men and women aged an average of 71 years at baseline over a 5-year follow-up study [38]. The results showed an average 5% loss of muscle tissue in both males and females, and this was associated with higher baseline body fat in males only.

1.2.3 Therapeutic Approaches Targeting Body Composition

1.2.3.1 Resistance Exercise

Resistance exercise training has been used as an intervention tool to improve skeletal muscle cross-sectional area, strength and function, and thereby improve functional capacity and reduce disease risk in aged individuals [39, 40]. A 10-week trial published in 1994 found that progressive resistance exercise training of 100 frail nursing home residents led to an increase in muscle strength of approximately 113%, an increase in gait velocity of 12%, and increased cross-sectional thigh area of 28% [41]. Two Brazilian studies on the effects of resistance training three times per week of females aged 60 and over found that both short (30 min) and long (50 min) training sessions led to increased skeletal muscle mass compared to non-exercising controls [42, 43]. However, only the 50-min session resulted in significantly increased strength and decreased fat mass. There have been many other studies conducted with mixed outcomes and cross-comparisons that have proven difficult due to study group heterogeneities, the different exercise regimes used, and the varying outcome measures reported. In line with this, the International Sarcopenia Initiative has indicated that well-defined populations, standardized training methods, and common outcome measures are needed to allow more accurate comparisons across studies [44]. In addition, the duration of the exercise period should be for at least 3 months and supervised programmes should be initiated for elderly people residing in community care. A recent study of 25 people with a mean age of 57 years found that 1 year of heavy resistance training resulted in increased muscle strength and mass but had no effect on visceral fat mass [45]. It is possible that reductions in visceral fat require other approaches for people in this age group. Another recent study of aged males with sarcopenia found that a 28-week resistance training programme, in combination with protein, vitamin D, and calcium supplementation, led to an improved sarcopenia Z-score, with increased skeletal muscle mass and hand grip strength, compared to the inactive control group [46].

1.2.3.2 Dietary Methods

In the elderly, sarcopenia may result from increased muscle protein breakdown and/or decreased basal muscle protein synthesis [47]. A few studies have suggested that older adults on diets containing 0.8 g protein/kg/day or less may not be receiving sufficient amounts of this nutrient [48, 49]. In support of this idea, a study of 70–79 year-old community-dwelling older adults found that those individuals receiving 1.2 ± 0.4 g protein/kg per day lost or maintained a higher proportion of lean mass compared to those on 0.8 ± 0.3 g protein/kg per day [50]. This finding was supported in a more recent systematic review [51]. Other investigations have found that distribution of the protein intake throughout the day in approximately equal portions may also be important factor in maintaining a favourable anabolic rate [52, 53]. In addition to protein, studies have found that diets enriched in n-3 long-chain polyunsaturated fatty acids (LCPUFAs) may improve muscle function, as shown by positive effects of these fats on measures of grip strength, knee-

extension strength, and muscle size [54]. Another study found that resistance training combined with an LCPUFA-rich diet favoured skeletal muscle hypertrophy in older females [55].

A systematic review of randomized control trials in elderly subjects found that vitamin D supplementation had a beneficial effect on lower leg muscle strength, body sway, and/or physical performance, although other studies reported no benefit [56]. However, a more recent meta-study found some evidence for additive effect of resistance training and vitamin D supplementation on the improvement of muscle strength in older adults, compared to vitamin D supplementation alone [57]. In addition, another review found that vitamin D supplementation (800–1000 IU/day) can improve muscle function, particularly in cases where vitamin D levels are low [58].

1.2.3.3 Pharmaceuticals and Natural Compounds

Coffee drinking has become a contender for healthy aging with known benefits against disorders such as cognitive impairment [59] and cardiovascular disease [60]. In addition, coffee drinking has been shown to be associated with lowering all-cause mortality in population studies [61, 62]. A recent meta-study identified 40 investigations of over 3.8 million subjects and found an inverse association between coffee consumption and all-cause mortality, cardiovascular disease, and cancer, with consumption of 3.5, 2.5, and 2 cups/day, respectively [63]. Any additional intake of coffee had no further effect on mortality and the effects were not modified by age, body weight, alcohol, smoking, or caffeine content. The active ingredients in coffee that appear to be associated with the protective effects are caffeine and certain polyphenol compounds which inhibit the mammalian target of rapamycin (mTOR) and reduce fat accumulation, respectively [64].

Metformin is an anti-diabetic drug with anti-hyperglycemic effects that are produced by reduction of hepatic glucose production and increased insulin sensitivity in peripheral tissues such as liver, skeletal muscle, and adipose tissue [65]. A clinical study found an anti-aging effect in diabetic patients on metformin with 7% lower all-cause mortality compared to non-diabetic controls [66]. Previous studies have suggested that metformin may have positive effects on a number of targets implicated in the aging process such as inhibition of inflammation, promotion of autophagy, and reduction of reactive oxygen species [67]. Furthermore, a 2-year double-blind clinical study of overweight patients with impaired glucose tolerance found that those individuals who received metformin had significant reductions in body weight and waist circumference in comparison to a reduced progression to diabetes [68]. However, it should be stressed that the potential anti-aging and anti-obesity benefits of metformin still requires further scrutiny.

1.2.3.4 Combination Approaches

A meta-analysis of studies which investigated body composition, metabolic health, and physical performance in individuals with sarcopenic obesity found that aerobic exercise decreased body weight and fat mass, whereas resistance exercise decreased fat mass and increased grip strength [69]. The same study found that a combination of aerobic and resistance exercise decreased fat mass and improved walking speed, although a low calorie diet provided no extra benefits in the case of either exercise regime. Another recent review found that a high protein diet can increase weight loss and preserve lean body mass by decreasing the rate of muscle protein breakdown [70]. Furthermore, consumption of dairy-specific protein increases insulin sensitivity by stimulating insulin release, fat oxidation, and the rate of muscle protein synthesis in aged adults and such effects are enhanced when a resistance exercise program is incorporated into the regime. A meta-analysis on frailty indices in older adults revealed that the combination of protein supplementation with multi-component exercise had significant positive effects on frailty status, and the resistance exercise led to an increase in lean mass, muscle strength, and physical mobility [71]. In contrast, another study found that a 2 day/week resistance exercise programme combined with a low-dose protein and micronutrient diet led to significant improvements in muscle

mass [72]. However the effects on physical performance in the combined exercise/diet programme were not significantly different compared to resistance training alone. Thus, further work on the types of exercise and protein/micronutrient supplementation is required to establish which programmes will have the most benefit on body composition and on reducing the risk of age-related disorders.

1.3 Biomarkers

Since aging is a major risk factor for most age-related diseases, there arises a need for valid biomarkers to aid in early identification and potentially guide therapeutic options. However, this is complicated by the heterogeneity found both within and across different human populations. Furthermore, no single biomarker has yet to be described that can be used to monitor the aging process. Therefore, it is expected that a biomarker algorithm will be required that represents the main physiological aspects of aging such as physical characteristics, body composition, and nutritional status, along with molecules and/or genetic polymorphisms representing metabolic, hormonal, and immune functions.

1.3.1 Physical Function and Body Composition

Physical performance assessments, such as handgrip strength, gait speed, timed up and go, and 6-min walk tests have been used in a number of studies as biomarkers of frailty and the aging process. Poor performance in some or all of these is associated with increased functional decline [73] or higher mortality rates [74, 75]. Poorer performance in some of these parameters has also been associated with higher risk of cardiovascular disease, dementia cognitive impairment, or disabilities [76]. As detailed above, decreased performance in such physical tests has been associated with changes in body composition due to decreased muscle mass and increased body fat. Furthermore, studies which have analysed muscle mass have reported that lower muscle mass is associated with increased occurrence of disabilities and functional impairment [76–78]. Therefore, it is important to include techniques, such as magnetic resonance imaging (MRI) and computer tomography, for assessment of various aspects of body composition.

1.3.2 Circulating Molecular Biomarkers

A number of systematic reviews have provided strong support for the use of blood lipids as predictors of mortality and age-related diseases [76, 79, 80]. Furthermore, high plasma levels of inflammation-related factors, such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, have been linked with lower handgrip strength and walking speed in aged adults [81], and C-reactive protein (CRP) and IL-6 have been linked with all-cause mortality [76]. Interestingly, studies of centenarians have shown lower levels of such inflammatory biomarkers compared with younger cohorts [82], with a concomitant increase in the levels of anti-inflammatory biomarkers like IL-10 [76, 83].

A number of hormones and hormonal signalling pathways are known to be perturbed in aging and age-related diseases. For example, impaired muscle strength and performance in aging has been linked to the perturbations of insulin signalling in regulating muscle protein metabolism. For these reasons, measurements of insulin resistance in clinical settings could provide an additional biomarker related to increased risk of morbidities and mortality [84]. Other age-related changes in hormones include decreased circulating levels of growth hormone and insulin-like growth factor-1 (IGF-1) as well as thyroid-stimulating hormone (TSH) and free thyroxine [85]. This also includes the sex hormones testosterone and estrogen in males and females, respectively [86, 87]. In addition, decreased levels of adiponectin occur with aging, and this has been linked with several adverse health outcomes [88]. This is consistent with the finding of another study which found higher levels of adiponectin in serum from long-

lived Greek (90+ years-old) individuals compared with younger controls [89].

A number of vitamins are known to be altered with aging and the strongest evidence for this is a decrease in the levels of vitamin D, which has been linked with age-related disorders such as mild cognitive impairment and sarcopenia [90, 91]. Finally, increased levels of cardiac troponin have been associated with myocardial damage, skeletal muscle aging, and may even have a role in cancer [92].

1.3.3 Genes

Recent studies have identified five genes with polymorphisms linked to longevity. These genes encode the forkhead box protein (*FOXO*), apolipoprotein E (*APOE*), the Klotho β-glucuronidase (*KL*), angiotensin-converting enzyme (*ACE*), and IL-6 (*IL6*). A meta-analysis identified five polymorphisms associated with cardiovascular health and exceptional longevity (85+ years), consisting of *ACE* rs4340, *APOE* ε2/3/4, *FOXO3A* rs2802292, *KLOTHO* KL-VS, and *IL6* rs1800795 [93].

1.3.3.1 FOXO

A recent study from Brazil found an association of *FOXO3* (rs2802292) polymorphisms with longevity in 220 participants aged 85+ years, compared to a control group of 234 individuals 70–75 years-old [94]. The *FOXO3* gene encodes a transcription factor which regulates the stress response, and the link to lifespan has been found in a number of studies [95, 96]. The *FOXO* pathway has therefore been suggested as a potential target for age-related diseases [97].

1.3.3.2 APOE

A genome-wide association study (GWAS) of nonagenarians compared to younger individuals identified significant associations for *APOE* variants [98, 99]. This was seen by an absence of *APOE4* and enrichment of the *APOE2* allele in the nonagenarians. *APOE* isoforms have been implicated as risk factors for cardiovascular disorders and Alzheimer disease, which have been attributed to effects on inflammation, oxidative stress, and lipid regulation [100].

1.3.3.3 KL

A recent study found that the *KL* rs9536314 polymorphism played a protective role in cancers and in determining human longevity [101]. The Klotho name comes from Clotho, one of three Fates of Greek mythology, who was associated with spinning the web of human life. Disruption of the *KL* gene in mice leads to a shorter lifespan and morbidities associated with age-related disorders [102]. The Klotho protein appears to act as a hormone in regulation of oxidative stress and inflammation, via inhibition of IGF1/PI3K and TGF-β signalling pathways, and has been shown to be a potential novel cardiovascular protective factor [103].

1.3.3.4 ACE

ACE is a component of the renin-angiotensin system, involved in regulation of blood pressure and sodium homeostasis [104]. A genetic evaluation study found that the DD genotype for *ACE* was significantly associated with Alzheimer's disease, as determined using Mini-mental State Examination (MMSE) scores [105]. This association was significantly stronger when the DD ACE allele was combined with the GG allele of *TNF* gene. Another study showed that the DD ACE genotype was associated with low muscle mass in elderly people in Jakarta [106]. Thus, ACE inhibitors are being tested in a number of disease areas [107]. The mechanism may involve the prevention of mitochondrial decline, as well as improvements in endothelial function and muscle metabolism [108].

1.3.3.5 IL-6

A recent study of a long-lived Chinese population showed a significantly lower frequency of the C-allele of the *IL6* rs1800796 locus compared to the control group, suggesting that this may be an unfavourable factor for longevity [109]. Furthermore, a meta-analysis of the PubMed, Embase, China National Knowledge Infrastructure, and Wanfang databases found that an IL6 polymorphism was associated with bone

mineral density and development of osteoporosis [110]. As IL-6 is known to stimulate inflammation and auto-immunity in diseases, such as type 2 diabetes, atherosclerosis, cardiovascular disorders, depression, Alzheimer's disease, systemic lupus erythematosus, prostate cancer, and rheumatoid arthritis, there is considerable interest in development of anti-IL-6 therapeutics for many of these conditions [111, 112].

1.3.3.6 Genetic Networks

A recent study constructed a functional interaction network leading to identification of 215 polymorphisms related to longevity of long-lived smokers compared to younger smokers [113]. The long-lived smokers were chosen on the possibility that their long survival was due to an innate resistance to the effects of cigarette smoking. The resulting network was used to generate a risk score that was significantly associated with a 22% increase in probability of being aged 90–99 years-old and a threefold increased probability of being over 100 years-old, as compared to being 52–79 years-old. This score was also linked with an 11% reduction in cancer prevalence. Pathways enriched in the network included PI3/AKT, insulin/IGF, and FOXO signalling, which have already been associated with aging and age-related diseases.

1.4 Conclusions and Future Perspectives

Research into increasing the healthspan is more critical than that of aiming to extend the lifespan. It is clear that healthy aging depends on a complex interaction between genetic predisposition and lifestyle factors. The combination of genetic and other molecular biomarkers may be used to assess risk of developing age-related disorders, which may allow interventions to delay or slow the aging process. Maintaining a healthy body composition consisting of higher muscular mass and lower adiposity appears to be important. A number of lifestyle interventions may also be helpful in this regard with the most promising approaches being the incorporation of a diet consisting of fewer calories and higher protein content and undertaking a combined resistance and aerobic exercise programme. There are also a number of natural products and pharmaceuticals undergoing testing which are mostly aimed at improving metabolism. Finally, several promising physiometric and molecular biomarkers have been identified which can be used to assess risk and pave the way for personalized approaches to maximize the chances of people achieving longer, healthier lives.

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Targeting Cancer Metabolism and Current Anti-Cancer Drugs

2

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Abstract

Several studies have exploited the metabolic hallmarks that distinguish between normal and cancer cells, aiming at identifying specific targets of anti-cancer drugs. It has become apparent that metabolic flexibility allows cancer cells to survive during high anabolic demand or the depletion of nutrients and oxygen. Cancers can reprogram their metabolism to the microenvironments by increasing aerobic glycolysis to maximize ATP production, increasing glutaminolysis and anabolic pathways to support bioenergetic and biosynthetic demand during rapid proliferation. The increased key regulatory enzymes that support the relevant pathways allow us to design small molecules which can specifically block activities of these enzymes, preventing growth and metastasis of tumors. In this review, we discuss metabolic adaptation in cancers and highlight the crucial metabolic enzymes involved, specifically those involved in aerobic glycolysis, glutaminolysis, de novo fatty acid synthesis, and bioenergetic pathways. Furthermore, we also review the success and the pitfalls of the current anti-cancer drugs which have been applied in pre-clinical and clinical studies.

Keywords

Cancer · Metabolism · Drug target · Enzymes · Bioenergetic

2.1 Introduction

Cancer is an abnormality of cells which lose the ability to regulate their growth. Due to the unlimited proliferation and acquired motility, cancer cells can further invade the surrounding or distant tissues, in a process known as metastasis, which is a major cause of death. Cancer cells are different from normal cells in several aspects. For example, they have the ability to sustain proliferative signaling, evade growth suppression, resist apoptosis, induce angiogenesis, enable replicative immortality, invade other tissues and suppress immune recognition. Recently, metabolic reprogramming has been added as another important cancer hallmark [1, 2].

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The metabolic reprograming in cancers has recently attracted attention over the last decade since the discovery of Warburg's effect, or aerobic glycolysis, which describes the phenomenon that cancers metabolize glucose to lactate, regardless of the presence of oxygen, because of a defect of mitochondria [3]. As a result of this phenomenon, pyruvate, the end product of glycolysis, is converted to lactate with little ATP production. Subsequent studies have shown that aerobic glycolysis provides several advantages for cancer cells [4, 5]. For example, glycolysis allows cancer cells to grow under conditions where the oxygen concentration fluctuates or during hypoxia [6, 7]. Glycolysis also provides ATP at a faster rate than oxidative phosphorylation [8]. Although glycolysis generates relatively little ATP per molecule of glucose, cancer cells can markedly increase glucose uptake and glycolysis such that ATP yield can meet their demands [9]. Regarding the increase of biomass, glycolysis provides several important biosynthetic precursors required during rapid proliferation, such as nucleotides, amino acids, and lipids [10, 11]. Lastly, glycolysis minimizes the production of reactive oxygen species (ROS) which are deleterious to cancer cells [12].

In addition to aerobic glycolysis, cancer cells also alter other metabolic pathways such as repurposing the tricarboxylic acid (TCA) cycle to support biosynthesis, increasing biosynthesis of lipids, amino acids and nucleotides to meet the high anabolic demand during rapid cell growth. Thus, targeting these metabolic pathways might be effective in the treatment of many cancers. Although several inhibitors targeting various metabolic pathways have been developed for decades, some have failed during the clinical trials because of their side effects and low efficacy. In recent years, novel anti-cancer drugs with excellent efficacy that target both glycolytic and bioenergetics pathways have been reported. Here we highlight and discuss some key metabolic enzymes which receive much attention as attractive anti-cancer targets (Table 2.1). We also review the recent anti-cancer drugs which have been developed to target those key enzymes, including their inhibitory mechanisms and anti-neoplasia action on cancer cells. The metabolic pathways and enzymes comprising these anti-cancer drug targets are shown in Fig. 2.1.

2.2 Glycolysis

Glucose is the easiest nutrient for most living cells to use because of its structure. Cancer cells also prefer to use glucose to support their growth. Increased glycolytic rate and expression of glycolytic enzymes clearly indicates that cancer cells rely on this pathway to support their growth and survival. Because glycolysis is an earliest catabolic pathway of glucose, inhibition of this pathway can effectively inhibit downstream pathways, thus providing an effective therapeutic means to block cancer growth.

2.2.1 Hexokinase

Hexokinase (HK) is the first regulatory step of glycolysis. HK catalyzes the phosphorylation of glucose to form glucose-6-phosphate. Four tissue-specific isozymes of HK, HK1, HK2, HK3, and HK4, are found in mammals. Although these HK isozymes catalyze the same reaction, they have some different kinetic properties [13]. HK1, HK2, and HK3 have a relatively low K_m (the substrate concentration at which the reaction rate is half maximal) for glucose, while HK4, also known as glucokinase, has a high K_m for glucose and is exclusively expressed in liver and pancreas [13]. HK1 is ubiquitously expressed in brain and kidney, while HK2 is expressed in skeletal and cardiac muscles. Since HK2 is aberrantly increased in several cancers such as hepatocellular carcinoma, pancreatic cancer, ovarian cancer, lung cancer, gastrointestinal cancer, breast cancer, and renal cancer [14–19], HK2 is regarded as a potential target for cancer therapy.

2-deoxyglucose (2-DG) is the substrate analog of glucose in which the hydroxyl group of carbon 2 (C2) is replaced with a hydrogen. After entering into cancer cells, 2-DG can still be phosphorylated by HK2 to generate 2-deoxyglucose-6-phosphate (2-DG6P) but cannot be further

Table 2.1 Target metabolic enzymes for anti-cancer drug development

Target	Drug	Effect	Development phase	Reference
Glycolysis				
Hexokinase	2-Deoxyglucose	Inhibition	In vitro: Breast cancer	[25]
			In vivo: Osteosarcoma, NSCLC	[30]
			Phase I/II trial: Glioblastoma	[32]
	3-Bromopyruvate	Inhibition	In vitro: Liver cancer, leukemia, nasopharyngeal carcinoma (NPC)	[34, 35, 39]
			In vivo: Liver cancer	[38]
	Benserazide	Inhibition	In vitro/in vivo: Colon cancer	[42]
	Xanthohumol	Inhibition	In vitro: Colon cancer, NSCLC, breast cancer, cervical cancer, colorectal cancer	[44–50]
			In vivo: Colorectal cancer	[44]
Pyruvate kinase	Shikonin	Inhibition	In vitro: Fibrosarcoma, leukemia, lung cancer, drug-sensitive and resistant cell lines, liver cancer, prostate cancer, Lewis lung carcinoma, melanoma, esophageal cancer, bladder cancer	[62, 64–68, 70–72]
			In vivo: Sarcoma, liver cancer, prostate cancer, leukemia, melanoma, esophageal	[64, 68, 70, 71]
			Clinical trial: Lung cancer	[69]
	DASA-58	Activation	In vitro: NSCLC	[75]
	TEPP-46	Activation	In vivo: NSCLC	[75]
	Micheliolide	Activation	In vitro: Leukemia, pancreatic adenocarcinoma, neuroblastoma	[76]
			In vivo: Leukemia xenograft zebrafish	[76]
Lactate dehydrogenase-A	Oxamate	Inhibition	In vitro: Nasopharyngeal cancer, gastric cancer, pituitary adenoma, Burkitt lymphoma	[79, 90–92]
			In vivo:Nasopharyngeal cancer,pituitary adenoma	[90, 92]
	FX11	Inhibition	In vitro: Lymphoma, pancreatic cancer, prostate cancer, osteosarcoma, gallbladder cancer	[88, 93–95]
			In vitro/in vivo: Lymphoma, osteosarcoma, gallbladder cancer	[88, 94, 95]
	Galloflavin	Inhibition	In vitro:Liver cancer, breast cancer, Burkitt lymphoma, endometrial cancer	[98–101]

(continued)

Table 2.1 (continued)

Target	Drug	Effect	Development phase	Reference
MCT-1 and MCT-4	AR-C155858	Inhibition	In vitro: Pancreatic ductal adenocarcinoma, Ras-transformed fibroblasts, multiple myeloma, breast cancer	[113, 119–123]
			In vivo: Ras-transformed fibroblasts, breast cancer	[119–123]
	AZD3965	Inhibition	In vitro: Breast cancer, small cell lung cancer, B-cell lymphoma and Burkitt lymphoma	[116, 124–126]
			In vivo:Breast cancer, small cell lung cancer, B-cell lymphoma and Burkitt lymphoma	[116, 124–126]
			Phase I trial: Several solid tumors	[128]
	α-Cyano-4-hydroxy cinnamate	Inhibition	In vitro: Multiple myeloma, glioma, breast cancer	[120, 129, 130]
			In vivo: Glioma, breast cancer, lung cancer, colorectal cancer	[129–131]
	Syrosingopine	Inhibition	In vitro: Cervical cancer, breast cancer, leukemia	[133]
	N,N dialkylcyanocinnamic acid	Inhibition	In vitro: Colorectal adenocarcinoma, breast cancer	[134]
	Lonidamine	Inhibition	In vitro: Melanoma	[135]
			In vivo: Melanoma	[136]
Mitochondrial metabolism				
Pyruvate dehydrogenase kinase	Dichloroacetate	Inhibition	In vitro:Prostate cancer, breast cancer, colorectal cancer, NSCLC, glioblastoma	[149–154]
			In vivo:Breast cancer, colorectal cancer, NSCLC, glioblastoma	[150, 152–154]
			Phase I trial: Glioblastoma	[155]
	CP-613	Inhibition	In vitro:NSCLC, breast cancer, kidney cancer	[156]
			In vivo: Pancreatic cancer, NSCLC	[156]
			Phase I trial: Relapsed or refractory AML, metastatic pancreatic cancer	[158–160]
			Phase II trial: Relapsed or refractory small cell lung cancer	[159]
			Phase III trial: Metastatic pancreatic cancer	[161]
TCA cycle				

(continued)

Table 2.1 (continued)

Target	Drug	Effect	Development phase	Reference
Isocitrate dehydrogenase 1 (IDH1)	BAY-1436032	Inhibition	In vitro/in vivo: Glioblastoma, AML	[179, 180]
			Phase I trial: AML, solid tumors	(NCT03127735, NCT02746081)
	IDH305	Inhibition	In vivo: Melanoma	[181]
			Phase I trial: Advanced malignancies that harbor IDHR132 mutations	[182, 359]
	Ivosidenib	Inhibition	In vitro: Chondrosarcoma	[185]
			Ex vivo: Primary human AML myeloblast	[184]
			In vivo: Glioma	[186]
			Phase I trial: Relapsed or refractory AML	[360]
	LY3410738	Inhibition	In vitro/in vivo: AML	[188]
Isocitrate dehydrogenase 2 (IDH2)	AGI-6780	Inhibition	In vitro: Human glioblastoma, erythroleukemia	[189–191]
	Enasidenib	Inhibition	Ex vivo: AML	[192, 193]
			In vivo: AML	[192]
			Phase I/II trial: Hematologic neoplasms	[361]
Isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2)	AG-881	Inhibition	Ex vivo/in vivo: Primary humanAML	[196]
			Phase I trial: Gliomas	[197]
Oxidative phosphorylation				
Mitochondrial respiratory complex I	Metformin	Inhibition	In vitro: Osteosarcoma, liver cancer, breast cancer, AML, colon cancer, prostate cancer	[204, 206–212]
			Ex vivo: AML	[207]
			In vivo: AML, colon cancer, prostate cancer	[207, 208, 211]
			Clinical study: Colorectal cancer	[215]
	Phenformin	Inhibition	In vitro: Rectal cancer	[218]
			In vivo: Breast cancer	[217]
Glutaminolysis				

(continued)

Table 2.1 (continued)

Target	Drug	Effect	Development phase	Reference
Glutaminase	DON	Inhibition	In vitro: Human lymphoblast, neuroblastomas, sarcoma	[240, 245]
			In vivo: Leukemia, murine mammary cancer, colon carcinoma, lung cancer, neuroblastomas, sarcoma	[243, 245]
			Clinical trials: Metastatic breast cancer, bronchogenic carcinoma, gastrointestinal adenocarcinoma, lung cancer, colorectal cancer, sarcoma, advanced refractory tumors	[243–245]
	Acivicin	Inhibition	In vitro: Leukemia, ovarian cancer	[241, 243]
			In vivo: Breast cancer, lung cancer	[241, 243]
			Phase II trial: Glioma	[243]
			Phase I trial: Several solid tumors	[362]
	BPTES	Inhibition	In vitro: Glioblastoma, human lung fibroblast and immortalized human kidney epithelial cell expressing conditional version of MYC, lymphoma	[250–252, 363]
			In vivo: Lymphoma	[251, 252]
	CB-839	Inhibition	In vitro: Breast cancer, NSCLC, leukemia, ovarian cancer, myeloma	[254–258]
			In vivo: Breast cancer, myeloma	[254, 258]
			Phase I/II trial: Several solid tumors	[259]
	Brachyanthera A8	Inhibition	In vitro: Breast cancer	[260]
	Physapubescink	Inhibition	In vitro/in vivo: Pancreatic cancer	[261]
De novo fatty acid synthesis				
ATP citrate lyase	SB-204990	Inhibition	In vitro: Liver cancer, NSCLC, prostate cancer, ovarian cancer	[276, 277]
			In vivo: Prostate cancer, lung cancer, pancreatic ductal carcinoma	[277]
	Cucurbitacin B (CuB)	Inhibition	In vitro: Breast cancer, pancreatic cancer, hepatocellular carcinoma, NSCLC, prostate cancer, HUVEC	[270, 280–282, 284–287]
			In vivo: Breast cancer, pancreatic cancer, chick chorioallantoic membrane	[282, 284, 287]
	NDI-091143	Inhibition	–	[289]

(continued)

Table 2.1 (continued)

Target	Drug	Effect	Development phase	Reference
Acetyl-CoA carboxylase	TOFA	Inhibition	In vitro: Colon cancer, lung cancer, prostate cancer, ovarian cancer, renal cell carcinoma, breast cancer	[301–305]
			In vivo: Ovarian cancer	[302]
	Soraphen A	Inhibition	In vitro:Breast cancer, prostate cancer, liver cancer	[295, 310, 311]
			In vivo: Breast cancer	[295]
	ND-630	Inhibition	In vitro: Liver cancer	[312]
	ND-646	Inhibition	In vitro/in vivo: NSCLC	[294]
	ND-654	Inhibition	In vitro/in vivo: Liver cancer	[317]
Fatty acid synthase	Cerulenin	Inhibition	In vitro: Breast cancer	[330]
			In vivo: Ovarian cancer	[331]
	C75	Inhibition	In vitro: Breast cancer	[336–339]
			In vivo: Breast cancer	[340]
	Orlistat	Inhibition	In vitro: Prostate cancer, breast cancer, ovarian cancer, stomach cancer	[343, 345–348]
	Fasnall	Inhibition	In vitro/in vivo: Breast cancer	[350]
	TVB-2604	Inhibition	In vivo: Colon cancer	[356]
			Phase I trial: Solid tumors	[357, 358, 364]

metabolized by the downstream steps in glycolytic pathway [20, 21]. Accumulation of 2-DG6P in the cells increases in organic phosphate trapping, resulting in depletion of cellular energy and causes cell death [22–26]. In vitro studies have shown that 2-DG treatment inhibits cell growth, cell cycle progression and lactic acid production in osteosarcoma cell line 143b, especially under hypoxic conditions [27–29]. Similarly, 2-DG restrains growth and clonogenicity of breast cancer cells through induction of apoptosis [25]. Although 2-DG possesses inhibitory effects on tumor growth in vitro, this compound alone does not show a significant anti-neoplastic effect in animal models [30]. However, 2-DG in combination with adriamycin or paclitaxel shows a significant inhibitory effect on growth of osteosarcoma or non-small cell lung cancer (NSCLC) in xenograft mouse models [30]. Further studies showed that long-term treatment with 2-DG can cause chemoresistance through the upregulation of the multidrug resistance P-glycoprotein (P-gp), which functions by outward transport of 2-DG [31]. A clinical study showed that the combination of oral administration of 2-DG with radiotherapy is safe and could be used in glioblastoma patients [32]. Nevertheless, chronic administration of 2-DG has adverse side effects including reduced food intake and reduced weight gain, increased cardiac vacuolization, and increased mortality in rats [33].

3-bromopyruvate (3-BrPA) is an alkylating agent and a potent HK2 inhibitor. 3-BrPA can inhibit HK2 activity and glycolysis in liver tumors and induce rat hepatoma cell death [34]. 3-BrPA treatment depletes ATP production and kills human leukemia HL-60 cells through apoptosis [35]. Mechanistically, HK2 is physically associated with the voltage-dependent anion channel (VDAC) on the outer membrane of mitochondria. This interaction blocks the access of two pro-apoptotic proteins, BAD and BAX, to the mitochondria. However, under glucose deprivation conditions, HK2 dissociates from the outer membrane of mitochondria, enabling BAD and BAX to access the mitochondria resulting in disruption of mitochondrial membrane integrity and initiation of apoptosis [36]. 3-BrPA triggers cancer cell death by modifying a cysteine residue in

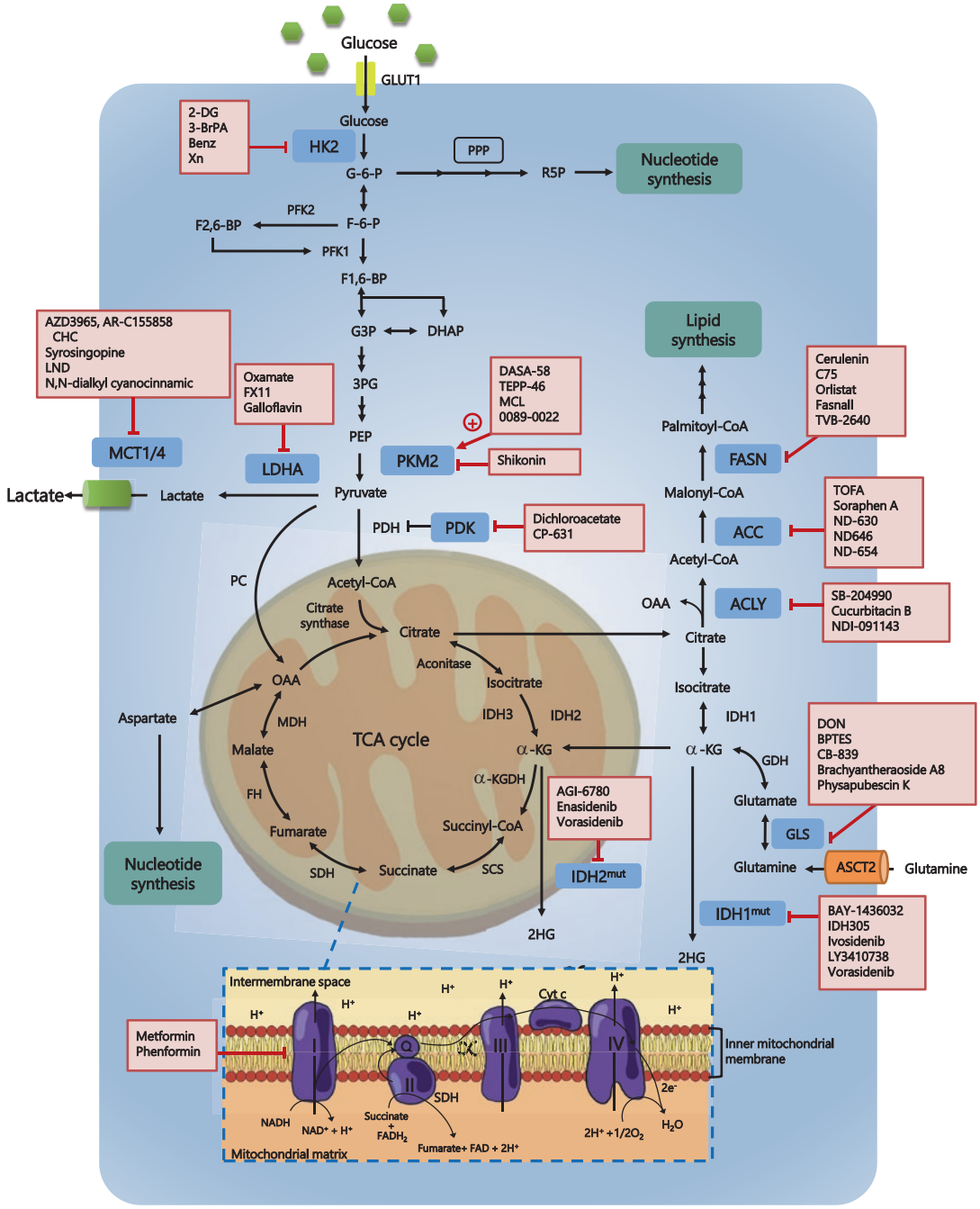

Fig. 2.1 A schematic diagram showing metabolic pathways, including glycolysis, oxidative phosphorylation, pentose phosphate pathway, and glutaminolysis in proliferating cells. The metabolic enzymes that are dysregulated in cancer cells to support their biosynthetic and bioenergetic demand can be exploited as potential therapeutic targets are shown in blue. The current anti-cancer agents are shown in red boxes. *GLUT1* glucose transporter 1, *G-6-P* glucose-6-phosphate, *PPP* pentose phosphate pathway, *R5P* ribose-5-phosphate, *F-6-P* fructose-6-phosphate, *F1,6-BP* fructose-1, 6-bisphosphate, *F2,6-BP* fructose-2, 6-bisphosphate, *PFK1* phosphofructokinase 1, *PFK2* phosphofructokinase 2, *G3P* glyceraldehyde-3-phosphate, *DHAP* dihydroxyacetone phosphate, *3PG* 3-phosphoglycerate, *PEP* phosphoenolpyruvate, *PDH* pyruvate dehydrogenase, *α-KG* α-ketoglutarate, *α-KGDH* α-ketoglutarate dehydrogenase, *SCS* succinyl-CoA synthetase, *SDH* succinate dehydrogenase, *FH* fumarase, *MDH* malate dehydrogenase, *OAA* oxaloacetate, *PC* pyruvate carboxylase, *GDH* glutamate dehydrogenase, *Cyt c* cytochrome c, *ASCT2* alanine, serine, cysteine transporter 2, *HK2* hexokinase 2, *PKM2* pyruvate kinase M2, *LDHA* lactate dehydrogenase A, *MCT1/4* monocarboxylate transporter 1 or 4, *PDK* pyruvate dehydrogenase kinase, *IDH* isocitrate dehydrogenase, *GLS* glutaminase, *ACLY* ATP citrate lyase, *ACC* acetyl-CoA carboxylase, *FASN* fatty acid synthase

the active site of HK2, resulting in the dissociation of HK2 from mitochondria, in a similar manner to that of glucose depletion, and thus induces apoptosis [37]. Intra-arterial injection of 3-BrPA into the rabbit VX2 model of liver cancer selectively inhibits tumor growth without any effect on the surrounding normal liver tissue and also suppresses the development of secondary metastatic lung tumors [38]. Recently, 3-BrPA was shown to reduce ATP production and increase intracellular ROS levels, accompanied by inhibition of proliferation and induction of apoptosis in the nasopharyngeal carcinoma (NPC) cell lines, HNE1 and CNE-2Z. In addition, 3-BrPA also exhibits an anti-tumor activity in mice bearing the CNE-2Z tumor [39]. Although 3-BrPA exhibits a potent cytotoxic effect against cancers both in vitro and in vivo, the clinical applications of 3-BrPA in cancer treatment are still problematic. For example, 3-BrPA-can induce a burning sensation in veins during intravenous infusion, a low diffusion rate to the tumor as well as inability to stay in the tumor mass due to enhanced permeability and a retention effect caused by abnormal vascular structure surrounding tumors. 3-BrPA can also rapidly attach to the thiol groups of glutathione and other proteins, reducing its efficacy to target cancer cells. However, formulating 3-BrPA with liposomes may help to overcome these obstacles in clinical oncology [40].

Benserazide (Benz) has recently been reported as a potent HK2 inhibitor and was approved by the Food and Drug Administration (FDA). Benz has long been used as an adjuvant for the treatment of Parkinson's disease [41] but has recently been used as an anti-cancer drug. This drug is predicted to partially occupy the binding site for glucose but selectively inhibits HK2 activity with a mixed mechanism of action [42]. Benz exhibits the strongest binding affinity to HK2 compared to 2-DG and 3-BrPA, [42]. In vitro studies showed that Benz reduces glucose uptake, lactate production, and ATP concentrations, leading to the loss of mitochondrial membrane potential and subsequent apoptosis. Benz also inhibits tumor growth and induces apoptosis in colorectal cancer xenograft mice [42]. Although Benz shows a good inhibitory effect on growth of tumors in in vitro and in animal models, there have been no reports of clinical trials of Benz in cancer patients.

Xanthohumol (2′,4′,4-trihydroxy-6′-methoxy-3′-prenylchalcone) (Xn), a prenylated flavonoid compound derived from hop plant (*Humulus lupulus*) has been reported as an HK2 inhibitor [43, 44]. Xn shows anti-proliferative, pro-apoptotic, and cytotoxic activities against colon cancer [45], NSCLC [46], breast cancer [47, 48], and cervical cancer [49, 50].

2.2.2 Pyruvate Kinase

Pyruvate kinase (PK) catalyzes the final step of glycolysis by conversion of phosphoenolpyruvate (PEP) to pyruvate, concomitant with the production of ATP. Four isoenzymes of PK, PKL, PKR, PKM1, and PKM2 are found in humans [51]. These four different isozymes exhibit different tissue distributions and kinetic properties. PKL and PKR are produced by alternative splicing of RNA from the same gene and are expressed in liver and erythrocytes, respectively. Similarly, PKM1 and PKM2 are produced by the same gene through an alternative splicing mechanism [52]. The tetramer form of PKM1 is the catalytically active form of the enzyme. PKM1 is expressed in muscle where it supports ATP production by coupling glycolysis with oxidative phosphorylation. Unlike other isoforms of PK, the PKM2 isoform tends to form a monomer or dimer which has a low enzymatic activity. Therefore, the expression of monomeric or dimeric form of PKM2 reduces the overall glycolytic rate, which causes the accumulation of glycolytic intermediates. The increased levels of these intermediates serve as biosynthetic precursors for nucleotides, lipids, and amino acids [52–56]. PKM2 is expressed in early embryonic tissues, proliferating cells and many cancers [53–55]. In addition, the monomeric/dimeric PKM2 has a non-enzymatic function by serving as a transcriptional co-activator and as a protein kinase that can modify the expression of oncogenes or tumor-suppressor genes [57–60]. Moreover, monomeric/dimeric PKM2 stimulates expression of cyclinD1 and

c-Myc, promoting cell cycle progression and metabolic reprogramming, respectively [58, 60]. Replacement of PKM2 with PKM1 can delay growth of human lung cancer xenografts in nude mice, confirming the role of PKM2 as a tumor-promoting isoform [53]. The overexpression and the roles of PKM2 in supporting biosynthesis in cancers suggest the exploitation of PKM2 as a therapeutic target for cancer treatment.

Shikonin, a naphthoquinone derived from the root of plant *Lithospermum erythrorhizon,* is one of the well-known PKM2 inhibitors [61]. Shikonin selectively inhibits PKM2 activity, but not that of other isoforms of PK, and can effectively inhibit glycolysis in both drug-sensitive and resistant cancer cell lines [62]. Clinical studies have shown that Shikonin is a potential anti-cancer agent in various malignancies [63–69]. Shikonin has been shown to be safe and effective in treating late-stage lung cancer patients who fail to respond to operation, radiotherapy, and chemotherapy [69]. Recent studies have shown that shikonin suppresses glycolysis, concomitant with inhibition of proliferation, and induction of apoptosis in Lewis lung carcinoma (LLC) and B16 melanoma cell lines. Moreover, shikonin inhibits B16 tumor cell growth in vivo [70]. This compound also decreases the expression of epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), hypoxia inducible factor (HIF)-1α and PKM2, and induces cell cycle arrest and apoptosis in esophageal cancer both in vitro and in vivo. On the other hand, overexpression of PKM2 enhances the resistance of esophageal cancer cells to shikonin [71]. A recent study showed that shikonin treatment overcomes cisplatin resistance and induces necroptosis in bladder cancer [72]. Because the monomeric or dimeric form of PKM2 can enter the nucleus and activate transcription of some oncogenes, several attempts have been made to convert dimeric PKM2 to tetrameric PKM2 which cannot activate oncogenes. N′,N′-diarylsulfonamides (DASA-58) and thieno [3, 2-b] pyrrole [3, 2-d] pyridazinone (TEPP-46), the first two compounds that were reported to selectively enhance the formation of PKM2 tetramer, reduce lactate production and inhibit growth of tumor xenografts in mice [73–75]. Other PKM2 activators including micheliolide (MCL) and 0089–0022 have also recently been reported to have anti-cancer activities [76, 77].

Although PKM2 has been an attractive therapeutic target for cancer treatment, inhibition of PKM2 activity may be a problem, because PKM2 is also expressed in other normal tissues [78].

2.2.3 Lactate Dehydrogenase-A

Lactate dehydrogenase (LDH) catalyzes the reversible conversion of pyruvate to lactate, concomitant with NAD^+ production [79]. LDH consists of two isozymes, LDH-A and LDH-B. The native forms of both isozymes are tetramers, which are capable of catalyzing the same reaction. However, LDH-A favors the conversion of pyruvate to lactate, while LDH-B favors the reverse direction of the reaction. Lactate is further secreted from cells via monocarboxylic acid transporters (MCTs) to maintain intracellular pH of cancer cells. The secreted lactate creates a slightly acidic microenvironment which promotes metastasis and suppresses immune cell function [80]. LDH-A is overexpressed in many cancers and supports aerobic glycolysis [81]. Overexpression of LDH-A is also associated with chemoresistance in many cancers [82–84], indicating that it is essential for survival of these cancers under this condition. Inhibition of LDH-A expression reduces proliferation of many cancers such as esophageal squamous cell carcinoma [85], neuroblastoma [86], and KRAS-induced NSCLC in a mouse model [83]. Mechanistically, inhibition of LDH-A increases the level of cytosolic pyruvate, which subsequently enters mitochondria to drive the TCA cycle and oxidative phosphorylation. However, because of restricted amounts of oxygen supply in solid tumors, the oxidative phosphorylation is not completed, resulting in overproduction of ROS which has a toxic effect on cancer cells [87–89].

Oxamate is an isoelectronic inhibitory analog of pyruvate and a first-generation LDH-A inhibitor. Oxamate inhibits LDH-A activity by competing with its substrate, resulting in an increased

concentration of pyruvate in mitochondria. Oxamate suppresses proliferation in association with cell cycle arrest, ROS production, and apoptosis in nasopharyngeal carcinoma cells [90], gastric cancer [91], and pituitary adenoma cell lines [92]. The inhibition of LDH-A expression also diminishes c-Myc-induced clonal transformation of human lymphoblastoid cells, and Burkitt lymphoma cells [79]. In addition to oxamate, 3-hydroxyl-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid (FX11) is another LDH-A inhibitor which competes with the binding of reduced nicotinamide adenine dinucleotide (NADH). FX11 has been shown to inhibit proliferation, invasion, and migration of PC-3 and DU145 prostate cancer cell lines [93], as well as osteosarcoma [94] and gallbladder carcinoma [95] cell lines. FX11 also induces oxidative stress and inhibits growth of human lymphoma and pancreatic cancer xenografts in mice [88]. Although, FX11 is an effective therapeutic, the highly reactive catechol portion of its molecule could produce off-target effects on non-tumor cells [96, 97].

Galloflavin, (CAS 568-80-9), a small molecule, inhibits LDH-A by direct binding without competing with substrate or cofactor binding [98]. This compound induces apoptosis and oxidative stress, inhibiting the growth of many cancers [98–101].

Although LDHA inhibitors have been extensively studied in several cancer cell lines and animal models, limited information regarding their efficacy and side effects in preclinical and clinical studies is available [102].

2.2.4 Monocarboxylate Transporters (MCTs)

Monocarboxylic acid transporters (MCTs) are proton-linked membrane proteins which function in the transport of monocarboxylic acids mainly, lactate and pyruvate, but not limited to ketone bodies. MCT-1, -2 and -4 are the main isoforms that carry lactate across the plasma membrane thereby maintaining intracellular pH [103]. There seems to be a functional redundancy between MCT-1 and MCT-4, by which both can transport lactate out of the cell. However, MCT-1 can also transport lactate into the cell for the subsequent conversion to pyruvate before entering mitochondria for oxidative phosphorylation. This difference between the two isoforms is further exemplified by the differential expression of these two MCTs in cancers with different metabolic phenotypes. MCT-4 is highly expressed in glycolytic or aerobic cancer cells which metabolize glucose to lactate. Under these circumstances, MCT-4 supports aerobic glycolysis by transporting lactate out of the cell so that the glycolytic pathway is not inhibited [102]. In contrast, oxidative tumors tend to produce ATP through oxidative phosphorylation using lactate as a nutrient source. Under these circumstances, the oxidative tumors express MCT-1, which can also transport extracellular lactate into the cell. Once inside the cell, lactate can be converted to pyruvate by LDH-B and used as a source of ATP production through oxidative phosphorylation [102]. Overexpression of MCT-1 and/or MCT-4 is associated with poor prognosis in many cancers [104–109], with metastasis in renal cancer and adenocarcinoma [110, 111]. Genetic suppression of MCT-1 or MCT-4 expression results in lactate accumulation accompanied by reduced proliferation, invasion, and migration in lung cancer [112]; pancreatic ductal adenocarcinoma [113]; oral squamous cell carcinoma [114]; and breast cancer [115, 116]. In a mouse model, suppression of MCT-1 and MCT-4 expression also inhibits the growth of human breast cancer xenografts [116, 117].

Because both MCT-1 and MCT-4 support bioenergetics of both glycolytic and hypoxic tumors, pharmacological inhibition of these two MCTs is a potential target of anti-cancer drugs. AR-C155858, a pyrrole pyrimidine derivative, specifically blocks MCT-1 activity and inhibits lactate export from the cells [118]. AR-C155858 has been shown to inhibit the growth of KRAS-transformed fibroblasts [119], myelomas [120], pancreatic ductal adenocarcinomas [113], and breast cancer cell lines [121, 122]. However, a recent preclinical study showed that AR-C155858 failed to reduce the growth of a murine breast cancer xenograft in nude mice [123].

AZD3965 is a selective and potent inhibitor of MCT-1, with well-studied pharmacokinetics [103]. In xenograft mouse models, AZD3965 treatment increases the intracellular concentration of lactate and inhibits growth of several tumors including small cell lung cancer [124], breast cancer [116], diffuse large B-cell lymphoma and Burkitt lymphoma [125]. The combination of AZD3965 with other anti-cancer drugs increases its efficacy against several cancers [126, 127]. AZD3965 is currently under phase I clinical trial in patients with advanced solid tumors such as prostate cancer, gastric cancer, and diffuse B-cell lymphoma [128]. While AZD3965 can effectively inhibit growth of MCT-1 over-expressing cancers, this drug does not work well with the cancers that overexpress both MCT-1 and MCT-4, because MCT-4 can counteract MCT-1 inhibition [124].

α-cyano-4-hydroxy cinnamate (αCHC) is another MCT-1 inhibitor which has been shown to inhibit growth and induce apoptosis in glioma cells [129], breast cancer [130], and myeloma cell lines [120]. αCHC can also inhibit the growth of lung carcinoma and human colorectal adenocarcinoma xenografts in mice [131]. There have been no further studies of this inhibitor in clinical studies.

As mentioned, the inhibition of MCT-1 alone is not sufficient to inhibit the growth of tumors that express both MCT-1 and MCT-4. For this reason, several efforts have been made to produce dual inhibitors for MCT-1 and MCT-4 such as Syrosingopine, Lonidamine (LND), and N, N-dialkylcyanocinnamic acid. Syrosingopine, an anti-hypertension drug, has recently been used to inhibit both MCT-1 and MCT-4 activities in several pre-clinical studies [131–133]. Combined treatment of Syrosingopine with metformin improves its efficacy for inhibiting growth of cervical, breast, and leukemic cancer cell lines [133]. N,N-dialkylcyanocinnamic acid has recently been reported as a dual MCT-1 and MCT-4 inhibitor. Inhibition of lactate export by this drug results in inhibition of glycolysis and oxidative phosphorylation in colorectal adenocarcinoma and triple negative breast cancer cell lines. It was also found to inhibit growth of colorectal and breast cancers in xenograft mouse models [134]. LND, a novel chemotherapy drug, has recently been reported to block MCT-1, -2, -4, and the mitochondrial pyruvate carrier, resulting in simultaneous inhibition of lactate efflux from the cell and pyruvate uptake into mitochondria [110]. This drug can inhibit growth of DB-1 melanoma in vitro and in xenograft mice [135, 136].

2.3 Mitochondrial Metabolism

Although aerobic glycolysis is a metabolic hallmark of many cancers, accumulating evidence has shown that some cancers such as breast cancer, Hodgkin lymphoma, diffuse large B-cell lymphoma and pancreatic ductal adenocarcinoma, use oxidative phosphorylation to support ATP production. This metabolic phenotype is known as the oxidative cancer cell [137]. Recent studies also showed that cancer stem cells with high tumorigenic and metastatic potentials use oxidative phosphorylation to support their growth [138, 139]. The bioenergetic switch from glycolysis to oxidative phosphorylation in this group of cancer makes mitochondrial metabolism an attractive target. Because mitochondria metabolism starts from oxidation of pyruvate to acetyl-CoA followed by the TCA cycle and oxidative phosphorylation, all key enzymes along these pathways are potential targets of anti-neoplastic agents.

2.3.1 Pyruvate Dehydrogenase Kinase

Pyruvate dehydrogenase complex (PDH) catalyzes the irreversible conversion of pyruvate to acetyl-CoA in the mitochondria. Since this reaction connects glycolysis and the TCA cycle, inhibition of this regulatory step results in the redirection of pyruvate to lactate or leads to alanine production [140]. PDH activity is regulated by reversible phosphorylation catalyzed by the pyruvate dehydrogenase kinase (PDK). Phosphorylation of PDH by PDK inactivates

PDH activity while dephosphorylation by phosphatases reactivates it. In mammals, PDK1, PDK2, PDK3, and PDK4 can phosphorylate PDH [141]. In most cancers, glycolysis is partly driven through the inactivation of PDH by PDKs [142, 143], enabling them to maintain the glycolytic phenotype. On the other hand, inactivation of PDK reverses the metabolic phenotype from glycolysis to oxidative phosphorylation. Increased entry of pyruvate into the TCA cycle caused by PDH activation can overwhelm TCA cycle activity and oxidative phosphorylation, resulting in an incomplete oxidation of reducing equivalents, leading to the overproduction of reactive oxygen species (ROS). In turn, increased ROS can create oxidative stress which can damage cancer cells [144–146]. Therefore, inhibition of PDK activity can be used to induce oxidative stress in cancers. Dichloroacetate (DCA) is a first generation PDK inhibitor [147, 148] first reported to induce oxidative stress, leading to cell death in many cancers, including prostate cancer [149], breast cancer [150], and colorectal cancer [151, 152]. DCA treatment can inhibit growth and angiogenesis of NSCLC and breast cancer xenografts [153]. Studies in glioblastoma patients showed that DCA treatment can switch metabolism of tumor cells from glycolysis to oxidative phosphorylation, accompanied by mild induction of apoptosis and reduction of angiogenesis surrounding the glioblastoma [154]. DCA is currently undergoing phase I clinical testing in patients with recurrent glioblastoma [155].

CPI-613 (devimistat) is an inhibitor of PDK. Because CPI-613 has a structure similar to lipoic acid, a co-factor of PDH, CPI-631 is thought to interfere with phosphorylation inhibition by PDKs and disrupt mitochondrial metabolism, leading to cell death [156]. Later studies also showed that CP-613 may also have an inhibitory effect on cancer growth by inhibiting the activity of α-ketoglutarate dehydrogenase which converts α-ketoglutarate to succinate [157]. CPI-613 is currently undergoing a phase I trial in patients with relapsed or refractory acute myeloid leukemia [158], as well as a phase II trial in patients with relapsed or refractory small cell lung carcinoma [159], and phase I and phase III trials in patients with metastatic pancreatic cancer [160, 161].

2.3.2 TCA Cycle

The TCA cycle is not only a central hub for oxidation of glucose, fatty acids, and amino acids it also functions as a biosynthetic hub in which its intermediates are used as biosynthetic precursors of non-essential amino acids, fatty acids, and nucleotides. In cancers, glutaminolysis is an important biochemical reaction that provides carbon skeletons into the TCA cycle, sustaining the levels of TCA cycle intermediates upon their removal for biosynthesis. Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG). IDH1 is located in the cytoplasm while IDH2 is located in mitochondria [162]. Mutation of arginine 132 to histidine in IDH1 (R132H) [163, 164] or mutations of arginine 140 or arginine 172 to histidine in IDH2 (R140H or R172H) [165, 166] alters their activity such that the mutant enzymes further convert α-KG to 2-hydroxyglutarate (2-HG) [167, 168]. The abnormal accumulation of 2-HG competitively inhibits α-KG-dependent dioxygenase, contributing to malignant transformation [169, 170]. Many tumors such as glioblastoma [163, 171], chondrosarcoma [172], osteosarcoma [173], myeloid leukemia [174], intrahepatic cholangiocarcinoma [175], breast cancer [176], and prostate cancer [177] appear to carry IDH1/IDH2 mutations [178]. Due to the strong effect of IDH1 and IDH2 mutations on tumorigenesis, IDH1 and IDH2 inhibitors have been developed to block the production of 2-HG by the mutant enzymes.

BAY-1436032 is an allosteric inhibitor of IDH1, which binds to the IDH1 mutant enzyme and interrupts dimer formation, inhibiting its activity [179]. BAY-1436032 lowers 2-HG production and reduces proliferation in glioma bearing an IDH1 mutation [179]. A similar result was also observed in acute myeloid leukemia (AML) bearing an IDH1 mutation, in which treatment of AML with this drug improved differentiation of myeloid progenitors to normal leukocytes both in

cell culture and in xenografted models [180]. BAY-1436032 is currently undergoing phase I clinical trials for AML, glioma, and intrahepatic cholangiocarcinoma (ClinicalTrials.gov NCT03127735, NCT02746081).

IDH305, a pyrimidin-5-yl-oxazolidine-2-one compound, is an allosteric inhibitor of mutant IDH1, with excellent capability of passing through the blood–brain barrier [181]. Pre-clinical studies showed that IDH305 lowers 2-HG levels in a patient-derived IDH1 mutant xenograft tumor model [181]. IDH305 is currently under phase I clinical trials, and the preliminary clinical data have already shown that IDH305 has a favorable safety profile and promising anti-tumor activity in AML harboring IDH1 mutations [182]. Further studies regarding the safety, tolerability, and anti-tumor activity as a single agent and in combination with others are on-going [182].

Ivosidenib (AG-120) (Tibsovo) is a highly selective inhibitor for mutant IDH1s without an inhibitory effect on IDH2. This drug inhibits the activity of the mutant enzyme by competing with cofactor binding [183]. In vivo pharmacokinetic studies have shown that this drug is rapidly absorbed, with low plasma clearance and a modest level of brain penetration [184]. Ivosidenib was found to markedly reduce the level of 2-HG and inhibit growth of primary human AML myeloblasts, human chondrosarcoma cell lines, and IDH1-R132H glioma xenografts [184–186]. Currently, Ivosidenib has been approved by the FDA for the treatment of adults with relapsed or refractory AML with IDH1 mutations in phase I clinical trials [187].

LY3410738, a new potent inhibitor of mutant IDH1 forms, was developed using a structure-based drug design approach. LY3410738 reacts with cysteine 269 in the allosteric binding pocket and rapidly inactivates the mutant IDH1 enzyme. LY3410738 can pass through the blood–brain barrier and exhibits prolonged pharmacokinetics. This drug can inhibit 2-HG production in glioma both in vitro and in vivo. Moreover, LY3410738 is more potent than AG-120 or Ivosidenib for inducing differentiation of myeloid progenitors to produce normal hematopoietic cells in patient-derived primary AML cells harboring IDH1 mutations [188].

AGI-6780, a urea sulfonamide compound, is the first small molecule selective inhibitor of IDH2 mutants [188]. It binds at the IDH2 dimer interface of the enzyme and also competes with binding of NADPH, resulting in inactivation of the enzyme [188–190]. AGI-6780 can suppress the production of 2-HG in human glioblastoma U87 and TF-1 cells harboring the IDH2-R140Q mutation, concomitant with restoration of normal differentiation of hematopoietic cells [189, 191]. Furthermore, 2-HG-induces DNA and histone hypermethylation in TF-1 erythroleukemia cells with the IDH2-R140Q mutation can be reversed by treatment with AGI-6780 [191]. Although AGI-6780 possesses an excellent inhibitory effect on normal differentiation of the hematopoietic cell lineage, further investigation of AGI-6780 on AML has been restrained due to the emergence of Enasidenib which is more potent than this drug.

Enasidenib is a selective inhibitor of IDH2. This drug preferentially binds to the IDH2 R172H mutant enzyme and lowers 2-HG production [184]. Both pre-clinical and clinical studies have shown that this drug lowers the levels of 2-HG in acute amyloid leukemia, in parallel with a high rate of differentiation of myeloid progenitors to normal leukocytes [192, 193]. Similar to Ivosidenib, Enasidenib was approved by the FDA for patients with relapsed or refractory AML bearing mutations of the IDH2 gene [194]. Enasidenib is currently undergoing phase II clinical testing.

AG-881 (Vorasidenib) is a triazine class compound, which is the first pan-inhibitor of both IDH1 and IDH2 mutant enzymes. AG-881 allosterically inhibits the activities of both mutant forms of IDH1 and IDH2 by binding to the allosteric pocket at the dimer interface. However, binding of this compound to mutant IDH-1 is much faster than to mutant IDH2 [195]. AG-881 also lowers the production of 2-HG in tumors bearing either IDH1 or IDH2 mutations, concomitant with improved differentiation of myeloid progenitors to normal hematopoietic cells [196]. A pharmacokinetic study showed that AG-881

exhibits the rapid oral absorption, low total body plasma clearance properties, and excellent brain penetration, supporting the clinical development of this compound [196]. AG-881 is currently being investigated in phase I clinical trial for safety, pharmacokinetics, and pharmacodynamics in glioma patients carrying mutations of IDH1 and/or IDH2 [197].

2.3.3 Oxidative Phosphorylation

As an anti-diabetic drug with glucose lowering effects, metformin has recently attracted attention as an anti-cancer drug. Metformin inhibits complex I of the respiratory chain, resulting in inhibition of ATP synthesis [198, 199]. This inhibition results in an increased ratio of AMP:ATP which, in turn, activates AMP-activated protein kinase (AMPK) [200, 201]. AMPK then activates phosphorylation of the transcription co-activator of gluconeogenic enzyme genes [201–203]. In cancers, metformin also depletes ATP synthesis through the same mechanism as reported in diabetic patients, resulting in cancer cell death [204, 205]. Further studies also showed that AMPK activation not only decreases protein synthesis through the inhibition of the mammalian target of rapamycin (mTOR) signaling pathway [206, 207] but it also activates the p53 tumor-suppressor gene [208, 209] and induces cell cycle arrest [210]. In addition, metformin diminishes TCA cycle activity, affecting cataplerosis and nucleotide synthesis [211, 212]. Metformin also reduces the risk of cancer in diabetic patients [213–215]. Owing to the uptake of metformin requiring the organic cation transporter 1 (OCT1) which is highly abundant on plasma membrane of hepatocytes, the transport of metformin into other cell types, such as cancer cells, is limited [216]. This has resulted in the use of another biguanide drug, phenformin, as a potential alternative anti-cancer drug. Phenformin, a more hydrophobic drug, can therefore penetrate through the plasma membrane of cancer cells better than metformin. Although this drug can cause lactic acidosis in diabetic patients, phenformin has been reported to have excellent anti-neoplastic effects in both cell culture and xenograft mouse models [217, 218]. The use of metformin as an anti-tumor drug in breast, prostate, esophageal, and uterus cancers is currently undergoing clinical trials [219].

2.4 Glutaminolysis

For decades, several studies have shown that glutamine, the most abundant amino acid in blood and muscle, is highly consumed in most cancers [220–224]. Glutaminolysis is the oxidative deamination of glutamine to glutamate by glutaminase (GLS) before subsequent conversion to α-ketoglutarate by glutamate dehydrogenase, enabling carbon skeletons of glutamine to enter the TCA cycle to support cataplerosis [11, 223, 225]. Moreover, glutamine is used to synthesize glutathione for maintaining cellular redox homeostasis [226] and is also an important modulator of mTOR [227, 228] and endoplasmic reticulum (ER) stress signaling pathways [229]. Most cancers are highly addicted to glutamine because the removal of glutamine dramatically reduces cell growth or induces cell death [230–234]. This information indicates that glutaminolysis is one of attractive targets for anti-cancer drugs.

2.4.1 Glutaminase (GLS)

Multiple lines of evidence show that GLS is overexpressed in many cancers, indicating that this pathway is essential for their growth and survival. In humans, there are two different isoforms of GLS, namely GLS1 and GLS2. GLS1 is mainly expressed in proliferating cells, while GLS2 is expressed in quiescent cells. During oncogenic transformation, there is a genetic switch from GLS2 to GLS1 expression in many cancers [235, 236]. Overexpression of GLS1 in cancer is also associated with poor prognosis and inhibition of GLS1 activity can inhibit cancer growth [237–239]. 6-diazo-5-oxo-L-norleucine (DON) and acivicin, the first generation of GLS inhibitors have been reported [240, 241]. These two compounds are L-glutamine analogs were isolated

from *Streptomyces* bacteria. They inhibit glutamine metabolism by irreversibly interacting with serine 286 in the GLS active site [242]. Although they are potent GLS inhibitors and can inhibit growth of several cancers [243–245], they produce severe off-target side effects by inhibition of enzymes such as NAD synthase [243, 246].

Bis-2-(5-phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulfide (BPTES) is a more selective inhibitor of GLS1 [247]. BPTES has been identified as a non-competitive allosteric inhibitor of GLS1. Binding of this compound at the allosteric pocket results in a dramatic conformational change that blocks the activation by phosphate [248] without off-target effects [249]. As a result of inhibition of TCA cycle input from glutamine, BPTES treatment lowers the levels of glutamate, fumarate, and malate, while increasing the level of glycolytic intermediates. Similarly, BPTES treatment was also found to diminish glutamate and other TCA cycle intermediates, concomitant with increased apoptosis in MYC-overexpressing cancers [250]. BPTES was reported to inhibit growth of P493 lymphoma xenograft in mice [251]. In addition, BPTES treatment prolongs the survival of mice bearing MYC-overexpressing cancer without toxic side effects [252]. However, BPTES is not used as anti-cancer drug because of its poor solubility and low bioavailability [253].

CB-839 is a BPTES derivative with an improved solubility [249, 254]. Inhibition of GLS by CB-839 diminishes glutaminolysis, concomitant with growth inhibition of triple negative breast cancer [254], NSCLC [255], leukemia [256], and ovarian cancer [257]. Inhibition of GLS1 by CB839 in ovarian cancer reduces phosphorylation of signal transducer and activator of transcription 3 (STAT3), making this cancer more sensitive to PI3K/Akt/mTOR inhibition [257]. CB-839 has also been used in combination with paclitaxel or pomalidomide to increase its efficacy in the tumor xenograft model [254, 258]. At present, CB-839 is the most effective small-molecule GLS1 inhibitor in phase I of clinical trials [259]. In addition to CB-839, the nor-oleanane triterpenoid compound, brachyantheraoside A8, has been reported as another GLS1 inhibitor [260]. Brachyantheraoside A8 has been shown to decrease migration and invasion and induced apoptosis in breast cancer [260]. Physapubescin K, a natural product from *Physalis pubescens*, has recently been reported to inhibit GLS1. This compound can inhibit growth of pancreatic cancer cell growth in vitro and in vivo [261]. Furthermore, physapubescin K also exhibits the synergistic inhibition of tumor growth with benserazide and erlotinib [261]. Although these new natural GLS1 inhibitors display potent anti-cancer activity, the molecular mechanism of inhibition of these small molecules and their side effects have not been elucidated. The long-term paradigm of glutaminase being a target of anti-cancer has recently been challenged by the study in the KRAS-driven NSCLC in which the rate of glutaminolysis was negligible when transplanted in nude mice [262, 263]. Furthermore, CB-839 also fails to block growth of NSCLC transplanted in nude mice [263] while it inhibits growth of this cancer in vitro, indicating that glutamine may not be required for in vivo growth.

2.5 De Novo Fatty Acid Synthesis

De novo lipogenesis (DNL) is the biosynthesis of fatty acids from glucose. This pathway combines glycolysis and long chain acyl-CoA synthesis [264] and is an important pathway for cancers because fatty acids are essential structural components of the plasma membrane. Numerous studies showed that rate of DNL is very high in many cancers [265–267]. Increased DNL in cancers is accompanied by increased activity or expression of several key lipogenic enzymes such as ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FASN).

2.5.1 ATP-Citrate Lyase

ACLY is the first key enzyme that links glycolysis and long chain acyl-CoA synthesis. The cytosolic ACLY catalyzes the cleavage of citrate to oxaloacetate and acetyl-CoA. Oxaloacetate is reconverted to malate before re-entry into mitochondria, while acetyl-CoA is used as a precursor

for long chain fatty acids and cholesterol synthesis (See Fig. 2.1) [268]. ACLY expression is elevated in stomach, prostate, liver, breast, kidney, and NSCLCs [269–274]. Recent studies showed that ACLY supports colon cancer metastasis by promoting the activity of CTNNB1, a key regulator of epithelial-mesenchymal transition [275]. Suppression of ACLY expression in breast, kidney, and NSCLCs markedly inhibits proliferation and induces apoptosis [272–274]. It also inhibits metastasis of colorectal cancer both in vitro and in vivo [275]. Among the ACLY inhibitors, SB-204990, cucurbitacin B (CuB), and NDI-091143 are the most potent inhibitors of DNL. SB-204990, a cell-penetrant γ-lactone prodrug of SB-201076, can inhibit both cholesterol and fatty acid synthesis in HepG2 cells [276]. Furthermore, this compound also inhibits proliferation and survival of highly glycolytic tumors [277]. Although SB-204990 shows an excellent anti-neoplastic activity in pancreatic ductal xenografts in a mouse model [277], there has been no clinical study of this compound in patients.

Cucurbitacins (CUs) are natural products isolated from cucumber, melon, squash, and pumpkins. These compounds contain a tetracyclic cucurbitane nucleus skeleton, namely, 9β-methyl-19-norlanosta-5-enea, which is traditionally divided arbitrarily into 12 categories, incorporating CUs A-T [278, 279]. Among several groups of CUs, CuB exerts anti-cancer activity by inhibiting proliferation and apoptosis in several human cancers including breast cancer [280–282], pancreatic cancer [283, 284], hepatocellular carcinoma [285], lung cancer [286], and prostate cancer [270]. CuB inhibits ACLY activity by reducing its expression and inhibits its phosphorylation [270]. In vivo studies showed that treatment with CuB can suppress growth in Panc-1 pancreatic [283], PC3 prostate [270], and H1299 NSCLC xenografts in nude mice [286]. The combination of CuB and gemcitabine improves the anti-proliferative effect in human breast cancer [282] and pancreatic cancer [284] xenografts in nude mice. Furthermore, CuB can inhibit tumor angiogenesis by suppressing human umbilical vascular endothelial cell proliferation, migration, and capillary-like structure formation, and induce apoptosis in vitro [287]. CuB has low oral bioavailability, but it could be distributed broadly into several organs such as lung, spleen, and kidney, with a high volume of distribution and tissue to plasma ratio [288]. Nevertheless, further clinical study of CuB is necessary to confirm their therapeutic effect in cancer patients.

NDI-091143 is the most recently identified ACLY inhibitor [289]. This compound shows an excellent potency and selectivity toward ACLY. NDI-091143 allosterically binds to ACLY and disrupts binding of citrate [289]. Although, there has been no report on the efficacy of NDI-091143 on cancer treatment, this compound or its analogs might have potential uses as anti-cancer agents due to their appealing allosteric inhibition mechanism to human ACLY activity [289].

2.5.2 Acetyl-CoA Carboxylase

ACC catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, an acetyl-group donor for de novo fatty acid biosynthesis [290]. In humans, ACC1 and ACC2 are encoded by two separate genes. Although, these two isoforms catalyze the same chemical reaction, their metabolic roles are distinct. ACC1 is a cytosolic enzyme that is expressed exclusively in lipogenic tissues such as liver and adipose tissue where it catalyzes the first committed step in the biosynthesis of long chain fatty acids [291]. On the other hand, ACC2 is expressed in oxidative tissues, that is, heart and skeletal where it controls oxidation of fatty acids through carnitine palmitoyltransferase I activity [292]. ACC1 is overexpressed in multiple human cancers, such as breast, liver, and NSCLC to support DNL [271, 293, 294]. Suppression of ACC1 expression or inhibition of ACC1 activity decreases phospholipid composition in the plasma membrane, reducing its integrity and receptor dimerization [295]. This combined effect in turn inhibits growth and progression of many cancers [294–296]. Several ACC1 inhibitors have been identified and studied. The most well-characterized ACC1 inhibitors are TOFA (5-(tetradecyloxy)-2-furoic acid), soraphenA, ND-646 and ND-654.

TOFA is a representative of fatty acyl-CoA mimetics that were first used for treatment of hyperlipidemia [297]. TOFA is further metabolized to 5-tetradecyloxy-2-foryl-CoA (TOFyl-CoA) which allosterically inhibits ACC1 by binding to the carboxyltransferase domain of the enzyme, preventing binding of acetyl-CoA [298]. This inhibitor can reduce fatty acid synthesis, triglyceride secretion and apolipoprotein B secretion in cultured hepatocytes [298, 299]. It reduces fatty acid, cholesterol, and triglyceride synthesis, increases fatty acid oxidation and decreases very low density lipoprotein (VLDL) production in rat livers perfused with TOFA ex vivo [300]. In cancers, TOFA blocks fatty acid synthesis, concomitant with inhibition of proliferation and induces apoptosis in several cancer cell lines such as HCT-8 and HCT-15 colon cancer [301], LNCaP prostate cancer [302], COC1 and COC1/DDP ovarian cancer [303], NCI-H460 lung cancer [301] and ACHN, and 786-O renal cell carcinoma [304]. TOFA also induces cell cycle arrest in ovarian cancer [303] and renal carcinoma [304]. In addition, it can inhibit growth of human ovarian tumors in xenograft mice [303]. TOFA treatment also reduces lipid droplet content and proliferation in breast cancer BT474, MCF-7, and T47D cell lines. However, the efficacy of TOFA in reducing lipid droplets and inhibiting proliferation were not observed in MDA-MB-231 cells [305].

Soraphen A is a macrocyclic polyketide natural product isolated from the myxobacterium strain *Sorangium cellulosum* [306]. This compound contains an unsaturated 18-membered lactone ring, an extracyclic phenyl ring, two hydroxyl groups, three methyl groups, and three methoxy groups [307, 308]. It was originally used as an anti-fungal agent in agriculture [307, 308]. Soraphen A inhibits ACC1 activity by binding at the dimer interface of biotin carboxylase domain which, in turn, perturbs oligomerization of the biotin carboxylase domain and inactivates ACC1 activity [309]. Soraphen A treatment reduces fatty acid synthesis, inhibits proliferation, and induces cell death in LNCaP and PC-3 M prostate cancer [310] and HepG2 [311] cell lines. Soraphen A affects composition, turnover, and activation of phospholipid membranes of SKBR3 breast cancer and Huh7 hepatocellular carcinoma cell lines, resulting in inhibition of their proliferation [295]. Soraphen A also inhibits the dissemination of breast cancer and hepatocellular carcinoma xenografts in a mouse model [295].

ND-630 was identified as an isozyme-nonselective, allosteric ACC inhibitor by structure-based drug design [312]. ND-630 binds to ACC1 in close vicinity to the AMPK phosphorylation site [313]. Binding of ND-630 at this position disrupts dimerization of ACC1 and inhibits enzymatic activity. Treatment with ND-630 diminishes fatty acid synthesis while stimulating fatty acid oxidation in human hepatocellular carcinoma HepG2 cells [312]. Presently, ND-630 is undergoing phase II clinical trials as a drug for non-alcoholic steatohepatitis, although the efficacy of ND-630 as anti-cancer agent has not been examined [314–316].

ND-646, another ACC1 inhibitor, inactivates ACC1 by interacting with several residues in the dimer interface of the BC domain, in a similar manner as that of ND-630. ND-646 inhibits fatty acid synthesis, proliferation, and induces apoptosis in a NSCLC cell line and inhibits growth of NSCLC xenografts in nude mice [294]. Recently, ND-654 has been reported as a new ACC1 inhibitor. Similar to ND-630 and ND-646, ND-654 binds to ACC1, interferes with dimer formation and inactivates enzymatic activity. However, unlike ND-646 which shows broad tissue absorption, ND-654 has been modified to increase hepatic uptake absorption for effective treatment of hepatocellular carcinoma. ND-630 inhibits human ACC1 with an IC_{50} of 3 nM and inhibits human ACC2 with an IC_{50} of 8 nM [317]. Treatment with ND-654 also inhibits fatty acid synthesis and proliferation in HepG2 cells. Finally, ND-654 treatment alone or in combination with sorafenib was found to reduce fatty acid synthesis, inhibit proliferation, and increase survival in a diethymitrosamine-induced hepatocellular carcinoma model in rats [317].

2.5.3 Fatty Acid Synthase

FASN catalyzes the condensation of two carbon units from malonyl-CoA to the growing chain of acyl-CoA. Similar to other lipogenic enzymes, FASN is overexpressed in most human carcinomas [318–322]. Elevated FASN expression has also been found to be correlated with poor prognosis in breast cancer [323], prostate cancer [324, 325], and NSCLC [326, 327], clearly demonstrating its importance in supporting the growth and survival of these cancers. Several first-generation FASN inhibitors such as cerulenin, C75, and orlistat compounds have been reported to inhibit growth of several cancers.

Cerulenin, an antibiotic derived from the fungus *Cephalosporum caerulens*, is a non-competitive inhibitor of FASN [328]. Cerulenin inhibits FASN activity by forming a covalent bond with a cysteine residue in the active site of the β-ketoacyl-synthase domain of the enzyme [328, 329]. Cerulenin inhibits proliferation and induces apoptosis in breast cancer cells in vitro [330]. Moreover, cerulenin delays tumor progression in a xenograft model [331]. However, the use of cerulenin still has some limitations, because the reactive epoxy group in its structure can react with other proteins and may affect cholesterol synthesis or proteolysis [332, 333].

C75 is the first synthetic FASN inhibitor that was developed to resolve the chemical instability of the reactive epoxide present in cerulenin [334]. C75 inhibits FASN activity by targeting the β-ketoacyl-synthase domain, in the same manner as cerulenin, and also inactivates the enoyl reductase and the thioesterase domains of the enzyme [335]. C75 treatment selectively inhibits tumor growth by inducing apoptosis in both cultured cancer cells and in xenograft mouse models [336–339]. Furthermore, long-term administration of C75 significantly delays breast cancer development in mouse models [340]. However, the use of cerulenin and C75 as anti-cancer drugs still has some problems due to side effects of weight loss and anorexia [341, 342].

Orlistat, an FDA-approved pancreatic and gastric lipase inhibitor, was originally developed as an anti-obesity drug. Orlistat inhibits FASN activity by forming a covalent adduct with the active site serine residue in the thioesterase domain of this enzyme [343, 344]. Orlistat inhibits proliferation and induces apoptosis in PC3-prostate cancer [343], breast cancer [345, 346], ovarian cancer [347], and stomach cancer [348] in vitro. Although orlitstat shows an excellent effect on the inhibition of cancer growth, it has poor solubility and low oral bioavailability [349].

Fasnall, a thiophenopyrimidine scaffold, has recently been discovered through a chemoproteomic platform fluorescence-linked enzyme chemoproteomic strategy. It has been reported as a potent and selective inhibitor with an IC_{50} of 3.71 μM [350]. Interestingly, Fasnall has potent anti-proliferative and pro-apoptotic activity against various breast cancer cell lines without any effect on normal cells [350]. Because malonyl-CoA produced by FASN is an inhibitor of fatty acid oxidation, inhibition of FASN by Fasnall reduces the level of malonyl-CoA, raising the concentration of palmitate. Increased cellular palmitate then reacts with serine to form ceramides, which potentially disrupts the integrity of the plasma membrane of cancer cells [351] and induces apoptosis [352–354]. An in vivo study showed that Fasnall reduces growth of virally induced and triple negative-breast cancer xenografts in mice with prolonged survival times [350]. Taken together, the potent anti-neoplastic activity of Fasnall suggests its further use in clinical studies.

TVB-2640 is an orally active, reversible FASN inhibitor. It inhibits the β-ketoacyl reductase (KR) activity of FASN [355]. TVB-2640 inhibits growth of human colon adenocarcinoma COLO-205 xenografts in rat, accompanied by reduction of Akt phosphorylation [356]. TVB-2640 is currently being investigated in phase I clinical trials with patients with NSCLC, ovarian cancer and breast cancer. This drug shows a good efficacy when combined with paclitaxel [357, 358].

2.6 Conclusion and Future Perspectives

Metabolic reprograming allows cancer cells to selectively alter their cellular metabolism to suit their needs during rapid proliferation. This allows us to design small molecules that inhibit the distinct metabolic pathways in cancer but do not affect normal cells. Even though specific targets have been identified, several drugs still possess limitations such as specificity, solubility, bioavailability, and adverse side effect while some fail during clinical trials. Currently, only a few anti-cancer drugs such as those that target glutaminase, MCTs, IDH1/2 and FAS are in clinical trials. The search for more inhibitors with exceptional specificity and more potency is challenging. However, with the application of high throughput technologies including transcriptomics, proteomics, and metabolomics, it should become feasible to investigate the specificity and efficacy of anti-cancer drugs at the cellular and organismal levels. Using the same systems biology tools, identification of specific responses to the drugs in individual patients is currently shifting the paradigm of future health care from cancer treatment to preventive and personalized medicine.

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A Review of Monoclonal Antibody-Based Treatments in Non-small Cell Lung Cancer

3

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Abstract

Non-small cell lung cancer (NSCLC) is one of the most common types of lung cancer worldwide. It metastasizes rapidly and has a poor prognosis. The first-line treatment for most patients is a combination of chemotherapy and radiation. In many subjects, using targeted treatments alongside chemoradiation has shown a better outcome in terms of progression and quality of life for patients. These targeted treatments include small biological inhibiting molecules and monoclonal antibodies. In this review, we have assessed studies focused upon the treatment of non-small cell lung cancer. Some therapies are approved, such as bevacizumab and atezolizumab, while some are still in clinical trials, such as ficlatuzumab and ipilimumab, and others have been rejected due to inadequate disease control, such as figitumumab.

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Keywords

Lung cancer · Non-small cell · Monoclonal antibodies · Targeted treatment

3.1 Introduction

Lung cancers are the most common cancers worldwide and the leading cause of cancer-related deaths globally. They are divided into three groups based on the microscopic appearance of the tumor cells: non-small cell lung cancers (NSCLCs), small cell lung cancers (SCLCs), and bronchial adenomas [1, 2]. NSCLCs are the most common, comprising more than 80% of lung cancers and have three distinct subtypes: squamous cell carcinoma (epidermoid); adenocarcinoma; and large cell (undifferentiated) carcinoma [1, 3].

Treatment of NSCLC consists of surgery, radiation, chemotherapy, targeted treatments, and immunotherapy, either alone or in combination. The most common drugs of choice for chemotherapy regimens include cisplatin, carboplatin, docetaxel, gemcitabine, paclitaxel, vinorelbine, and pemetrexed. Unlike chemotherapy drugs, targeted agents are specifically designed to attack cancer cells, causing less damage to normal cells, and are thus being used alone or in combination with chemotherapeutic agents. The common targeting agents used in NSCLC treatment are biological inhibitors or monoclonal antibodies that are aimed at different receptors and ligands, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), programmed cell death ligand 1 (PD-L1), type 1 insulin-like growth factor receptor (IGF-1R), receptor activator of nuclear factor kappa-B ligand (RANKL), hepatocyte growth factor (HGF), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Monoclonal antibodies against each target are shown in Table 3.1, and some of the approved drugs that have indications in other types of cancer are shown in Table 3.2. In Table 3.3, the common adverse effects of each agent are mentioned [1–6].

Table 3.1 MABs and biological inhibitors in NSCLC

Class of drug	Generic name	Brand name
TKI	Erlotinib	Tarceva®
	Gefitinib	Iressa®
	Crizotinib	Xalkori®
	Afatinib	Gilotrif®
PARP	Veliparib	–
	Olaparib	Lynparza®
PI3K	Buparlisib	–
	Alpelisib	–
EGFR	Cetuximab	Erbitux®
	Nimotuzumab	TheraCIM®
	Matuzumab	–
	Necitumumab	Portrazza®
	Panitumumab	Vectibix®
VEGF EGFR	Bevacizumab	Avastin®
VEGFR	Ramucirumab	Cyramza®
PD-1	Pembrolizumab	Keytruda®
	Nivolumab	Opdivo®
PD-L1	Atezolizumab	Tecentriq®
	Durvalumab	Imfinzi®
	Avelumab	Bavencio®
IGF-1R	Figitumumab	–
RANKL	Denosumab	Prolia®, Xgeva®
HGF	Ficlatuzumab	–
	Rilotumumab	–
CTLA-4	Ipilimumab	Yervoy®
	Tremelimumab	–
TR-2 (DR5)	Conatumumab	–

3.2 Targeted Therapies and their Approved Antibodies

3.2.1 Epidermal Growth Factor Receptor (EGFR)

One of the potential targets in NSCLC is the epidermal growth factor receptors (EGFRs). They are widely expressed on the cell surface (up to 85%) of lung cancer patients and affect cell cycle progression, apoptosis, angiogenesis, tumor cell motility, and metastasis [7, 8]. The EGFR group is divided into four tyrosine kinase receptor subtypes: EGFR, HER2, HER3, and HER4 [9]. The most important ligands that bind to these receptors are epidermal growth factor (EGF) and transforming growth factor α (TGFα). Therapeutic agents directed at these receptors include monoclonal antibodies, vaccines against

Table 3.2 Approved monoclonal antibodies for lung cancer and their indications

Drug	Indications
Bevacizumab	Colorectal cancer Lung cancer Kidney cancer Cervical cancer Ovarian cancer
Ramucirumab	Metastatic NSCLC Gastric cancer Colorectal cancer
Pembrolizumab	Advanced non-small cell lung cancer Advanced melanoma Head and neck squamous cell cancer Classical Hodgkin's disease Lymphoma Microsatellite instability-high cancer Advanced urothelial bladder cancer Advanced gastric cancer Advanced cervical cancer Primary mediastinal B-cell lymphoma
Nivolumab	Advanced non-small cell lung cancer Melanoma Advanced kidney cancer Head and neck squamous cell cancer Advanced bladder cancer Advanced liver cancer Colorectal cancer (MSI-H/dMMR) Classical Hodgkin lymphoma
Atezolizumab	Patients with previously treated metastatic non-small cell lung cancer Certain patients with advanced urothelial carcinoma

EGF, ligand-toxin conjugates, and tyrosine kinase inhibitors (TKIs). The most common of these are monoclonal antibodies such as cetuximab, nimotuzumab, panitumumab, matuzumab, necitumumab, and TKIs, such as erlotinib, gefitinib, crizotinib, and afatinib [7, 9–12]. Studies have shown that patients with EGFR mutations show a better response to targeting agents than to chemotherapy. The mutations are categorized into three classes, being identified only by direct sequencing of polymerase chain reaction analyses of the EGFR gene. It should be noted that mutations are more common in women and non-smokers [8, 13]. How therapeutic agents bind to EGFR is shown in Fig. 3.1.

3.2.1.1 Cetuximab

Cetuximab is a chimeric IgG1 antibody directed against EGFR. It works by blocking the binding of EGF and TGFα to the receptor. Studies have addressed the efficacy of cetuximab alone or in combination with other drugs or radiation therapy for the treatment of NSCLC [11]. However, the data from multiple clinical trials indicates that cetuximab does not benefit overall survival. It was therefore removed from the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) guidelines in 2016 [14, 15]. It should be noted that cetuximab is still used in head and neck cancers, as well as in metastatic colorectal cancer, and the most common adverse effects of this agent are acne-like rash, diarrhea, and rare infusion reactions [7, 10–12, 16, 17].

3.2.1.2 Nimotuzumab

Nimotuzumab is a humanized anti-EGFR IgG1 monoclonal antibody that has approval for the treatment of advanced head and neck tumors, glioma, and esophageal cancer [18]. There have been several clinical studies addressing the effect of nimotuzumab in combination with radiother-

Table 3.3 Common side effects of MABs used in NSCLC

Drug name	Common side effects
Cetuximab	Acne-like rash, diarrhea, and rare infusion reactions
Nimotuzumab	Skin rash, pruritus, and diarrhea
Matuzumab	Skin and gastrointestinal events (e.g., rash, diarrhea)
Necitumumab	Acneform rash, skin dryness, fissures, and hypomagnesemia
Panitumumab	Skin toxicity including dry skin and acne, diarrhea, deep vein thrombosis, paronychia and stomatitis
Bevacizumab	Hemorrhage, hyper-tension, proteinuria, and headache
Ramucirumab	Febrile neutropenia and pneumonia
Pembrolizumab	Fatigue, pyrexia, and diarrhea
Nivolumab	Pruritus, diarrhea, and nausea
Atezolizumab	Fatigue, nausea, decreased appetite, asthenia, pneumonia, and increased aspartate aminotransferase
Durvalumab	Cough, fatigue, upper respiratory tract infections, and rash
Avelumab	Treatment-related pneumonitis and immune-related events
Figitumumab	Hyperglycemia, rash, diarrhea, decreased appetite, and asthenia
Denosumab	Osteonecrosis of the jaws
Ficlatuzumab	Diarrhea, acnei-form dermatitis, and paronychia
Rilotumumab	Anemia, lymphopenia, diarrhea and acnei-form rash
Ipilimumab	Skin and gastrointestinal events (e.g., rash, pruritus, and diarrhea)
Tremelimumab	Diarrhea, nausea, anorexia, colitis, vomiting, dyspnea, asthenia, and dry skin
Conatumumab	Increased amylase and lipase, peripheral neuropathy, diarrhea, vomiting, and headaches

apy or TKIs against NSCLC which have had promising results. Nimotuzumab is mostly used when there is tolerance against TKIs. With the low dose and high efficacy of this drug, it appears to be a promising monoclonal antibody (MAB) for the treatment of EGFR-associated NSCLCs. It should be noted that unlike other anti-EGFR MABs, nimotuzumab rarely binds to EGFR expressed in normal tissue. The most common adverse effects of nimotuzumab include skin rashes, pruritus, and diarrhea [19–26].

3.2.1.3 Matuzumab

Matuzumab is a humanized IgG1 monoclonal antibody directed against EGFR. Matuzumab has been assessed in a number of clinical studies, confirming both its patient tolerance and antitumor activity. These trials have included phase 1 and 2 for use of matuzumabin combination with paclitaxel or pemetrexed. The side effects include cutaneous and gastrointestinal events (e.g., rash, diarrhea) which are common among anti-EGFR antibody therapies [27–29].

3.2.1.4 Necitumumab

Necitumumab is a fully human IgG1 anti-EGFR monoclonal antibody. Several clinical trials have investigated necitumumab as a therapeutic agent for NSCLC patients with encouraging results [30, 31]. The SQUIRE phase 3 clinical trial compared the use of necitumumab alongside gemcitabine and cisplatin against gemcitabine and cisplatin alone in stage 4 previously untreated NSCLC patients and the results showed an improvement in overall survival in patients on the necitumumab regimen [30, 32–36]. Due to its success in the SQUIRE clinical trial, a phase 2 clinical trial comparing the use of necitumumab with paclitaxel and carboplatin as an alternative chemotherapy choice, compared to paclitaxel and carboplatin alone, was undertaken. The results of this trial support the use of necitumumab alongside chemotherapeutic agents for NSCLC patients [37]. Although promising results have been shown in previous clinical trials, by contrast, the phase 3 INSPIRE clinical trial, in which necitumumab was used alongside pemetrexed and cisplatin versus pemetrexed and cisplatin alone in stage 4 previously untreated NSCLC patients, no advantage of addition of necitumumab on survival of NSCLC patients was seen [38]. The overall adverse effects of necitumumab are similar to this group of antibodies and include acneiform rash, skin dryness, fissures, and hypomagnesemia [31, 35].

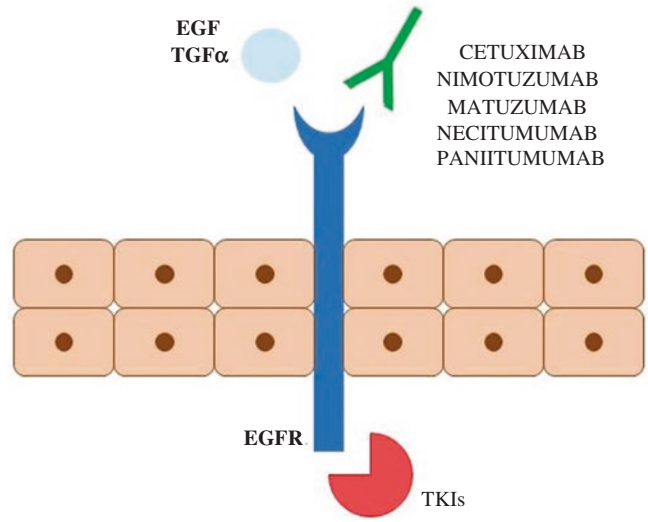

Fig. 3.1 EGFR and its associated monoclonal antibodies

3.2.1.5 Panitumumab

Panitumumab is a fully human IgG2 monoclonal antibody which targets EGFR and is used as a therapeutic agent either alone or in combination for various types of cancers [39, 40]. A phase 2 clinical trial with the addition of panitumumab to paclitaxel and carboplatin showed that there was no improvement in overall survival and time to progression in NSCLC patients, despite acceptable toxicity [41]. Another phase 2 clinical trial (CHAMP) showed that the use of panitumumab alongside pemetrexed and cisplatin resulted in increased toxicity and lowered the quality of life of NSCLC patients [42]. Another phase 2 clinical trial showed that the use of panitumumab alongside carboplatin and paclitaxel had no advantage compared to carboplatin and paclitaxel alone [43]. A similar phase 2 trial compared panitumumab with carboplatin and pemetrexed to carboplatin and pemetrexed alone, with the same result [44]. Only one phase 3 clinical trial showed efficacy from adding panitumumab to erlotinib and bevacizumab as a second-line treatment for NSCLC [45]. The adverse effects of this agent include skin toxicity, including dry skin and acne, diarrhea, deep vein thrombosis, paronychia, and stomatitis [41, 45, 46].

3.2.2 Vascular Endothelial Growth Factor (VEGF)

VEDF receptors are a group of three tyrosine kinase receptors that are highly expressed on tumor cells and have an important role in cancer angiogenesis. VEGF includes eight ligands that induce angiogenesis by signaling through the tyrosine kinase receptors [47, 48]. Monoclonal antibodies against VEGF prevent the proliferation of vascular tumor cells by inhibiting the physiological pathway of angiogenesis. The only monoclonal antibody approved for inhibiting vasculature growth via VEGF in NSCLC is bevacizumab [48–50]. How these agents bind to the VEGFR is illustrated in Fig. 3.2.

3.2.2.1 Bevacizumab

Bevacizumab is a recombinant humanized anti-IgG1 antibody against VEGF, approved by the Food and Drug Administration (FDA) in 2006 as a first-line treatment for non-squamous NSCLC in combination with carboplatin and paclitaxel, carboplatin and pemetrexed, or cisplatin and pemetrexed. VEGF is the major regulator of angiogenesis in normal and malignant tissues. It should be noted that a history of hemoptysis is a contraindication for use of this drug, and it is not

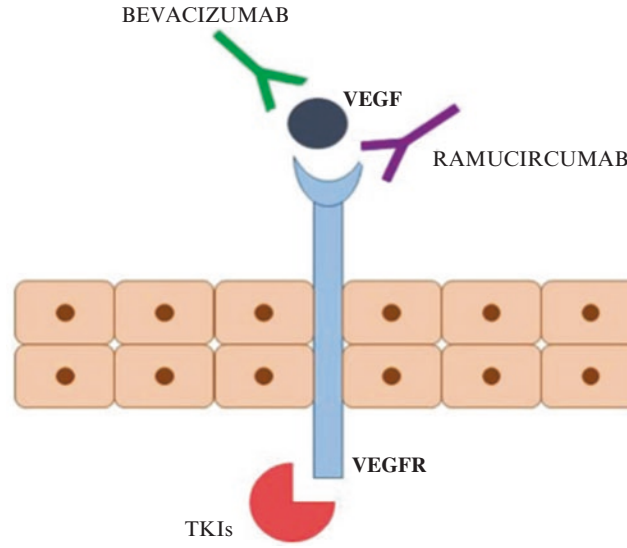

Fig. 3.2 VEFG and its associated monoclonal antibodies

recommended for patients with squamous cell cancer. Bevacizumab can also be used as a single agent for maintenance therapy. The most serious adverse effect which can also be fatal is hemorrhage, which can be pulmonary or gastrointestinal related. Other common side effects include hypertension, proteinuria, and headache [51–54].

3.2.2.2 Ramucirumab

Ramucirumab is an FDA approved human IgG1 antibody directed against VEGF receptor 2. It is administered intravenously with a 10 mg/kg dose before docetaxel for patients with metastatic NSCLC. The most common adverse effects are febrile neutropenia and pneumonia. In 2014, at the conclusion of the REVEL clinical trial, it was also approved for gastric and colorectal cancers [55–57].

3.2.3 PD-1 and PD Ligands 1 and 2 (PD-L1 and L2)

One of the potential checkpoints for therapeutic targets directed against tumor cells are programmed cell death ligand 1 (PD-L1, also known as CD274) and 2 (PD-L2), that are present on tumor cells with their receptor on T-cells. PD-L1 and PD-L2 bind to the programmed cell death 1 receptor on T-cells, causing a downregulation of apoptotic molecules, and thus increasing the survival of tumor tissue. Pembrolizumab and nivolumab are antibodies that bind to PD-1 on T-cells, and atezolizumab, durvalumab, and avelumab are antibodies that bind to PD-L1, and these agents are used to treat NSCLC [2, 58–60]. The use of anti-PD-1 is more effective against tumors that express both PD ligands [61–63]. The interactions of PD-L and PD-L1 and their associated drugs are illustrated in Fig. 3.3.

3.2.3.1 Pembrolizumab

Pembrolizumab is a humanized IgG4 monoclonal antibody directed against PD-1 for use in advanced NSCLC. Based on guidelines from the NCCN for NSCLC, the FDA has approved the use of pembrolizumab as a first-line treatment for patients with more than 50% expression of PD-L1 and for patients with metastatic non-squamous NSCLC, combined with carboplatin and pemetrexed. Pembrolizumab is used in patients with negative or unknown test results for EGFR mutations and anaplastic lymphoma kinase (ALK) rearrangements. High-dose IV corticosteroids should be administered for patients with immune-mediated adverse events. The recommended dose of pembrolizumab is 200 mg administered as an intravenous infusion over 30 min every 3 weeks. Based on the

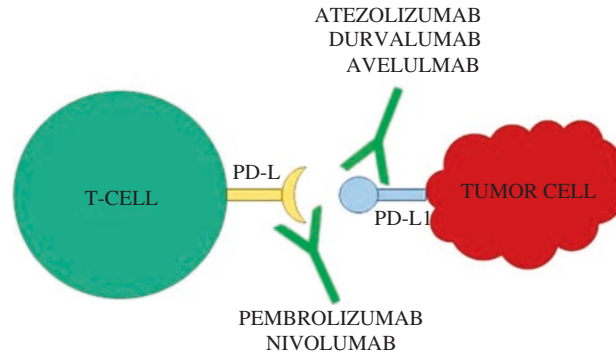

Fig. 3.3 PD-L and PD-L1 and their associated monoclonal antibodies

KEYNOTE-024 trial, pembrolizumab showed a longer progression-free period with less adverse effects than with combined platinum-based chemotherapy agents. The common adverse effects of pembrolizumab include fatigue, pyrexia, and diarrhea [64–67].

3.2.3.2 Nivolumab

Nivolumab is a human IgG4 monoclonal antibody directed against PD-1receptors on T-cells and is used for the treatment of various cancers, including metastatic NSCLC. The safety of nivolumab as a second-line treatment in metastatic NSCLC was evaluated by the CHECKMATE-017 and CHECKMATE-057 clinical trials. The recommended dose of nivolumab is 240 mg every 2 weeks or 480 mg every 4 weeks, administered as an intravenous infusion over 30 min. The most common side effects are pruritus, diarrhea, and nausea [68]. Nivolumab can be used for the treatment of both squamous and non-squamous NSCLC [59, 67, 69–71].

3.2.3.3 Atezolizumab

Atezolizumab, a humanized monoclonal IgG1 antibody, is the first FDA-approved antibody directed against PD-L1, with approval granted in 2016. The Fc region of atezolizumab is modified so it eliminates antibody-dependent cell cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [67, 72]. It is used as a second-line therapy in patients with metastatic NSCLC who have disease progression during or after platinum-based chemotherapy [61]. Several clinical trials (BIRCH, POLAR, OAK) have demonstrated that adding atezolizumab to the chemotherapy regimen of cancer patients improves their overall survival. However, side effects are common and include fatigue, nausea, decreased appetite, asthenia, pneumonia, and increased aspartate aminotransferase [61, 67]. Unlike pembrolizumab, the use of atezolizumab is independent of the percentage of PD-L1 expression [73, 74]. The recommended clinical dose for atezolizumab is 1200 mg every 3 weeks, and it has a half-life of 27 days [75].

3.2.3.4 Durvalumab

Durvalumab is an approved human IgG1 monoclonal antibody for urothelial carcinoma and stage 3 NSCLC that binds to PD-L1. The recommended dosing for treatment of stage 3 NSCLC is 10 mg/kg every 2 weeks. The most common adverse effects are cough, fatigue, upper respiratory tract infections, and rash. Following the PACIFIC clinical trial, which established the safety and efficacy of durvalumab, it gained approval in 2017 [72, 76–80].

3.2.3.5 Avelumab

Avelumab is a fully human IgG1 monoclonal antibody that binds to PD-L1 and blocks its binding to PD-L and CD80 [58]. According to a phase 1 clinical study (JAVELIN), avelumab demonstrated acceptable safety and efficacy in patients with recurrent NSCLC who were previously treated with platinum-based therapies. Its side effects include treatment-related pneumonitis and immune-related adverse events [81, 82].

3.2.4 IGF-1R

IGF receptors are a group of three tyrosine kinase receptors consisting of IGF-1R, IGF-2R, and the insulin receptor (INSR) that play a role in cell growth and proliferation. IGF-1, IGF-2, and insulin-like binding proteins are the ligands for these receptors [83, 84]. These ligands and their receptors are over expressed in malignant tumors, resulting in proliferation, metastasis, and drug resistance [84, 85]. The interaction of the IGF-1 receptor with its ligand is illustrated in Fig. 3.4.

3.2.4.1 Figitumumab

Figitumumab is a human IgG2 monoclonal antibody that inhibits IGF-1R. IGF receptors play important roles in cell growth and development. IGF-1R is expressed on the majority of lung cancer cell lines, and thus affects cancer progression. Blocking this receptor aids the inhibitory effect of chemotherapy agents. Figitumumab was added as a first-line therapy to carboplatin plus paclitaxel and showed good results in phase 2 clinical trials but failed to increase overall survival in NSCLC patients in phase 3 clinical trials due to adverse events such as hyperglycemia, rash, diarrhea, decreased appetite, and asthenia. Thus, further development of figitumumab was discontinued [86–88].

3.2.5 RANKL

Skeletal lesions and bone metastases are common in NSCLC patients, causing extreme pain and impacting the patient's quality of life. Bisphosphonates and denosumab are both recommended for treatment. The RANKL present on osteoblasts binds to the RANK receptor on osteoclasts, inducing bone resorption through signal transduction [89–91]. This interaction is illustrated in Fig. 3.5.

3.2.5.1 Denosumab

Denosumab is a novel, fully human IgG2 monoclonal antibody specific to RANKL present on osteoblasts, which causes inactivation of bone resorption by osteoclasts [92]. Several clinical trials have evaluated the efficacy of denosumab compared to bisphosphonates, although data to date show no difference between them [93–96]. Even though there have been reports of osteonecrosis of the jaw when using this agent, it is recommended that 120 mg denosumab be administered subcutaneously every month for treatment of metastatic bone disease [90, 97].

3.2.6 HGF

HGF, or scatter factor, is a cellular growth factor that activates tyrosine kinase signaling after binding to mesenchymal epithelial transition

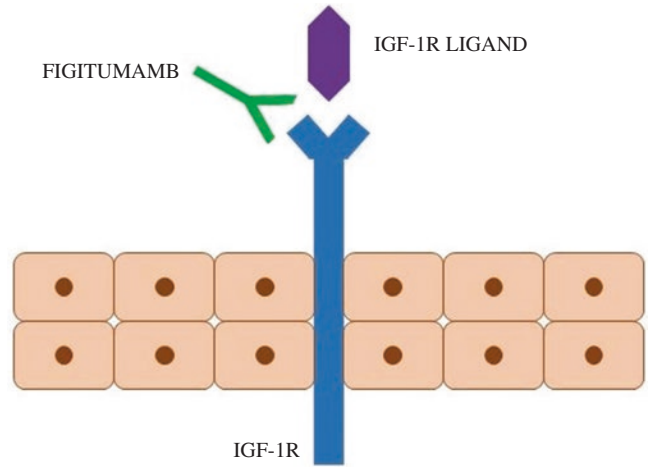

Fig. 3.4 IGF-1 receptor and ligand and the effects of figitumumab

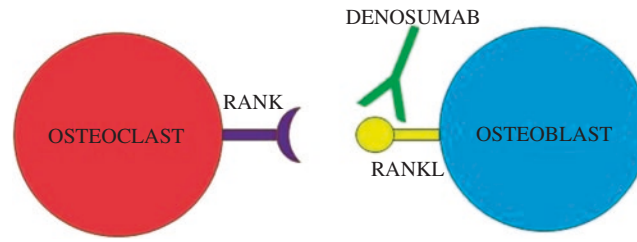

Fig. 3.5 RANK and RANKL and their associated monoclonal antibodies

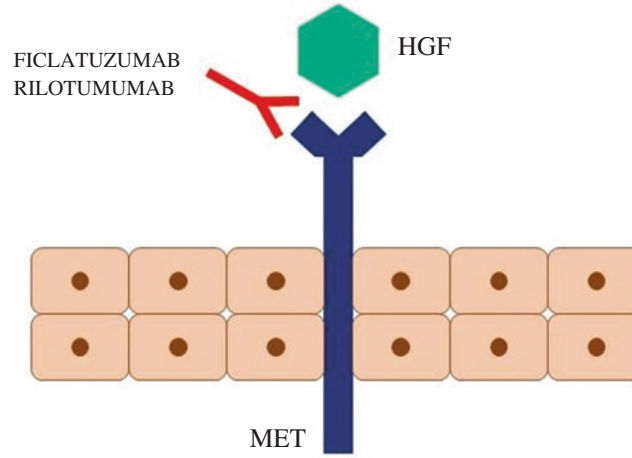

Fig. 3.6 HGF and MET

factor (c-MET) or the HGF receptor (HGFR). MET is expressed in epithelial cells and its activation can lead to resistance to EGF receptor inhibitors [98–100]. A schematic illustrating the interaction between MET and HGF is shown in Fig. 3.6.

3.2.6.1 Ficlatuzumab

Ficlatuzumab (AV299) is a humanized IgG1 monoclonal antibody that binds to HGF, thus inhibiting signaling of the c-MET receptor. Studies show that combination therapy with ficlatuzumab and cetuximab or erlotinibcan have a good patient outcome, but combination therapy with ficlatuzumab and gefitinib offers no benefit over gefitinib alone. The most common adverse events of ficlatuzumab include diarrhea, acneiform dermatitis, and paronychia [101, 102].

3.2.6.2 Rilotumumab

Rilotumumab (AMG 102) is a fully human IgG2 monoclonal antibody directed against human HGF. A clinical trial studied the effect of adding rilotumumab to erlotinib in patients with previously treated NSCLC [98]. Despite side effects such as anemia, lymphopenia, diarrhea, and acneiform rash, the safety profile as well as the disease control rate were determined to be acceptable.

3.2.7 CTLA-4

CTLA-4, also known as CD152, is an immune modulator which downregulates and inhibits immune responses, specifically those of T-cells. The homolog of CTLA-4 is CD28, which stimulates response. Both receptors act when bound with CD80 (B7–1) and CD86 (B7–2) on antigen presenting cells (APCs). CTLA-4 is normally expressed on regulatory T-cell lymphocytes, but in cancers and immune diseases, it is also expressed on normal T-cells resulting in a downregulation of the immune response [103–105]. A schematic illustrating the interaction between CTLA-4 and CD80/CD86 is shown in Fig. 3.7.

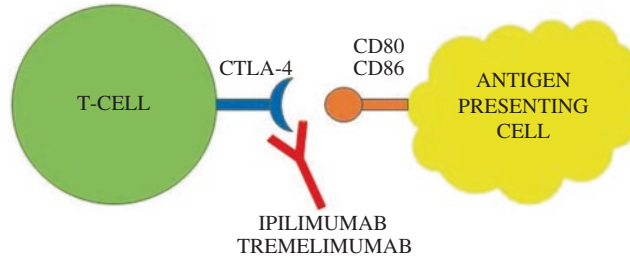

Fig. 3.7 CTLA-4 and its associated monoclonal antibodies

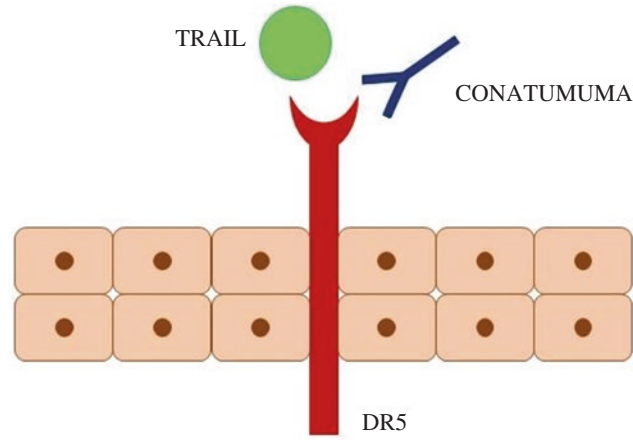

Fig. 3.8 Death receptor 5 (DR5) and TRAIL

3.2.7.1 Ipilimumab

Ipilimumab is a fully human IgG1 monoclonal antibody that inhibits the attachment of CTLA-4 to its ligands (CD80 and CD86). Clinical studies comparing the use of ipilimumab alongside paclitaxel and carboplatin versus paclitaxel and carboplatin alone showed that adding ipilimumab to the treatment regimen improves progression-free survival. The most common adverse effects involve the skin and gastrointestinal tract (e.g., rash, pruritus, and diarrhea) [106, 107]. Ipilimumab is an approved agent for patients with metastatic melanoma [107, 108]. Different clinical trials (CHECKMATE 032 and CHECKMATE 012) have shown promising results for the use of ipilimumab alongside nivolumab for patients with both SCLC and NSCLC who have previously been treated with platinum-based therapies [68, 109]. Ipilimumab is also suggested alongside carboplatin and etoposide as first-line treatment for extensive-stage SCLC, but results to date have been inconclusive [108, 110].

3.2.7.2 Tremelimumab

Tremelimumab is a fully human IgG2 monoclonal antibody and inhibitor of CTLA-4. A phase 1 clinical trial carried out between 2013 and 2015 showed manageable tolerance for durvalumab (20 mg/kg every 4 weeks) plus tremelimumab (1 mg/kg), thereby dictating the doses for current phase 3 clinical trials [78, 111]. The ARCTIC phase 3 clinical trial is currently on-going for studies of durvalumab with or without tremelimumab for previously treated NSCLC patients [77]. The most common observed adverse effects are diarrhea, nausea, anorexia, colitis, vomiting, dyspnea, asthenia, and dry skin [111].

3.2.8 Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) Receptor 2 (TR-2) or Death Receptor 5 (DR5)

TR-2 or DR5 causes cell apoptosis when in contact with TRAIL, a protein that functions as a ligand [112, 113]. The interaction of TRAIL with DR5 is illustrated in Fig. 3.8.

3.2.8.1 Conatumumab

Conatumumab is a fully human IgG1 monoclonal antibody directed against the extracellular domain of death receptor 5, imitating the activity of TRAIL and thus inducing cell apoptosis. A randomized clinical trial was undertaken to study the effect of adding conatumumab to paclitaxel and carboplatin as a first-line treatment in NSCLC. Even though the treatment was tolerated, despite side effects such as increased amylase and lipase, peripheral neuropathy, diarrhea, vomiting and headaches, no significant improvement in outcome was observed [113, 114].

3.3 Conclusions

Treatment of NSCLC is still a challenging area for caregivers, due to specific patient and tumor characteristics that are associated with this disease. However, pharmaceutical companies worldwide have shown interest in developing targeted therapies to improve patient survival. Studies have shown that the use of monoclonal antibodies for cancer treatment has improved the overall survival of cancer patients, due to direct targeting of the tumor cells. Some therapies have been approved by drug administrations but most are still in clinical trial and research phases. Despite having shown significant clinical effects, extensive studies are still needed to determine safety, efficacy, and when their use is warranted, especially in terms of specific genetic alterations and long-term adverse effects.

Conflict of Interest The authors have no conflicts of interest.

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4 Parkinson's Disease and Impairment in Mitochondrial Metabolism: A Pathognomic Signature

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Abstract

Mitochondrial bioenergetics is vital for the proper functioning of cellular compartments. Impairments in mitochondrial DNA encoding the respiratory chain complexes and other assisting proteins, accumulation of intracellular reactive oxygen species, an imbalance in cellular calcium transport, or the presence of organic pollutants, high fat-ketogenic diets or toxins, and advancing age can result in complex disorders, including cancer, metabolic disease, and neurodegenerative disorders. Such manifestations are distinctly exhibited in several age-related neurodegenerative diseases, such as in Parkinson's disease (PD). Defects in complex I along with perturbed signaling pathways is a common manifestation of PD. Impaired oxidative phosphorylation could increase the susceptibility to PD. Therefore, unraveling the mechanisms of mitochondrial complexes in clinical scenarios will assist in developing potential early biomarkers and standard tests for energy failure diagnosis and assist to pave a new path for targeted therapeutics against PD.

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Keywords

Mitochondria · Complex I · Mitochondrial DNA · Genomic DNA · OxPhos · Parkinson's disease

4.1 Introduction

Mitochondria are the chief energy production sites in eukaryotic cells and play a critical role in cell growth, differentiation, cellular signaling, apoptosis, and cell cycle control. They are double layered organelles, located in the cytoplasm of the cells and are among the largest cell organelles. The interaction between outer membrane proteins, such as hexokinase and the voltage-dependent anion channel (VDAC 1), and the inner membrane proteins including the adenine nucleotide translocator, connect both membranes together. Each membrane is a phospholipid bilayer with embedded proteins. The outer membrane is smooth, while the inner membrane has many inverted folds called cristae, which increase the surface area and form working spaces for mitochondrial reactions. The inner membrane is selectively permeable to certain molecules, such as pyruvic acid and adenosine triphosphate (ATP) [1]. The mitochondrial process of oxidative phosphorylation (OxPhos) aids in metabolizing car-

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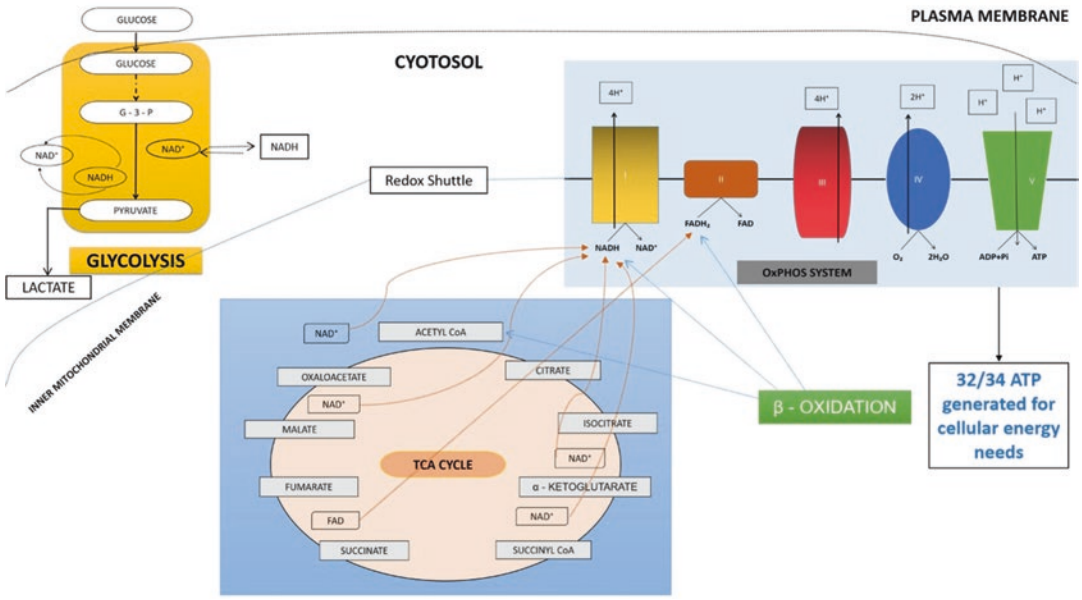

Fig. 4.1 Figure showing the link between the three most important modes of energy production pathways: glycolysis, tricarboxylic acid (TCA) cycle, and electron transport chain (ETC). The oxidative phosphorylation system acts as the pivotal connection between cellular bioenergetics (glycolysis) and the mitochondrial TCA cycle, yielding maximum number of ATP molecules (32/34)

bohydrates and fatty acids. It acts as a link between cellular bioenergetics (glycolysis) and the mitochondrial Kreb's cycle and electron transport chain (ETC) (Fig. 4.1). The ETC is considered as the synonym of OxPhos as this produces the maximum energy (32 ATP/glucose molecule), compared to the other two processes [2, 3].

The ETC is multi-protein complex encompassing electron donors to an electron acceptor (oxygen) in the redox reaction and releases energy in the form of ATP. The four protein complexes in the ETC are known as complex I, II, III, and IV. Several steps are involved in the transfer of the redox energy through nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) to oxygen. The non-integral protein of ETC is the cytochrome C (CYC) situated on the inner mitochondrial membrane. Coenzyme Q helps in the energy transfer from complex I to III bypassing complex II. Complex I consists of NADH succinate dehydrogenase complex that pumps four hydrogen ions across the membrane into the intermembrane space, thereby creating a hydrogen ion gradient. Complex II consists of FADH2 and does not pump protons to the intermembrane space but participates indirectly in ETC by donating electrons. Ubiquinone, being the first electron acceptor for both complex I and II, transfers them to complex III and enhances the proton gradient. The electrons are then delivered to CYC, which transports them to complex IV. Complex IV consists of proteins and enzymes that finally accepts the electrons and reduces oxygen by accepting two protons from the surrounding medium to generate water. Due to the pumping of protons by the complexes, ATP synthesis occurs at complex V by the electrochemical gradient through the transfer of an inorganic phosphate to adenosine diphosphate (ADP) [3, 4].

The whole process involved in oxidation of high energy compounds (NADH, FADH2) along with the transfer of inorganic phosphates to ADP via the proton gradient is called OxPhos. During OxPhos, a cascade of biochemical reactions takes place that leads to the production of huge amount of reactive oxygen species (ROS), such as reactive hydroxyl, nitrogen, oxygen, and hydrogen

species during the whole process [4, 5]. ROS can indiscriminately react with a wide variety of organic substrates causing peroxidation of lipids, cross-linking and modification of proteins and mutations in DNA, and can cause significant alterations in the structure and function of cell organelles [4–7]. Substantial ROS generation in the mitochondria due to OxPhos can lead to the onset and progression of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) because of the excessive demand of energy by the neurons that need to be persistently metabolically active. Such diseases can manifest in a wide variety of clinical symptoms and vary in their pathophysiology, with some causing memory and cognitive impairments and others affecting a person's ability to move, speak, or breathe [5–9]. PD is one such neurodegenerative disorder that has deep links with perturbed OxPhos. Disturbances in OxPhos could increase susceptibility to PD through incremental increases in oxidative stress, mutation-induced failures in the ETC, transfer of limited energy to the neurons, and deposition of fats leading to plaque accumulation.

Here, we review the mechanisms of the mitochondrial complexes in clinical scenarios with the aim of providing insights into the development of potential early biomarkers and standard tests for energy failure diagnosis and to pave a new path for targeted therapeutics against PD.

4.2 Impaired Mitochondrial Metabolism Etiologies

Some of the major causes of aberrant mitochondrial metabolism are attributed to ROS and gene mutations. Several studies have reported certain "large scale deletions or point mutations" in the mitochondrial DNA (mtDNA) that severely affect mitochondrial energy metabolism by hampering the OxPhos process [9]. Hence, the gradual deterioration of mitochondrial ETC will lead to the production of less ATP, thereby affecting vital cellular processes, in favor of more catabolic pathways, such as autophagy and lysosomal degradation, resulting in the accumulation of waste products in the cell (Fig. 4.2). The etiologies of the mitochondrial dysfunction can be conferred by intrinsic or

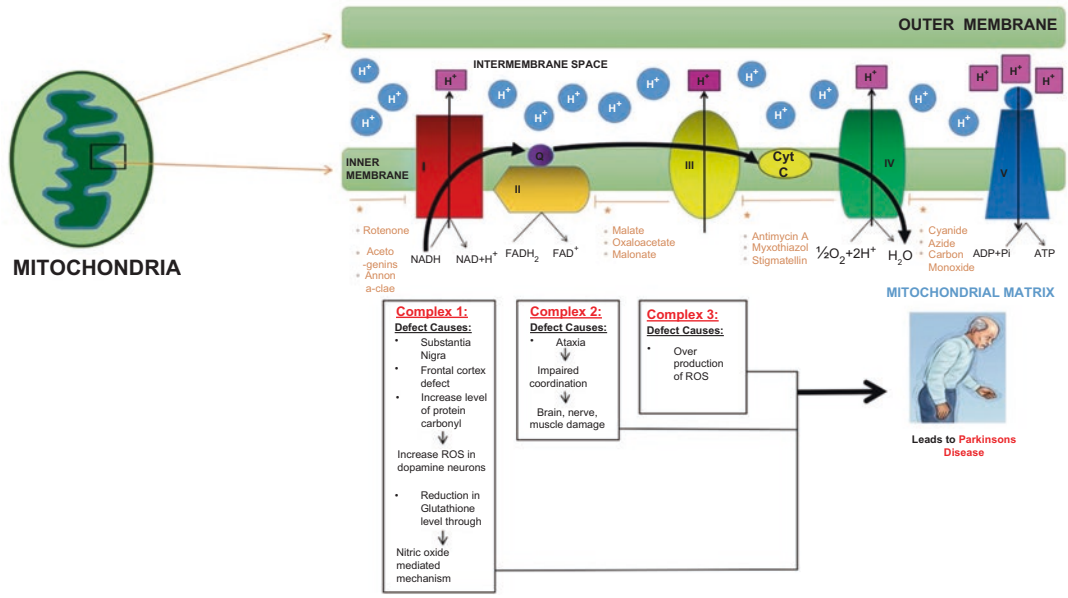

Fig. 4.2 Figure demonstrating several factors responsible for inducing irregularities in the electron transport chain (ETC) complexes of the mitochondria, thereby leading to Parkinson's disease

extrinsic factors, some of which are summarized below.

4.2.1 Mutations in the OxPhos System

The integrity of OxPhos system is imperative for optimal cellular energy production. However, the functional assembly of OxPhos is a complex process. The main factors causing mitochondrial impaired mechanism include mutations. MtDNA and nuclear DNA (nDNA) encode several subunits of complex I, II, III, IV, and V. Complex I [NADH dehydrogenase chain 1 (ND)-1–6 and ND-4 L], complex III (CYB), complex IV [CYB oxidase (COI-III)], and complex V (ATP 6 and ATP 8) comprise the 13 mtDNA-encoded subunits [10]. If there happens to be any abnormalities in any of the genes, this may cause mitochondrial dysfunction, which might eventually lead to a condition such as Parkinsonism [11]. A small change, deletion, or duplication in either of the genes will affect the other. For example, mutations in polymerase gamma (POLG) is one of the most common nDNA mutations that influences the regulation of mtDNA [12]. A study revealed that an increase in the number of mtDNA deletion or rearrangements in substantia nigra pars compacta (SNpc) are observed in PD patients. One study found 47% more protein carbonyl modifications on catalytic subunits in cortical mitochondria isolated from PD brain tissues, indicating an increased burden of oxidative damage [13]. MtDNA haplotype analysis revealed that certain haplogroups reduced the risk of PD. Maternally inherited mutations in mtDNA were detected in one family with PD. Using single molecular polymerase chain reaction (PCR) of individual pigmented neurons of the substantia nigra in aged people and PD patients, somatic deletions in mtDNA were found to be increased to high levels [11, 14]. MtDNA can also undergo single base deletion, and duplication, as well as tandem duplication and depletion, thereby decreasing the amount of mitochondria in the cell which, in turn, could lead to mitochondrial dysfunction. Such defects in the mtDNA or nDNA genes of enzymes can eventually decrease the ATP:ADP ratio [15]. Adult OxPhos diseases can be inherited as autosomal recessive and dominant traits with a milder phenotype and include mtDNA deletions. Mutations in LRRK 2 (leucine-rich repeat kinase 2) are the most common cause for development of PD. The five enzyme complexes of OxPhos are synthesized from 13 polypeptides encoded by the mtDNA and 50 polypeptides encoded by the nDNA. Complex I defects are the most common of the respiratory chain defects and are frequently exhibited as a result of impaired assembly of the enzyme. Such defects are seen to induce the fatal neurodegenerative disorder Leigh Syndrome (LS) with or without cardiomyopathy. Complex III exhibits frequent mutations in CYB and BCS1L genes, which leads to excessive ROS production that can damage DNA and tissues [15, 16]. These mutations can further impair the formation of the complex III enzyme, thereby reducing its activity. Several gene defects have been reported in complex IV, which are inherited as an autosomal recessive deficiency and exhibited in critical diseases like LS, fatal infantile CYC oxidase deficiency, and late-onset neurodegenerative disease. In addition, ATP6 gene mutations in complex V have been manifested in neuropathy, ataxia, and LS [15].

4.2.2 Defects in Complex 1 and PD Prognosis: A Pathognomic Signature

It is now well-known that the activity of complex I is reduced in the substantia nigra of PD patients [16, 17]. A recent study showed that a PD-specific complex 1 deficiency was also found in the frontal cortex. A number of catalytic subunits of complex I were seen with increased levels of protein carbonyls in the Parkinsonian brain, resulting in excessive oxidative damage of complex I subunits that could lead to complex disassembly and dysfunction [18]. The authors suggested that sporadic PD is a result of the decrease in complex I activity. However, it appears that the substantia nigra is more vulnerable to impairments of com-

plex I activity than the other brain regions and peripheral organs, possibly due to the increased levels of ROS generated within dopamine neurons as a result of dopamine metabolism and iron content [19, 20]. In a mouse model, the mitochondrial mass was found to be lower in dopaminergic neurons of the SNpc compared to dopaminergic neurons located in the adjacent ventral tegmental area (VTA) [19]. Similar studies have shown that humans also have a low mitochondrial mass in SNpc dopamine neurons, with complex I inhibition and selective degeneration in PD [20]. Increased burdens of oxidative stress can be reproduced by the incubation of control brain mitochondria with NADH in the presence of rotenone and administration of exogenous oxidants. Decrease in the antioxidant capacity also leads to acute reduction of cellular and mitochondrial glutathione levels that results in decreased mitochondrial complex I dysfunction through a nitric oxide-mediated mechanism [1]. In addition, the succinate dehydrogenase complex flavoprotein subunit A (SDHA) gene present in complex II exhibits missense mutations that cause ataxia, myopathy, and optic atrophy. Mutations in *mt-CYB* and *BCS1L* genes of complex III can also lead to the overproduction of ROS that can damage the mtDNA of neurons [20].

4.2.3 Intracellular ROS

Because electron flow through the ETC is an inefficient process, wherein only 0.4–4% of the oxygen is reduced, this leads to production of "primary" ROS, like the superoxide anion. When the superoxide anion has accumulated to excessive amounts in the cells, this gradually leads to the generation of "secondary" ROS, such as highly reactive hydroxyl radicals that can damage DNA components (purines and pyrimidines, the deoxyribose backbone) and induce mutations. The imbalance between ROS production and cellular antioxidant activity leads to oxidative stress. ROS can affect mitochondrial DNA by causing modulations in ATP production [21, 22]. ROS requires high energy, large numbers of mitochondria, and high presence of fatty acids. Thus, they can damage the susceptible neurons. ROS also stimulates aging and causes cell damage in the body. Oxidative stress is the imbalance between the generation of ROS (free radicals) and antioxidant defenses. Complexes I and III are considered as the major sites involved in the production of superoxide and other ROS [20–24]. This oxidative stress can lead to mitochondrial dysfunction that will ultimately lead to the reduction in ATP, calcium influx, and increased permeability of mitochondrial permeability pore, eventually resulting in apoptosis as exhibited in PD [21, 22]. From this, it can be seen that oxidative stress can cause dopaminergic neurotoxicity. Studies have shown that induction of oxidative stress can not only cause deleterious changes in mitochondrial function but can also induce an innate immune response, resulting in a diminution in cellular antioxidant defenses [16, 20–28]. In addition, a number of environmental factors have been found to cause overproduction of ROS that can damage DNA components and induce mutations [16, 27–32]. In this regard, ROS generation in the neuronal cells can have a significant negative impact. Because the brain is metabolically highly active, it is more susceptible to oxidative stress-related injuries than most other tissues. Hence, the increased ROS production and decreased production of antioxidant enzymes can further lead to brain cell death and neurodegeneration as found in PD.

4.2.4 Impaired Calcium Balance

Calcium acts as a second messenger in signal transduction. The transport of calcium ions across biological membranes is vital for the proper functioning of enzymes and processes. Perturbations in this process have been associated with irregularities in membrane permeability that can lead to increased respiratory rates and altered balance between oxygen consumption and OxPhos [33]. Mitochondrial swelling occurs due to increased influx of calcium ions because of improper mitochondrial cristae unfolding, caused by the opening of the mitochondrial per-

meability transition pore (MPTP). This can result in physiological responses leading to the loss of mitochondrial membrane potential and abnormalities in cellular calcium homeostasis, elevated oxidative stress, and reduced ATP. Consumption of calcium-rich food enhances MPTP opening leading to the increased entry of calcium ions into the mitochondria, thereby causing swelling. Thus, calcium deregulation is known to impair mitochondrial function that can lead to MPTP opening and apoptosis [26, 34]. This process has been implicated in the progression of PD and other neurodegenerative disease [35]. In addition, the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) is the membrane that joins the mitochondria with the ER and is responsible for calcium transport for signal transduction pathways. The excessive efflux of Ca^{2+} from the ER is regulated by mitochondria. If the efflux is beyond the limit, then Ca^{2+} is transported and binds with protein kinase C (PKC) and activates the transcription factor, thereby leading to apoptosis and disassembly of the mitochondrial complexes [33, 34]. However, if the influx of calcium ion in the mitochondrial matrix decreases, this may affect the proton ion gradient of the ETC, thereby affecting ATP synthesis by lowering the rate. Eventually, such dysregulated Ca^{2+} transport across the mitochondrial membranes will lead to the generation of excessive ROS, lower ATP production, and disassemble the mitochondrial complexes, leading to apoptosis. Such phenomena have been consistently observed in PD cases [35].

4.2.5 Mitochondrial ETC Inhibitors and Environmental Toxicants

Several drugs and toxins impart "off-target" effects to mitochondrial metabolism. In particular, they can inhibit and uncouple the ETC, generate and exacerbate ROS production, and alter mitochondrial protein synthesis [36]. One of the signature mitochondrial toxins is the broad-spectrum pesticide, rotenone. Rotenone interrupts the OxPhos system by inhibiting the NADH dehydrogenase enzyme, thereby blocking the electron transport in mitochondria, mainly by inhibiting electron transfer from Fe-S centers in complex I to ubiquinone, creating a cloud of electrons within the mitochondrial matrix. This ultimately prevents oxygen availability for cellular respiration. The cellular oxygen will be finally reduced to free radical form, generating ROS that will eventually hamper mitochondrial DNA and function. An example of this is adriamycin (doxorubicin), a chemotherapeutic used as an antineoplastic drug that acts by intercalation within DNA bases and inhibition of topoisomerase II and induces irreversible and amplified cardiomyopathy in the mitochondria. This drug easily enters the mitochondria due to its lipophilic nature and interacts with cardiolipin, a signature mitochondrial inner membrane lipid [36–39], and acts as an uncoupler of OxPhos, leading to ROS generation and decreased ATP production.

Environmental toxicants including both organic and inorganic compounds have also been implicated in mitochondrial toxicity. For example, paraquat acts like a mito-inhibitor by modulating the redox cycling in a similar manner as adriamycin [40]. Also, cyanide and carbon monoxide act as inhibitors of complex IV [33] and halt the aerobic cellular respiration abruptly. Situations in which there is a simultaneous occurrence of folate deficiency with either cyanide or methanol exposure have resulted in mitochondrial toxicity-mediated optic neuropathy in approximately 50,000 people [38–42], suggesting the impact of such toxicants via an environment–environment interaction mechanism. The implications of the mitochondrial effects posed by these toxicants/chemicals have also been exhibited in several clinical syndromes and pathologies associated with mitochondrial abnormalities. For example, the mitochondrial defects posed by rotenone, paraquat, and manganese are all associated with PD and other age-related neurodegenerative diseases [38, 39, 42, 43].

4.2.6 Increased Fat Diet Uptake

Hepatocyte fatty acid oxidation and ketone body production can be induced by high fat diets. In

such diets, the nutrient supply and cellular energetic needs enhance the function of mitochondria. Such diets can lead to obesity, which is a major cause of impaired mitochondrial metabolism and behavior [44]. A fat-rich diet decreases $FADH_2$-associated cellular respiration and increases proton leak, thereby compromising mitochondrial energetic efficiency in addition to an elevation in ROS production. Due to improper mitochondrial energetic cristae unfolding, there is an increased influx of calcium ions that enhances mitochondrial swelling. As a result, the MPTP opens, which can induce increased oxidative stress, decreased ATP production, and abnormalities in cellular calcium homeostasis, as well as other effects [45]. The dysregulation of calcium can lead to impaired mitochondrial function that can result in MPTP opening and apoptosis. This finding has led to the suggestion that the increased mitochondrial swelling could be a major cause for MPTP opening in neurodegenerative diseases, such as PD. Several models of PD have been generated and shown to have enhanced oxidative stress upon intake of a fat-rich diet [44, 46, 47]. Such an inadequate maternal nutrition can increase maternal and fetal oxidative stress through the increased production of ROS [48].

4.3 PD and Perturbed Mitochondrial Metabolism

The brain consumes more than 30% of the total energy generated by an organism because it needs to maintain synaptic homeostasis. Therefore, neurons require the most energy among all cell types in the body and harbor huge numbers of mitochondria to support proper function. In line with this, several studies have demonstrated that mitochondrial diseases frequently manifest along with neuromuscular abnormalities [6, 49–51]. A gradual fall in ATP production due to impaired mitochondrial metabolism with advancing age is currently a typical hallmark of neurodegenerative disease, such as in case of PD.

PD is an age-related neurodegenerative disease marked by locomotory disturbances exhibiting rigidity, bradykinesia, and resting tremor, with an average incidence of around 25 per 100,000 people that increases sharply with age after age 60 [52]. The etiology of PD is marked by the damage to dopaminergic receptors in the substantia nigra region of the brain. The most notable cause of such damage is the accumulation of ROS (Fig. 4.3) that are produced considerably in the glial cells and mitochondria of the neurons (mainly highly pigmented neurons), referred to as the "hot spots" of brain ROS generation [53]. Mitochondrial profiling of PD patients has demonstrated defects in the mitochondrial OxPhos system in platelets, muscle, and substantia nigra, thereby suggesting that mitochondrial function assessment should be an early predictor of the progression of PD. In addition, interactions between the intermediates of the quinol cycle with hydrogen peroxide to form hydroxyl radicals might also lead to the development of PD. In various studies around the world, it has been found that oxidative stress and complex I inactivity causes the OxPhos impairment and increased risk of PD. Mainly, the inhibitors and pesticides are considered to be the causative agent of the failure of complex I. Recent studies have also found that complex I defects in the ETC of the mitochondria severely affect neurons, leading to the development of PD by over production of ROS [54, 55]. Mutations in the phosphatase and tenson homologue (PTEN)-induced putative kinase 1 (PINK1) gene responsible for the regulation of osmotic pressure and mitochondrial membrane potential causes irregularities in the management of cellular oxidative stress and causes a huge production of ROS in the neurons. This gene mutation is considered as one of the genetic hallmarks of PD. The PTEN gene mutation also induces impairment of the alpha synuclein protein, which directly interrupts mitochondrial complex I activity, thereby leading to decreased ATP production and mitochondrial dysfunction. In addition, exogenous agents like environmental pesticides could be related to PD development, such as the effect seen with MPTP. Classical PD cases (5–10%) result from

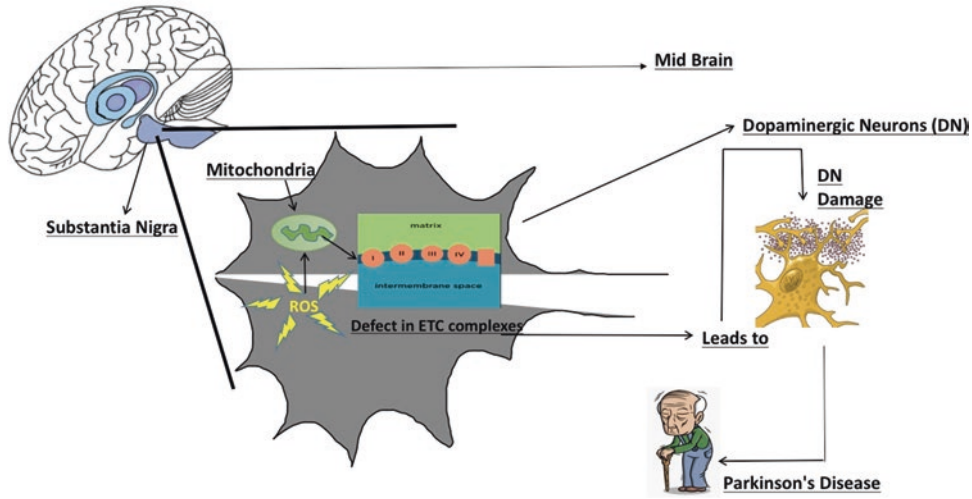

Fig. 4.3 Figure showing the implication of "ROS" in inducing perturbed mitochondrial metabolism leading to dopaminergic neuron damage and eventually Parkinson's disease

monogenetic mutations, although the remaining cases have no known etiology. When MPTP enters astrocytes, it converts into 1-methyl 4 phenylpyridinium (MPP+) that enters into the dopamenergic neurons in the SNpc and causes damage. MPP+ gets selectively absorbed by the pigmented dopaminergic neurons, thereby suppressing complex I, increasing ROS formation and oxidative stress-induced neuronal death. Similarly, rotenone that acts as a complex I inhibitor has been used to create PD animal models. An analysis of the association between pesticide exposure and in PD in 436 cases and 854 controls found an increased risk of PD after long-term exposure to pesticides. The calculated combined adjusted odds ratio (OR) was 1.94 (1.49–2.53) and similar ORs were observed in studies conducted in the United States, Asia, Europe, and Canada. It was also observed that the risk of PD increased with longer exposure times, with an adjusted OR of 5.81 (1.99–16.97) for ≥10 years of exposure [56]. Subsequently, another study reviewed 31 case-control studies published up to 2003 and found that about half of them reported significant associations between pesticide exposure and PD risk [57]. An epidemiological study from 2000 to 2011 found that seven out of the eight prospective studies provided evidence of an association between pesticide exposure and PD, reporting risk estimates of twofold or higher. Among 23 case studies, 13 studies found an increased risk of PD [58, 59]. In support of the epidemiological evidence, increased levels of some pesticides have been quantified in post-mortem brains from PD patients. High concentrations of pesticides including dieldrin, lindane, and p-p-DDE have been observed in PD cases compared with controls [60, 61]. Furthermore, complex IV inhibitors like carbon monoxide have also been implicated in the suppression of the activity of motor neurons in PD-related cases [62]. Mitochondrial profiling of PD patients has demonstrated defects in the mitochondrial OxPhos in the platelets [63], muscle [64], and substantia nigra [65], thereby suggesting that mitochondrial function assessment should be an early predictor of PD. In addition, interactions between the intermediates of the quinol cycle with hydrogen peroxide to form hydroxyl radicals that act as ROS might also lead to the development of PD. Complex I impairment may also cause accumulation of alpha synuclein, which results in increased oxidative stress damage and dysfunction of this complex [66]. Conversely, a key regulator of energy metabolism, peroxisome proliferator-activated receptor-γ coactivator (PGC-1) plays a role in neuroprotection [67]. This has been suggested by studies which showed

that its inhibition decreases the activity of complex I.

As suggested above, the most important agent for oxidative damage is the accumulation of ROS [68–71] that are produced at high levels in glial cells and mitochondria of the neurons (mainly the highly pigmented neurons), and these are referred to as the "hot spots" of brain ROS generation. In line with this, annonaceous acetogenins [72, 73], one of the most powerful lipophilic complex I inhibitors [74, 75], was found to cause neuronal cell death [76] and induce redistribution of tau protein [77] (a feature of Parkinsonism) in primary cultures of striated neurons [73].

4.4 Conclusions and Future Perspectives

Considering the fact that mitochondrial OxPhos plays an imperative role in shaping the clinical outcome of a neurodegenerative disorder, answering questions pertaining to the role of perturbed energy metabolism in PD will be crucial for developing early diagnostics and prompt therapeutics for improving the clinical manifestations. Some questions that could be posed for discussion are: (1) what are the different mitochondrial proteins that mediate the advent and progression of PD; (2) what are the mitochondrial energy cascades involved in PD; (3) how do the energy metabolism regulation work in PD; (4) what are the specific biomarkers for the early detection of PD; (5) how does perturbed redox biology aid in the progression of PD; and (6) how can natural dietary compounds aid in ameliorating pertubed mitochondrial energy metabolism in PD? Presently, there is a scarcity of therapeutics for the specific management of neurodegenerative diseases. This has enhanced the quest to identify compounds aimed at improving mitochondrial energy metabolism in clinical medicine, with particular reference to neurodegenerative diseases, such as PD. Hence, human clinical trials focused on evaluating the therapeutic potential of non-specific energy-boosting compounds in clinical scenarios are welcomed and are currently being pursued [78]. Naturally occurring compounds, such as plant-based products, mushrooms, organic supplements, and probiotics having antioxidant properties are currently being investigated as ways of improving mitochondrial function and energy deficit. Among these, oral creatine and creatine analogs, or amino acid derivatives and peptides derived from biological sources have shown interesting outcomes. Some studies have demonstrated low-to-medium positive therapeutic potential of organic creatine for improving energy metabolism in HD and PD [78, 79]. However, little or no information is currently available on the role of several other similar compounds on mitochondrial uptake and bioenergetic behavior in clinical interventions. Therefore, more studies focusing on the role of mitochondrial energy metabolism in PD and other neurodegenerative disease are warranted, particularly in the direction of developing early diagnostic biomarkers and generating alternative, effective therapeutics.

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5 Targeted Treatment of Age-Related Fibromyalgia with Supplemental Coenzyme Q10

Iain P. Hargreaves and David Mantle

Abstract

Fibromyalgia is a common chronic pain condition of unknown aetiology, although mitochondrial dysfunction, oxidative stress, and inflammation have been implicated in the pathophysiology of this disorder. Treatment generally involves physiotherapy, anticonvulsants, and antidepressant therapy; however, the symptomatic relief conferred by these treatments can be very variable, and there is a need for additional therapeutic strategies. One such treatment which is gaining a lot of interest is the use of coenzyme Q10 (CoQ10) supplementation. The therapeutic efficacy associated with CoQ10 supplementation is thought to arise from the ability of supplementation to restore an underlying deficit in CoQ10 status which has been associated with fibromyalgia together with the ability of CoQ10 to improve mitochondrial activity, restore cellular antioxidant capacity, and ameliorate inflammation. This chapter outlines the evidence supporting the therapeutic utility of CoQ10 in the treatment of fibromyalgia.

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Keywords

Fibromyalgia · Mitochondrial dysfunction · Oxidative stress · Coenzyme Q10 · CoQ10 · Supplementation

5.1 Introduction

Fibromyalgia is a chronic disabling disorder, affecting up to 5% of the U.K. population. In addition to the cardinal symptoms of muscle pain and fatigue, fibromyalgia patients also suffer from a wide range of co-morbidities, including headache, anxiety/depression, sleep deprivation, memory and concentration disturbances, as well as digestive dysfunction [1]. The reason people develop fibromyalgia is not fully understood and conventional prescribed drug treatments may be of limited effectiveness. Treatment has progressed from inappropriate use of non-steroidal anti-inflammatory drugs (NSAID) and opioid type pain relieving drugs, through the use of anticonvulsants, such as pregablin and gabapentin, and antidepressants such as amitriptyline, to non-pharmacological treatments such as cognitive behavioural therapy or specific exercise regimes [1]. However, the symptomatic relief conferred by these various treatments can be very variable, and there is a need for additional therapeutic

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strategies, including those based on nutritional supplementation. Therefore, the aim of this chapter will be to provide information on nutritional treatment strategies targeted to pain relief in fibromyalgia, focussing upon coenzyme Q10 (CoQ10) supplementation and the evidence supporting its therapeutic utility in the treatment of this disorder.

5.2 Pathogenesis of Fibromyalgia

The pathological mechanisms underlying fibromyalgia are thought to involve mitochondrial dysfunction, oxidative stress, and dysregulation of the inflammatory response [2]. However, there is a growing consensus that the oxidative stress and inflammation associated with fibromyalgia originate from the mitochondrial dysfunction [3, 4]. Evidence of oxidative stress in fibromyalgia has been indicated by increased levels of the lipid peroxidation production, malondialdehyde in blood mononuclear cells (BMC), and plasma of patients with this condition in conjunction with decreased activity of the antioxidant enzyme, catalyse [5]. Furthermore, an increase in mitochondrial reactive oxygen species (ROS) generation was reported in the BMC of patients with fibromyalgia in a study conducted by Cordero et al. in 2013 [6]. Indices of the dysregulation of inflammation in fibromyalgia have been denoted by increased serum/plasma levels of the pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α) [7], interleukin (IL)-8 and IL-1Ra [8] and IL-1B and IL-18 [4]. Decreased levels of ATP [5, 7, 9], a decreased mitochondrial DNA content relative to that of nuclear DNA [5, 9] together with a diminution in the status of the mitochondrial respiratory chain (MRC) carrier, CoQ10 [3, 5, 7] and a decreased mitochondrial membrane potential have all been reported in the BMC of patients with fibromyalgia strongly supporting evidence of mitochondrial dysfunction in this disorder.

Within the confines of this chapter, it would not be possible to outline all of the mechanisms that have been proposed to account for the oxidative stress, inflammatory response, and mitochondrial dysfunction reported in fibromyalgia. However, a paradigm will be offered based on the results of studies from the current literature to account for these pathological anomalies in fibromyalgia.

Mitochondrial dysfunction appears to be one of the primary events initiating both ROS generation and inflammation in fibromyalgia [7, 10]. The cause of the MRC impairment with deficiencies in the activities of the MRC enzymes, complex I,II, III, and IV (Fig. 5.1) together reduced expression in the protein levels of complexes I and III as well as a diminution in the expression of the electron carrier cytochrome c [4]. It has been suggested that the MRC impairment may be related to the downregulation of genes encoding for the regulatory proteins, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), transcription factor A, mitochondrial (TFAM), and nuclear respiratory factor 1 (NRF1) that has been reported in fibromyalgia and which would be expected to impair mitochondrial biogenesis [4]. An impairment in mitochondrial biogenesis may account for the decrease in both mitochondrial DNA copy number and the activity of the mitochondrial marker enzyme, citrate synthase, in BMC isolated from patients with fibromyalgia [5, 9]. However, the deficit in CoQ10 status which has been widely reported in fibromyalgia may also be an important causative factor for the mitochondrial dysfunction determined in this order. A deficit in cellular CoQ10 status has been associated with multiple MRC enzyme deficiencies, decreased mitochondrial membrane potential, and a concomitant reduction in ATP levels [11] which have all been documented in fibromyalgia [12].

In addition to its role as an electron carrier in the MRC, CoQ10 also serves as a potent lipid soluble antioxidant [13] and, accordingly, a deficiency in cellular CoQ10 status has been associated with an increase in mitochondrial ROS generation which has also been reported in the BMC of fibromyalgia patients [4, 11]. This increase in ROS generation together with the accumulation of the products of mitochondrial

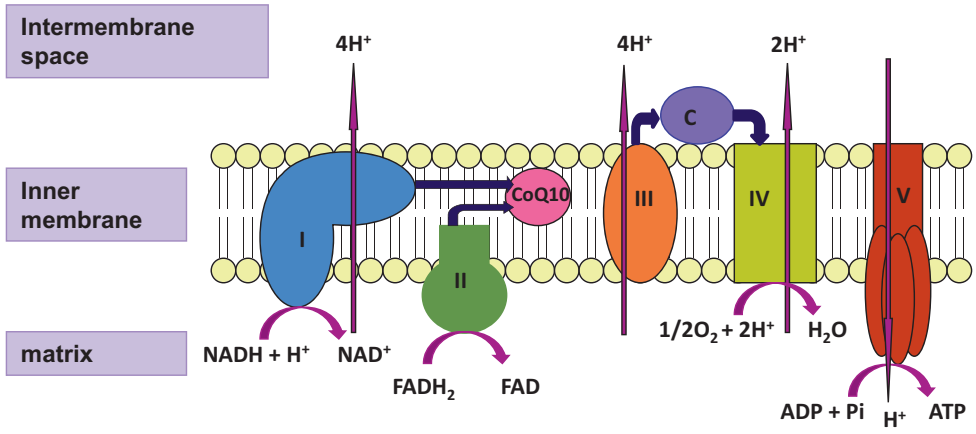

Fig. 5.1 Diagram of the mitochondrial respiratory chain (MRC) showing the enzyme complexes I–V and the electron carriers coenzyme Q_{10} (CoQ10) and cytochrome c (C)

DNA oxidation have been reported to be potent activators of the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome, a cytosolic oligomeric protein complex present in the cells of the immune system which regulates the innate immune response [14].

In vitro and in vivo studies using BMC isolated from fibromyalgia patients have reported increased gene expression of the NLRP3 protein, a component of the inflammasome, as well as caspase-1, an inflammatory response initiator, together with increased serum and cell culture levels of the inflammatory cytokines, IL-1β and IL-18 in association with a deficit in cellular CoQ10 status [4]. Pharmacologically induced CoQ10 deficiency in BMC from healthy volunteers has also been reported to cause an increase in the synthesis of the pro-inflammatory cytokine, TNF-α, accompanied by an increase in mitochondrial ROS production [7]. As well as promoting the inflammatory response by generating ROS and oxidized mitochondrial DNA products, a CoQ10 deficiency may also remove the inhibitory effect of this molecule on inflammasome activation [7]. A high positive correlation has been reported between serum IL-1β and IL-18 levels and the pain scores in fibromyalgia patients indicating the importance of inflammation in the pathophysiology of this disorder [4]. Furthermore, inflammatory cytokines have been reported to induce fatigue, fever, sleep, and myalgia, which are symptoms reported in fibromyalgia [8].

The putative mechanisms that have been implicated in the generation of oxidative stress and inflammation in fibromyalgia are outlined in Fig. 5.2. Although there is some evidence to indicate that a deficiency in cellular CoQ10 status may be an important triggering event in the mitochondrial dysfunction, oxidative stress and inflammation associated with fibromyalgia, the actual factors responsible for this deficiency, have yet to be elucidated.

Overall, Fibromyalgia is thought to result from a self-reinforcing, increasingly destructive process of impaired energy production, free radical damage, and inflammation.

5.3 Fibromyalgia and Aging

Fibromyalgia is a disorder that can occur at any age in both men and women. However, fibromyalgia is considered by some medical practitioners as a condition primarily affecting middle aged women. Thus, fibromyalgia in older patients is understudied, and because of the likelihood of other age-related problems, diagnosis of fibromyalgia in the elderly may be overlooked. Only six clinical studies relating specifically to fibromyalgia in the elderly have been published in the medical literature over the past 30 years. In 1988,

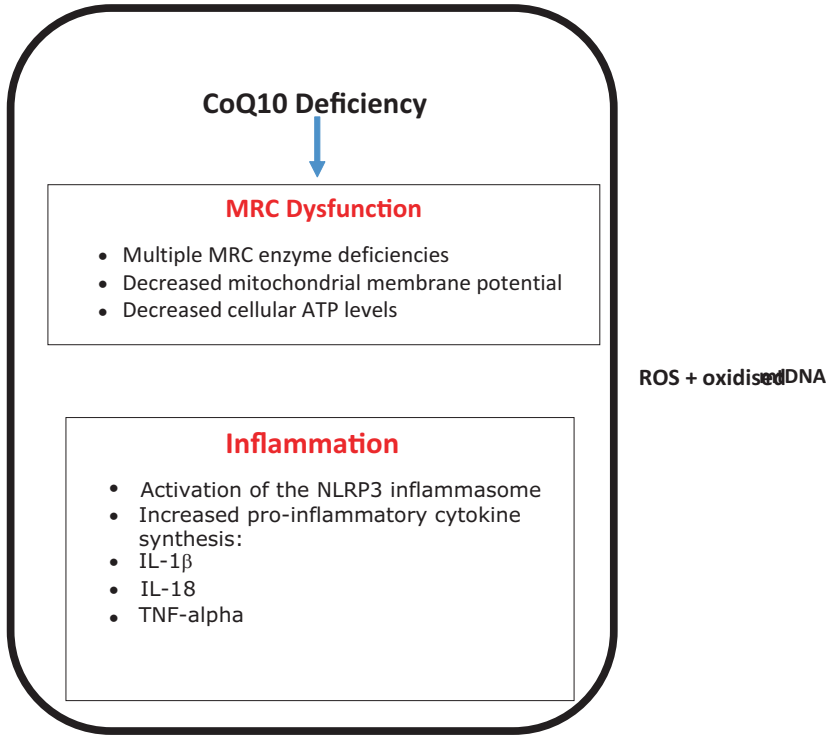

Fig. 5.2 Putative mechanism of oxidative stress and inflammation generation in fibromyalgia. *COQ10* coenzyme Q_{10}, *MRC* mitochondrial respiratory chain, *ROS* reactive oxygen species, *mtDNA* mitochondrial DNA

Yunus et al. first reported that fibromyalgia in the elderly was often unrecognised and treated with inappropriate medications such as steroids [15]. The most recent study by Jacobsen et al. (2015) found that more than 80% of older (55–95-years-old) patients with fibromyalgia were subject to pain, lack of mobility, and sleep disruption resulting from under-treatment [16]. In addition, many of these patients were using ineffective and potentially harmful opioid- or steroid-type medications.

Two non-pharmacological interventions known to benefit fibromyalgia are exercise and nutrition [17, 18]. Exercise is an important part of the treatment in fibromyalgia, and it also helps to keep the bodyweight down, which reduces the stresses on the joints. However, older individuals may find taking part in such exercise regimes challenging. With regard to nutrition, supplementation may benefit the primary symptoms or co-morbidities associated with fibromyalgia. Fibromyalgia patients are at increased risk of disorders such as diabetes, thyroid dysfunction, cardiovascular disease, and osteoporosis. In view of its MRC electron carrier and antioxidant functions, supplementation with CoQ10 may benefit the cardinal symptoms of muscle pain and fatigue, as well as headache and migraine which have been associated with cellular energy failure and oxidative stress [5, 13]. In addition, nutritional supplementation may benefit co-morbidities, such as gastrointestinal dysfunction (probiotics), osteoporosis (calcium, vitamin D3, vitamin K2), sleep problems (melatonin), and thyroid dysfunction (selenium) [19].

5.4 Nutritional Supplementation and Aging

Nutritional supplementation may be of particular importance in the elderly. The body requires a range of nutrients to maintain normal functioning. Some of these, such as CoQ10 and glucosamine,

are manufactured wholly or mainly within the body, while many (such as vitamins) must be derived from the normal diet. As people age, their bodies become less efficient at manufacturing nutrients, such as CoQ10, or in absorbing dietary nutrients from the digestive tract. As an example, optimum production of CoQ10 occurs around 25 years of age, production then gradually declines with increasing age, such that production at age 65 is approximately half that of 25. Thus, supplementation with CoQ10 in older individuals with fibromyalgia addresses two issues, a deficiency known to occur in fibromyalgia and a deficiency known to result from the normal aging process [20].

Similarly, the elderly are at risk of the potential deficiency in a wide range of other essential nutrients, and a study by Borg et al. in 2015 identified deficiencies of vitamin D3, vitamins B1, B2, and B12, calcium, magnesium, and selenium as being of particular public health concern [21]. Selenium is a trace element essential for the activity of 25 selenoproteins involved in the regulation of the inflammatory response and cellular antioxidant capacity, and therefore a deficiency in this trace element may lead to a weakening of the immune system and a compromised antioxidant capacity which are both integral to the pathophysiology of fibromyalgia [22]. As noted above, many of these nutrients have been found to be depleted in fibromyalgia, with a corresponding benefit on symptoms following supplementation. Again, supplementation addresses two issues: (1) deficiency due to fibromyalgia and (2) deficiency due to aging [19, 23].

5.5 CoQ10

CoQ10 is a lipid soluble molecule consisting of a benzoquinone nucleus derived from tyrosine and an isoprenoid side chain which is synthesised in all the cells of the body apart from red blood cells, as the presence of mitochondria are required for its synthesis [13]. As previously mentioned, the major function of CoQ10 is that of an electron carrier in the MRC where it transports electrons derived from complex I and complex III to complex III enabling a continuous passage of electrons within the MRC, a prerequisite for oxidative phosphorylation and cellular ATP generation (Fig. 5.1) [13]. CoQ10 also serves as an important lipid soluble antioxidant molecule protecting cellular membranes and circulatory lipoproteins against free-radical-induced oxidative damage [13, 24]. The antioxidant function of CoQ10 is attributed to its fully reduced ubiquinol form, in addition to acting as an antioxidant in its own right, and it is also involved in the regeneration of other antioxidants, such as α-tocopherol (the active antioxidant form of vitamin E) and vitamin C [25]. One of the principle enzymes involved in reducing CoQ10 to its ubiquinol form is the selenium-containing enzyme thioredoxin reductase. Therefore, a deficit in selenium status may compromise cellular antioxidant capacity [26]. Furthermore, selenium also serves as a prosphetic group for the antioxidant enzyme glutathione peroxidase [22]. In addition to its role as an electron carrier and antioxidant, CoQ10 has also been shown to directly affect the expression of a number of genes, including some of those involved in inflammation [24]. It has been suggested that CoQ10 is able to elicit an anti-inflammatory response by both its antioxidant function as well as by controlling the gene expression of the nuclear transcription factor, NFF-kappaB1 which has a key role in regulating the immune response to infection [27].

An adequate supply of CoQ10 is essential for normal functioning of mitochondria. Although some CoQ10 is obtained from the normal diet (approximately 5 mg/day), most of the daily CoQ10 requirement (estimated at 500 mg) is synthesised within the body [24]. As noted above, as people age, the capacity of the body to synthesise its own CoQ10 decreases. Optimal production occurs around the mid-20s, with a continual decline in tissue levels thereafter [20]. In addition to the normal aging process, CoQ10 levels have also been shown to be depleted in a variety of disorders, including fibromyalgia, as well as by statin-type drugs. Dietary supplementation with CoQ10 therefore provides a mechanism to maintain adequate levels within the body [13, 24].

5.6 Clinical Studies on CoQ10 Supplementation in Fibromyalgia

Fibromyalgia patients have been shown to have depleted tissue levels of CoQ10 (up to 40–50% of normal), together with increased levels of mitochondrial dysfunction, oxidative stress, and inflammation, both in adult [7, 9] and juvenile [28] patients. A recent Norwegian clinical study has highlighted the link between fibromyalgia and inflammation [29]. The study comprised 150 women aged 18–60, divided equally into three groups: (1) fibromyalgia patients; (2) chronic fatigue syndrome patients; and (3) healthy controls. Blood samples from the study were taken from all participants and analysed for levels of high-sensitivity C-reactive protein (hsCRP), a sensitive biochemical marker of inflammation.

Both the fibromyalgia and chronic fatigue syndrome patients had significantly increased mean levels of hsCRP (1.3 mg/L and 0.94 mg/L, respectively), compared to the levels in healthy controls (0.60 mg/L). The results from this study are noteworthy since inflammation is a well-known cause of pain and fatigue [30].

A number of clinical studies have been undertaken to investigate the effect of CoQ10 supplementation on fibromyalgia symptoms. The rationale for using CoQ10 in the treatment of fibromyalgia is multifaceted. This includes replenishing the underlying CoQ10 deficiency associated with the disorder, increasing electron flow in the MRC, enhancing cellular antioxidant capacity, and modulating the inflammatory response.

Cordero et al. correlated headache symptoms with reduced CoQ10 levels and increased oxidative stress [5]. Following CoQ10 supplementation (300 mg/day for 3 months), there was a significant decrease in oxidative stress as indicated by an increase in the activity of antioxidant enzyme, catalase, and a significant decrease in level of the lipid oxidation product, malondialdehyde, in the BMC of fibromyalgia patients. These effects were accompanied by significant improvement in headache symptoms in the fibromyalgia patients. Similarly, a randomised, double-blind, placebo-controlled clinical study in 20 fibromyalgia patients found that supplementation with CoQ10 (Pharma Nord Bio-Quinone, 300 mg/day for 40 days) significantly reduced (by more than 50%) pain and fatigue [4]. There was a corresponding improvement in mitochondrial energy generation as indicated by BMC ATP levels and reduced oxidative stress and inflammation, as assessed by the circulatory levels of the inflammatory cytokines, IL-1b and IL-18. In the latter study, psychopathological symptoms (including depression) were significantly improved following CoQ10 supplementation compared to placebo, and this improvement was linked to the effect of CoQ10 supplementation in reducing oxidative stress and inflammation and increasing levels of the neurotransmitter, serotonin [31, 32].

Several studies have reported abnormal blood lipid profiles in patients with fibromyalgia. In a study of 80 women with fibromyalgia, Gurer et al. reported increased blood levels of total and LDL-cholesterol compared to normal control subjects [33]. In a study carried out in Spain at Seville University, Cordero et al. evaluated the blood lipid profiles of 180 patients with fibromyalgia [34]. Approximately two-thirds of these patients had increased levels of total cholesterol and low density lipoprotein (LDL)-cholesterol, which correlated with the severity of their fibromyalgia symptoms assessed using the Fibromyalgia Impact Questionnaire (FIQ) and Visual Analogue Scales (VAS) of pain. These increases in cholesterol may result in part from genetic factors as well as from lack of exercise and increased body mass index (BMI). The lack of physical activity and increased total/ LDL-cholesterol blood levels may explain the increased risk of cardiovascular disease in fibromyalgia patients noted by Acosta-Manzano et al. [35].

Various studies have demonstrated that coenzyme Q10 can help to control cholesterol levels in the blood. First, CoQ10 can reduce cholesterol levels by directly inhibiting the genes responsible for the biosynthesis of LDL-cholesterol. Second, CoQ10 is circulated in the blood using LDL-cholesterol as a carrier. At the same time, the antioxidant action of CoQ10 helps to prevent the LDL-cholesterol from being oxidatively damaged by free radicals, thereby reducing the risk of atherosclerosis. Third, in addition to inhibiting

cholesterol synthesis, statin drugs also inhibit the body's production of CoQ10, which is generated via the same biochemical pathway [24, 36]. Supplementation with CoQ10 can reduce statin-associated adverse effects, such as muscle pain or the increased risk of diabetes. Randomised controlled clinical trials have shown supplemental CoQ10, both alone or in combination with other supplements, can significantly reduce total blood or LDL-cholesterol levels in hypercholesterolaemic subjects. Thus, studies by Schmelzer et al. in 2011 [37], using 150 mg CoQ10/day for 2 weeks, and by Mohseni et al. in 2014 [38], using 200 mg CoQ10 for 12 weeks, reported significant reductions of approximately 15% in LDL cholesterol levels following CoQ10 supplementation.

A study by Miyamae et al. in 2013 also reported that ubiquinol treatment (100 mg/day for 12 weeks) of patients with juvenile fibromyalgia resulted in decreased circulatory levels of free cholesterol and cholesterol esters, indicating that ubiquinol supplementation improved cholesterol metabolism and chronic fatigue scores, as measured by the Chalder Fatigue Scale [28].

The beneficial effects of CoQ10 as an adjunct therapy to the commonly used anticonvulsant, pregabalin (commonly used to reduce the pain sensation in fibromyalgia), was recently demonstrated in a study by Sawaddiruk et al. [39]. In this randomised placebo-controlled clinical study, 11 fibromylagia patients were randomly allocated to pregabalin alone or to pregabalin with CoQ10. The results of the study indicated that although pregbalin treatment alone reduced pain and anxiety in the patients, pregbalin combined with CoQ10 treatment reduced pain, anxiety, as well as mitochondrial oxidative stress in BMC and inflammation in the fibromyalgia patients, compared to baseline.

5.7 Safety and Bioavailability of CoQ10

CoQ10 is generally well-tolerated, with no serious adverse effects reported in long-term use [40]. In rare cases, individuals may experience mild gastrointestinal disturbance. There are no known toxic effects, and CoQ10 cannot be overdosed. The safety of CoQ10 has been confirmed in more than 200 randomised controlled trials, covering a wide range of disorders. Several case studies have suggested that CoQ10 may interfere with the action of warfarin, although a randomised controlled clinical trial showed that CoQ10 supplementation at 100 mg/day had no effect on the clinical action of this anticoagulant medication [41].

Bioavailability is defined as the proportion of an ingested substance that reaches the blood circulation. Because of its relatively large molecular size and lipid solubility, the bioavailability of CoQ10 is intrinsically low. CoQ10 is absorbed from the intestinal tract via the same mechanism as other lipid soluble nutrients. This occurs via a lipid carrier through mucosal cells initially into the lymph, and thence into the bloodstream. Thus, absorption is optimised when CoQ10 is dissolved in a carrier oil (preferably soya or palm oil). When supplemental CoQ10 is first produced (via a yeast fermentation process), it is obtained in the form of crystals which cannot be absorbed from the digestive tract. It is essential that these crystals are dispersed into single CoQ10 molecules (and remain dispersed during the product shelf-life) to enable optimum bioavailability. Adding CoQ10 crystals to a carrier oil without such dispersal, a cost-saving technique used by some manufacturers, is inadequate. Disparity in the findings of clinical trials supplementing CoQ10 undoubtedly result from inadequate bioavailability and insufficient dosage or treatment duration.

5.8 Conclusions

In view of the ability of CoQ10 supplementation to restore an underlying CoQ10 deficiency in fibromyalgia patients together with its ability to improve MRC activity, restore cellular antioxidant capacity and ameliorate inflammation, all of which are factors associated with the pathophysiology of fibromyalgia, CoQ10 therapy should be considered as an appropriate adjunct treatment for this chronic pain disorder as shown in Fig. 5.3. However, larger controlled clinical trials are still required to provide further data of the effectiveness of CoQ10 in the treatment of fibromyalgia.

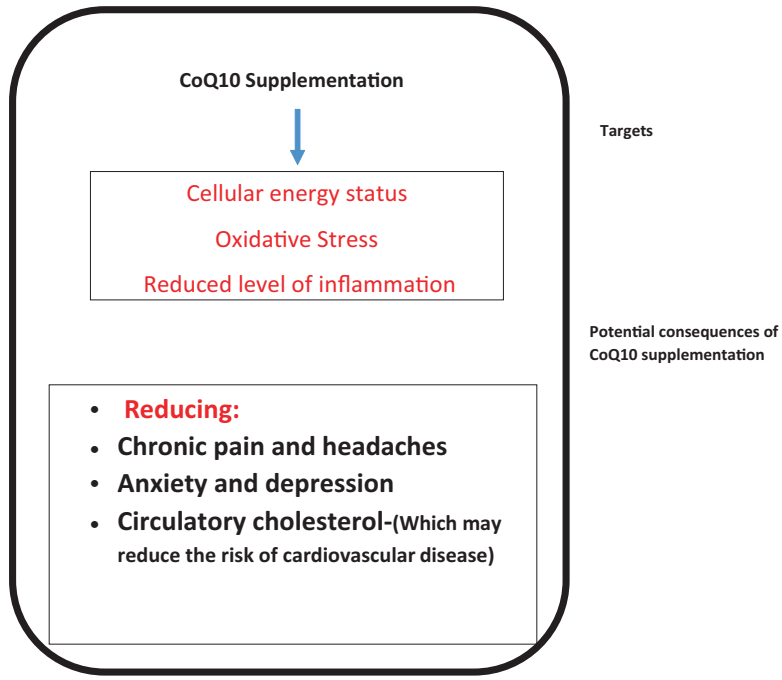

Fig. 5.3 The potential targets and consequences of CoQ_{10} supplementation in fibromyalgia. *COQ10* coenzyme Q_{10}

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6 Immunoregulatory Effects of Tolerogenic Probiotics in Multiple Sclerosis

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Abstract

Gut microbiota has essential roles in the prevention and progression of multiple sclerosis (MS). The association between the gut microbiota and the central nervous system (CNS) or immune system response of MS patients has been documented in many studies. The composition of the gut microbiota could lead to sensitization or resistance against promotion and development of MS disease. Probiotics are the major part of gut microflorapopulation and could be substituted with tolerogenic pro-

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biotics that protect the CNS against autoimmune responses. Tolerogenic probiotics with anti-inflammatory and immuno-modulatory properties have effects on intestinal flora and can reestablish regulatory mucosal and systemic immune responses. Probiotics are able to prevent and restore excessive activation of inflammatory responses, especially autoreactive T cells and inflammatory cytokines. Tolerogenic probiotics, through induction of regulatory T cells and increase of anti-inflammatory cytokines, play a crucial role in controlling inflammation and maintaining tolerance and hemostasis. Therefore, probiotics can be considered as a preventive or therapeutic tool in MS. In the present review, we focus on the immunoregulatory effects of tolerogenic probiotics on the severity of disease, as well as Th1, Th2, and Treg populations in different experimental and human studies of MS.

Keywords

Tolerogenic probiotics · *Lactobacillus* · *Bifidobacterium* · Multiple sclerosis · Experimental autoimmune encephalomyelitis

6.1 Introduction

Multiple sclerosis (MS) is a complex autoimmune disorder of the central nervous system (CNS) characterized by generation of autoantibodies and autoreactive T cells against myelin proteins leading to focal inflammation, axonal degeneration and damage, or dysfunction of neurons and oligodendrocytes, followed by progressive, long-term physical and/or mental disability [1, 2]. Around 2.5 million people are currently diagnosed with MS worldwide [3]. In recent years, MS mortality has increased, rising from 12,000 in 1990 to 20,000 in 2012 [4]. The median life expectancy is approximately 5–10 years lower for MS patients compared with an age-matched general population [5].

Similar to many other autoimmune diseases, women are much more susceptible to MS with a female to male ratio of 2:1 to 3:1, although men with MS tend to have more severe clinical outcomes and poorer recovery after the initial disease relapse [6]. Since all regions of CNS can be affected, MS patients can exhibit diverse manifestations varying greatly case by case and over the course of disease, representing a challenge to clinicians [7]. The signs and symptoms tend to debut between the ages of 20 and 40 years [8]. Patients complain frequently about gait instability, sensory disturbances, fatigue, weakness, spasticity, loss of balance, tremor, vision problems and depression, as well as bladder and bowel dysfunctions that seriously reduce the quality of their lives [7, 9, 10].

Although neither the primary cause nor the pathogenesis of MS is known definitively, the interplay between environmental factors and genetic factors has been demonstrated to contribute to the dysregulation of immune tolerance, involving an orchestrated attack of the innate and adaptive immune system towards components of the brain or spinal cord [11]. It has been reported that, during the disease course in both patients and animal model of MS, experimental autoimmune encephalomyelitis (EAE), immune regulatory defects in synergy with increased migration of autoagressive myelin antigen specific CD4+ effector T cell promote a key process in the pathogenesis of the disease [12]. The presence of plaques are the pathologic hallmark of MS, which consists of a defined hypocellular area and axonal oligodendrocyte damage accompanied by variable gliosis and inflammation, relative preservation of axons, formation of an astrocytic scar and

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cell death, as well as the presence of inflammatory cells (mostly lymphocytes) infiltrating into the CNS [13, 14].

EAE is a T-cell-mediated model of autoimmune demyelination of the CNS, which widely serves an experimental model for MS in order to provide insights into the possible cellular and molecular factors involved in the pathogenesis [15]. It is well established that the EAE model can be developed in susceptible sensitized animals through immunization by different myelin-derived proteins emulsified in complete Freund's adjuvant (CFA) such as myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) or myelin basic protein (MBP), or by adoptive transfer of autoreactive T cells to naive animals [15, 16]. A growing number of studies using MS animal models have led to the suggestion that B cells have no critical role in the induction of EAE [17] but play a critical role in controlling the onset and severity of the EAE immunopathogenesis [18]. The autoimmune etiology of MS and EAE has been shown to involve breakdown of the blood–brain barrier (BBB) and autoimmune response as a result of dysregulated innate and adaptive immune pathways, resulting in myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction that causes clinical manifestations of these diseases [19, 20].

Based on the clinical disease pattern, MS pathology is commonly classified into four major clinical subtypes characterized by increasing severity, including relapsing-remitting (RR), secondary-progressive (SP), primary-progressive (PP), and progressive-relapsing (PR). Interestingly, subtypes of MS have dissimilar underlying neuropathologies, suggesting that MS may represent a heterogeneous group of autoimmunity disorders [21]. RR is the most common subtype of MS and accounts for 85% to 90% of cases, which is characterized by relapses usually followed by periods of recovery or remission [22]. PPMS is estimated to represent about 10–15% of patients with MS, in which clinical disability progression occurs continuously with no distinct period of remission or remissions. This subtype of MS has been most commonly diagnosed in older subjects compared to relapsing onset MS, with no gender predominance [23–25]. Within a span of 10–15 years in disease onset, up to 60% of patients with RRMS will subsequently go on to develop SPMS, which is followed by a phase of uninterrupted disease progression (Table 6.1) [21, 26].

Table 6.1 Common four major clinical forms of MS (based on pathology)

Type	Disease course
Relapsing/remitting multiple sclerosis (RRMS)	The most common subtype of MS and characterized by acute neurological deficits or worsening of relapses generally followed by periods of incomplete or complete recovery
Secondary progressive multiple sclerosis (SPMS)	Second most common type of MS, characterized by initial relapses, followed by progressive neurological decrease without any remission
Primary progressive multiple sclerosis (PPMS)	Accounts for approximate 10–15% of patients with MS. characterized by steady functional decline from the onset of the disease independent of relapses (inflammation)
Progressive relapsing multiple sclerosis (PRMS)	The lowest common type of MS types, characterized by progressive disability from the onset of symptoms, with later superimposed acute attacks. PRMS and PPMS cannot be distinguish during early stages, until the relapses occur

6.2 MS and the Immune System

Despite extensive efforts aimed at defining MS immunopathology, the precise mechanisms that initiate immune responses to auto-antigens or derived peptides remain debatable [27]. MS is known as an autoimmune disorder, in which the host immune system fails to distinguish the self-peptides from foreign ones, leading to an aggressive immune response against the myelin sheath surrounding axon terminals, and consequently to myelin destruction and loss of oligodendrocytes and axons [28, 29]. Under normal conditions, the CNS is considered as an immune-privileged site that accessibility of T cells and other immune cells is restricted by the endothelial BBB and epithelial blood-cerebrospinal fluid barrier

(BCSFB) [30, 31]. Environmental factors in genetically susceptible subjects are thought to play an important role in disruption of BBB-mediated immune surveillance, whereby activated cells readily pass through the inflamed barrier [27].

In the initiation of CNS inflammation, under certain conditions autoantigens or microbial antigens are presented by professional antigen-presenting cells (APCs) to T cells in lymphoid tissues that can provoke peripheral activation of proinflammatory lymphocytes [32]. After activation in periphery, myelin-specific T cells are capable of transmigration across the BBB into the CNS which is regulated by adhesion of integrins on the leukocyte surface and endothelial cells then be reactivated by APCs presenting self-antigens in the brain [33]. After reactivation in the CNS, autoreactive T cells trigger an inflammatory cascade which consequently activates macrophages and microglial cells [34]. In turn, macrophages and microglia secrete chemokines that contribute to the recruitment of additional T cells, DCs, and macrophages followed by oligodendrocyte death, demyelination, and ultimately neuronal loss [33–35]. It is important to point out that antibodies and B cells can also have the capacity to migrate across the CNS followed by demyelination and inflammation by complement-mediated cytotoxicity [20, 36]. With a key role in recruitment of adaptive immune cells to the CNS, astrocytes and microglial cells, as the main innate immune cells of MS lesions, critically contribute to the demyelination and neurodegeneration process of MS through functional changes associated with their activation [9, 37, 38]. Additionally, astrocytes as the most abundant cell population in the CNS are able to modulate CNS inflammatory responses by secreting cytokines and chemokines at multiple levels [39]. These insights emphasize the importance of the innate immune response as strong mediators of MS pathogenesis.

6.2.1 Th1 and Th17 in MS

Recent evidence has revealed that pro-inflammatory T cells, such as Th1 and Th17 subtypes, are crucial immunological participants in the neural-immune mechanisms underlying MS/EAE with distinct clinical signs and pathological features [40]. There have been extensive studies in favor of both Th1 and Th17 cells that imply complementary roles in the pathogenesis of EAE by inducing an inflammatory milieu resulting in demyelination within the brain and spinal cord, with axonal damage [40]. Th17 cells are characterized by the production of proinflammatory cytokines, such as interleukin (IL)-22, IL-21, IL-17A-F and IL-23, which have been recognized as key contributors to MS by increasing inflammation in myelin sites [38]. Another Th lineage, the autoreactive Th1 type lymphocytes that generate IFN-γ and TNF-α, have been shown to increase MS by developing inflammation or by exerting toxic or pro-apoptotic effects on oligodendrocytes. However, it should be noted that production of IFN-γ and IL-17 are the indicator cytokines of Th1 and Th17 cells, respectively [41, 42]. There is accumulating evidence demonstrating an increased frequency of IFN-γ -producing Th1 and IL-17-producing Th17 cells during EAE/MS relapse [42–44] which has been shown to be closely related to infiltrating monocytes and macrophages into the CNS following increases in the permeability of the BBB. This has been further supported by other studies that have shown high levels of inflammatory mediators secreted by infiltrating Th17 cells that could drive disruption of BBB tight junctions, demyelination, and hamper nerve conduction [45, 46]. Additional studies have revealed that impairment of all of the factors involved in Th17 cell development or deficiency consistently either attenuate or abrogate EAE [47]. On the other hand, TNF-α and IFN-γ have all been shown to exert direct myelinotoxic properties and elevated amounts of these cytokines coincide with disease activity [48].

6.2.2 Th9 and Th22 in MS

Two additional subsets of effector T cells Th9 and Th22 have been recognized, and the capacity of these T-cell subsets to induce EAE is currently

being investigated. Multiple lines of evidence have recently pointed to a role of Th9 and Th22 in the immunopathogenesis of MS [38, 49]. The frequencies of Th22 and the related cytokine IL-22 have been detected to be increased in the blood and CSF of patients with MS, especially during the active phases of the disease. In addition, it is important to note that IL-22 concentrations, the signature cytokines of Th22, were found to be increased during a relapse phase of MS, suggesting a role in the immunopathogenesis [49]. The role of Th9 in EAE was further supported by studies which showed that Th9 cells induce EAE and inflammation and IL-9 knockout mice are protected from developing EAE, indicating that Th9 cells may provoke the inflammatory process [38]. However, the accurate role of these subsets in EAE and MS is not clearly understood and has been the subject of on-going investigations.

6.2.3 CD8+ T Cells and B Cells in MS

Supported by the EAE animal model, growing evidence points to the pathogenic involvement of $CD8^+$T cells in the pathophysiology of MS, although it has traditionally regarded to be a $CD4^+$-mediated autoimmune disease [50]. Recent neuropathological studies have convincingly demonstrated that, within the T cell population, $CD8^+$ T-cells are the most predominant T-cells in CNS lesions [51], where their numbers strongly correlate with the extent of acute axonal damage [50]. $CD8^+$ T cells are believed to mediate pathogenic processes in numerous cells types by direct cytotoxicity or production of pro-inflammatory mediators including IL-17, IFN-γ, and TNF-α [51, 52]. Besides the involvement of T cells in MS pathogenesis, limited attention has been directed to another important immune cell type, i.e., B cells. B cells and humoral immunity contribute to initiation, progression, and subsequent tissue damage in the autoimmune pathogenesis of MS by different mechanisms such as abnormal production of antibodies, co-stimulating T cells to produce and release inflammatory factors, and by secretion of inflammatory cytokines, such as IL-6, TNF-α, and IL-12 [53]. Studies have also demonstrated accumulation of B cells and plasma cells in the brains and CSF of patients with MS [54].

6.2.4 T Regulatory (Treg) and Th2 Cells in MS

Along with the upregulation of proinflammatory mediators following induction of EAE, immunosuppressive activity of Tregs has been reported to be impaired in MS [55]. Tregs play an important role in maintaining immune homeostasis, the prevention of autoimmunity, and suppressing deleterious inflammatory responses [56]. Treg is a unique CD4+ T-cell subset defined by expression of transcription factor Foxp3 and secretion of the suppressive cytokines TGF-β, IL-10, and IL-35 along with a protective role in MS by modulation of inflammation [57]. The mechanism by which Tregs have been implicated in immunosuppressive functions on various effector cells, especially pathogenic autoreactive T cells, is directly through production of the above-mentioned suppressive cytokines or in a contact-dependent fashion or indirectly via inhibiting maturation of APCs [19]. Another important anti-inflammatory CD4+ T-subset that has been proposed to possess a protective function in the disease is Th2. Alteration of Th2-related is recognized as a primary contributing factor to an inflammatory demyelinating disease [2]. Th2 cells are a source of anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β and are also supposed to be involved in the attenuation of neuro-inflammatory processes by down-regulating various aspects of the Th1 inflammatory response [58]. There is abundant evidence that upregulation of Th2-derived cytokines can reduce the severity of inflammatory demyelinating diseases, such as MS, and is associated with the recovery from disease [2, 59]. Additionally, IL-$4^{-/-}$and IL-$10^{-/-}$mice have been demonstrated to have increased susceptibility to EAE [2].

To summarize, switching immune cells from an anti-inflammatory to a pro-inflammatory state, in particular with an increased ratio of Th17/Treg

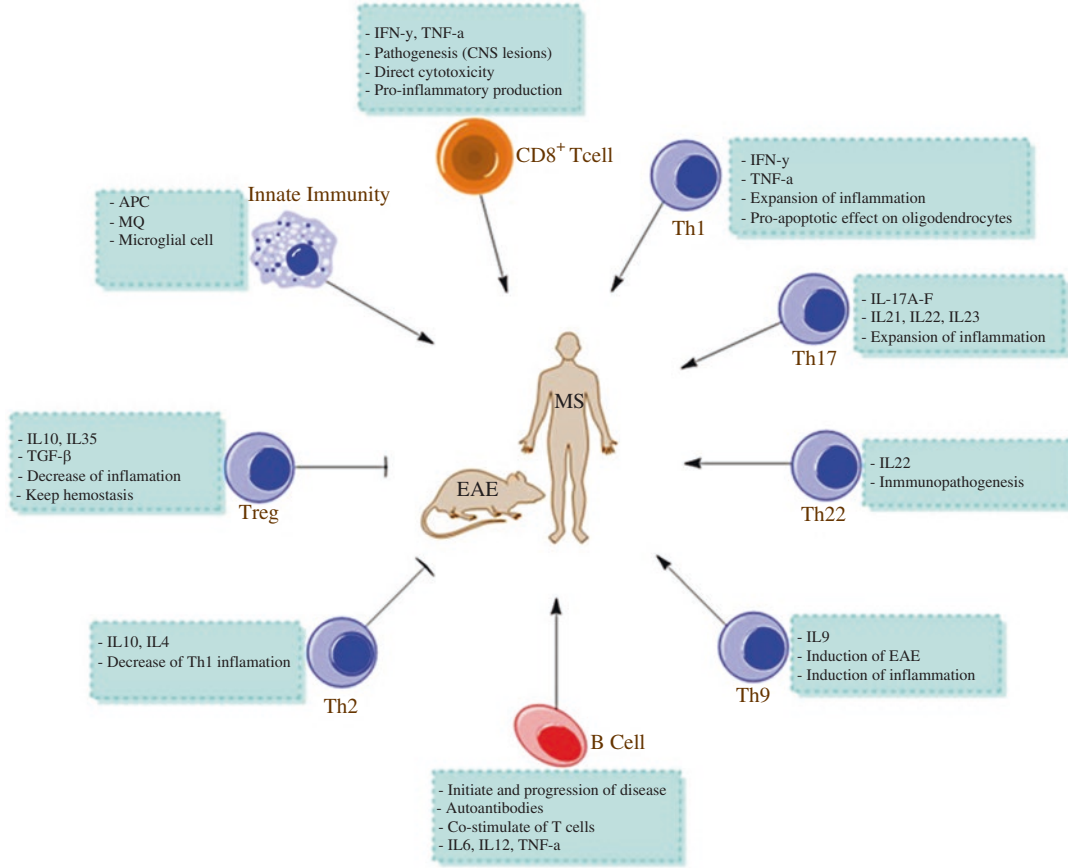

Fig. 6.1 Schematic figure of different immune cells and their inhibitory or activation roles in control or progression of disease in MS patient and EAE mice

and Th1/Th2, which causes a high inflammatory state in the CNS, may precede the clinical and laboratory symptoms of the disease activity (Fig. 6.1).

6.3 Risk Factors of MS

As mentioned above, MS is a complex disease in which both genetic and environmental factors contribute to its etiology and clinical course [60]. In most autoimmune/inflammatory disorders, the human leukocyte antigen region has the strongest genetic associations with MS [61]. Evidence for the importance of genetics comes from epidemiologic studies that show first-degree relatives have 20–40 times greater chance of developing MS in comparison to the general population [62]. Second- and third-degree relatives also have a clearly elevated risk of MS incidence [63]. Furthermore, twin studies from several populations have consistently indicated that dizygotic twins (~5% concordance) display a significantly lower clinical concordance rate than monozygotic twins (~30% concordance), supporting a high heritability [63]. The risk of developing MS increases from typically seven- to 26-fold in parents, from 12- to 38-fold in siblings, and from 6- to 25-fold in children of MS patients [64]. However, the hypothesis that genetic predisposition alone is not sufficient for promoting MS has been drawn from twin studies showing that monozygotic twins (100% genetic similarity) have an approximately 25–30% lifetime risk of developing MS when one of them has been diagnosed, suggesting the influence of both genetic

susceptibility and environmental triggers in the evolution of MS [10, 65].

One of the most intriguing aspects of MS is that lifestyle and environmental factors may have a strong influence on progression and course. A large number of environmental risk factors including smoking and sedentary lifestyle [66], physical activity [67], obesity [68], indigenous geography, and migration patterns, exposure to infectious agents [68], and sleep restriction [69] have been identified to play an important role in the prevention and management of this condition.

Environmental contributions are further supported by analysis of the ratio of hormone levels in MS patients. It was found that sex hormone have potential association with course and susceptibility to MS [70, 71]. Of note, it was proposed that the incidence of MS may be decreased with migration from low risk to high risk countries before 15 years of age [72]. This latitude gradient in MS has been suggested to be driven by environmental rather than genetic factors, especially exposure to sunlight and decreased levels of vitamin D [69, 73].

In addition to these environmental risk factors, the dietary habits and the gut microbiota have been frequently recognized to be associated with an increased risk of MS [74]. Epidemiological studies have reported that diets rich in low-calorie meals based on vegetables, whole cereals, legumes, fruit, and fish pose beneficial effects in the prevention and alleviation of MS [75] through inhibiting production of proinflammatory molecules and restoring or maintaining a diverse symbiotic gut microbiota [76]. Conversely, eating high-salt diets, animal fat, hypercaloric diets, red meat, saturated animal fat, sugar-sweetened drinks, and fried food has been shown to be associated with increased risk of the disease by elevating proinflammatory pathways and subsequent promotion of a dysbiosis gut microbiota state [75, 76]. It is noteworthy that influence of the many MS risk factors may be related to the gut microbiota dysbiosis [10]. A bidirectional connection between the gut microbiota and CNS disease supports the concept that dysbiosis of the gastrointestinal microbiota can also influence the clinical manifestations and inflammatory markers in MS/EAE [10]. Current evidence from different clinical studies has revealed striking variations in the composition of the intestinal microbiome in MS patients compared to that in healthy controls [77]. It has also been indicated that more than 70% of MS patients suffer from at least one gastrointestinal tract disease [78]. The overall evidence indicates that a comprehensive program of lifestyle modification can appear as a non-invasive and viable approach for preventing and controlling MS and possibly other neurological conditions.

6.4 Therapeutics Approaches for MS

During the past decade, there have been considerable efforts to develop efficient therapeutic approaches for MS [79]. Owing to the growing evidence from both animal and human studies suggesting pathophysiology of autoimmunity and immune-mediated mechanisms involved in the disease, research focuses on developing a promising therapy to modulate or suppress inflammatory responses [80]. Despite tremendous scientific efforts, the available preventive disease-modifying therapies (DMTs) have failed to treat MS completely and are aimed at slowing the progression of the disease, protection of functional capability, reducing individual symptoms and delaying or preventing progressive MS onset. Of these, the interferons and glatiramer acetate are the best treatments currently in use, and these appear to work by decreasing immune-mediated inflammation [3, 4, 81]. On the other hand, these drugs are only effective against the RRMS subtype and exhibit some drawbacks, such as inconvenient methods of administration, significant side effects, and low adherence rates. Therefore, more efficient preventive treatment strategies need to be developed [82, 83].

Currently, the ability of probiotics to impact on many aspects of physiological and pathological processes of the host has opened up new therapeutic prospects for the treatment of CNS inflammation [84]. The most common types of

microorganisms used as probiotics are *Lactobacillus (L)* and *Bifidobacterium (B)* [85]. A number of health improving properties have been attributed to the consumption of some probiotic strains, which leads to protection of the function of the gastrointestinal epithelial barrier, increased adhesion to intestinal mucosa, interference with the ability of pathogens to colonize, competitive exclusion of pathogenic microorganisms, and enhancement of antimicrobial peptide production and immunomodulatory functions [86, 87]. Treatment using probiotic therapy has been found to exhibit broader spectrum of action mechanisms to achieve immune modulation compared to conventional immunosuppressant drugs [88]. In contrast to immunosuppressant drugs, adverse reactions upon long-term treatment of probiotics have been rare [88]. Surprisingly, treatment with some probiotics could have profound effects on CNS functions, conferring the potential therapeutic effect of probiotics on neurodegenerative diseases [87, 89].

Probiotic-based therapies have been shown to have immune-modulating effects as prophylactic agents or dietary supplements to reverse immunological disturbances which, in turn, modulate the progression of EAE and probably MS. The immunomodulatory and beneficial effect of probiotic treatments has often been suggested to be related to inhibition of Th1/Th17 responses while increasing the immunoregulatory responses of Treg cells [9, 88, 90]. Another potential mechanism behind the efficacy of probiotic administration has also been attributed to decreasing the production of pathogenic cytokines (IL17, IFNγ, TNFα, and IL-12) and increasing anti-inflammatory cytokines (IL-4, IL-10, and TGFβ) [88, 91]. More importantly, another underlying molecular mechanism through which probiotic administration may modulate CNS function is through mitigating neurotoxin-induced oxidative dysfunctions and neurotoxicity [92]. Histological analysis of the CNS showed that probiotic administration decreased recruitment of inflammatory cells into the spinal cord and subsequently suppressed clinical paralysis in EAE mice [91]. All of these results point to the use of dietary supplemented with probiotics as an alternative approach to conventional therapy in MS patients. However, there is a necessity for further studies to fully quantify their clinical efficacy in this disease.

6.4.1 Probiotics and Autoimmune Diseases

Probiotics are live microorganisms that colonize different sites of the mucosal gut barrier. The beneficial effects of non-photogenic probiotics have been shown in many studies [93]. Probiotics can help in keeping the integrity of intestinal barrier [94], modulation of immune responses [95], and better absorption of foods [96]. These live microorganisms can produce antimicrobial compounds with anti-pathogenic effects and maintain the balance of the microbial population in the intestine [97]. The important roles of probiotics are modulation of immune responses in different diseases. Probiotics increase the strength of immune response in diseases such as cancer and infection but tolerogenic probiotics regulate the immune response and help in keeping tolerance in inflammatory and autoimmune diseases [98]. In fact, tolerogenic probiotics could directly and indirectly affect all autoimmune responses, through the generation of tolerance mechanisms and inhibitory cytokines. Research in this area has shown the effects of tolerogenic probiotics in autoimmune conditions such as systemic lupus erythematosus (SLE) through increase of inhibitory cytokines, decrease of inflammatory cytokines, and production of modulatory DCs [99, 100]. Administration of *L. delbrueckii* and *L. rhamnosus* probiotics were found to generate tolerogenic DCs and Treg in in vitro and amelioration of symptoms in a pristane-induced lupus mouse model [100–102]. These beneficial immunomodulation effects also changed the feature of immune response to tolerance in irritable bowel disease (IBD) [103], rheumatoid arthritis [104], and allergy [105]. Multiple sclerosis is one of the most injurious autoimmune diseases that has

been found to be linked with dysregulated immune responses and imbalance of gut microbiota [106]. It seems that the use of probiotic strains can maintain and restore the microbial balance with reduction of inflammatory responses in MS patients. All studies on dysbiosis in patients and animal models with MS have confirmed the homeostatic role of gut–brain axis in the host [107]. The present literature review was intended to evaluate the regulatory effects and the therapeutic impacts of probiotic strains on immune system responses in MS patients.

6.4.2 Cross-Talk of Multiple Sclerosis and Microbiota

Several cross-sectional studies have demonstrated that an altered gut microbiota composition is associated with some types of neurological and autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, Parkinson's disease, MS, and EAE, [108, 109]. Owing to the intimate relationship between commensal microbes and the immune system, it is not surprising that an aberrant gut microbiome plays a crucial role in dysregulation of immune responses and immunological tolerance and subsequently triggers and/or exacerbates the spontaneous development of EAE [110]. Much evidence has accumulated to show the importance of the microbiota in regulation of the development and/or function of different types of immune cells, especially with respect to the balance between potentially pro-inflammatory cells, Th1 and Th17 cells, and anti-inflammatory T cells, Th2, and Tregs, which represent a potential role of dysbiosis in autoimmune and inflammatory diseases [111, 112]. A key mechanism by which commensal microbiota affects the homeostasis of immune cell populations in lamina propria involves stimulation of innate immunity by microbial "pattern recognition receptors" [112]. Various microorganisms have been found which have been implicated in the development and/or maintenance of the gut immune system in MS. Some microorganisms seem to facilitate beneficial immune responses while others promote harmful ones [10]. In the case of human autoimmune diseases, a comparison between individuals afflicted with MS and healthy controls showed that MS patient microbiota populations were different compared in gut microbiota, which could mean that this is a risk factor in disease exacerbation [10, 109]. Supportive of this postulate, the dysbiosis found in patients suffering with RRMS can be a result of either increased levels of *Mycoplana, Pseudomonas, Blautia, Haemophilus* and *Dorea genera*, or decreased levels of *Parabacteroides, Adlercreutzia*, and *Prevotella* [113]. Furthermore, by comparing the sample patients and healthy controls, a decrease has been found in *Bacteroides*, *Prevotella*, *Anaerostipes*, *Faecalibacterium*, and *Clostridia XIVa* and *IV* clusters were observed [10], as well as an increase in the proportions of *Actinobacteria*, *Bifidobacterium Akkermansiamuciniphila*, *Mycoplasma*, *Acinetobactercalcoaceticus* in the gut microbiota of MS patients [10, 109, 114, 115]. Notably, a shorter time to relapse was associated with absence of *Fusobacteria*, as well as with the presence of the *Archaea Euryarchaeota* and an increased abundance trend of *Firmicutes* and *Euryarchaeotawere* [116]. Collectively, a rise in incidence and prevalence rate of MS in developed countries has been attributed to altered profiles of the intestinal bacterial flora.

Another mechanism suggested through which the gut microbiota can play a central role in MS is the bidirectional microbiota–gut–brain axis that transfers signals between the gastrointestinal system and the CNS. It is known that a dysregulated microbiota homeostasis is associated with several CNS developmental problems, confirming the effect of the gut commensal microbiota composition and the development and function of the CNS [110, 117]. In addition, human studies have pointed out that an increase of intestinal barrier and BBB permeability, caused by bacterial dysbiosis, can initiate an immune-inflammatory response in MS [10, 28]. This was supported by a result which showed that the

absence of microbiota might induce disruption of BBB tight connections and thus elevate BBB permeability [118]. Interestingly, a number of relevant processes are constantly controlled by gut microbiota such as the maturation and function of microglia [119], the limitation of astrocyte pathogenicity, stimulation of microglia, and expression of myelin genes [10].

6.4.3 Modulatory Effects of Tolerogenic Probiotics on CNS

The CNS is a privileged site, which must be protected from immune responses and immune cells. Therefore, any strategy or treatment that can help this security is crucial. Probiotics are able to protect the CNS barrier by different indirect mechanisms. One study showed that a mixture of three probiotic Lactobacilli on EAE mice decreased inflammation in the CNS following reduced autoreactive T cell responses. Also, clinical scores were significantly reduced after 20 days of probiotics therapy [90]. Treatment of female C57BL/6 EAE mice with two tolerogenic *lactobacillus* strains showed reduction in clinical scores, delays in the time of disease onset, and infiltration of mononuclear cells into the CNS [120]. A study on the EAE rat model colonized with *Bifidobacteriumanimalis* showed that the severity of EAE was delayed [121]. Oral administration of *P. acidilactici R037*, as a prophylactic or treatment, could suppress severity of disease in the EAE model [122]. In addition, prevention in the development of experimental autoimmune encephalomyelitis was observed in C57BL/6 mice after administration of a lipopolysaccharide (LPS)-free Hsp65 producing recombinant *Lactococcuslactis* strain was found to reduce infiltration of inflammatory cells and injury signs in the CNS [123]. In another study, it was shown that in MS patients and healthy people, the gut microbiota is different which could potentially affect the disease course. In fact, distinct microbial flora in RRMS patients compared to healthy people altered the disease course and susceptibility to MS [124]. It has been shown that TGF-α as inhibitory and VEGF-β as promoting factors of inflammation and cell damage are significantly associated with gut microbiota, and any change or breakdown in this flora population leads to excessive inflammation and progression of disease [125]. Some studies showed that the gut microbiota could regulate the integrity of the BBB, and breakdown in normal gut microbiota of germ-free mice caused the elevated permeability [126]. Furthermore, a change in gut microbiota by antibiotic therapy in EAE mice showed that the altered population of flora led to decreased severity and progression of MS [127].

6.4.4 Modulatory Effects of Tolerogenic Probiotics on Treg Cells

One of the most important strategies for controlling and treating autoimmune diseases with increased uncontrolled immune responses focuses on increasing Treg cells and related anti-inflammatory cytokines to restore homeostasis of immune responses. Tolerogenic probiotics could affect immune cells through unknown mechanism and increase the shift towards Treg cells. In vitro and in vivo studies showed that tolerogenic bacteria could reduce inflammation and control the Th1, Th17/Treg axis. An in vivo study in EAE mice showed that the mixture of three probiotic Lactobacilli (*L. plantarum DSM 15312*, *L. paracasei DSM 13434,* and *DSM 15313*) could induce $CD4^+CD25^+Foxp3^+$ Treg cells in mesenteric lymph nodes (MLN), as well as TGF-β1 in serum, and IL-10 in MLN, spleen and blood [90]. The colonization of EAE mice with *Bacteroidesfragilis* decreased the symptoms in mice through induction of $Foxp3^+$ Treg cell differentiation [128]. *Lactobacillus plantarum A7* and *Bifidobacterium animalis PTCC 1631* strain, as tolerogenic microbiota, have been shown to increase anti-inflammatory cytokines, such as IL-10 and TGF-β, along with reduction of T cell prolifera-

tion in spleen and lymph nodes of a C57BL/6 EAE female mouse model separately and in combined treatment [120].

Colonization of mice by *Pediococcusacidilactici R037* provided a significant elevation in the number of $CD4^+$ T cells in both spleen and MLN. Administration of *R037* showed that it had beneficial effects on EAE through induction of FOXP3-IL10-producing Tr1 cells [122]. In an in vivo study, consumption of Hsp65-producing *Lactococcuslactis* by C57BL/6 mice showed significant elevation in the number of $CD4^+$ $FOXP3^+$ natural-Treg and inducible-Treg (also $CD4^+$ LAP^+ Treg) in spinal cord, spleen, and MLN. The study showed that CD4+ LAP+ Treg cells play an effective role in controlling the EAE [123]. Anti-inflammatory responses were also observed following cell culture of lymph node and spleen cells through increased IL-10 and reduced IL-17 production [123]. In an EAE mouse model, colonization of germ-free mice by gut microbiota from MS patient was shown to reestablish MS susceptibility through reduction in function and proportion of Treg cells and also decreased IL-10 levels [129, 130]. Oral administration of purified polysaccharide A derived from *B. fragilis* could decrease inflammation in EAE mice and may serve as a preventive and therapeutic tool by induction of Treg and IL10-producing cells [131]. *Prevotella histicola* use in a transgenic mouse model could reduce the severity of disease through elevation of the number of tolerogenic cells, such as Treg ($CD4^+FoxP3^+$), DCs, and macrophages [132]. In a human study, MS patients fed a probiotic cocktail (*Bifidobacterium, Lactobacillus,* and *Streptococcus*) daily for 2 months showed a significant reduction in the number of inflammatory monocytes and decreased CD80 and HLA-DR expression on classical monocytes and DCs, when compared with control group. In the control group, the administration of the probiotic cocktail showed reduced expression of HLA.DQA1 and HLA. DPB1 as MS risk alleles [132].

6.4.5 Modulatory Effects of Tolerogenic Probiotics on Th1 and Th17

Th1 and Th17 cells are involved in the pathogenesis of MS, with the immunopathology occurring through this inflammatory axis. Therefore, many approaches have targeted reduction of the inflammation of this autoimmune branch, and probiotics are able to decrease the inflammatory activity of Th1 and Th17 cells. In an in vitro study, the influence of segmented filamentous bacterium (SFB) on Th17 cell differentiation demonstrated that colonization of mice with a single commensal microbe induced Th17 cells and production of both IL-22 and IL-17 in the intestinal *lamina propria*. This suggested that the microbiota of MS patients has a pro-inflammatory effect [133]. The suppressive effects of *L. plantarum DSM 15312*, *L. paracasei DSM 13434*, and *DSM 15313* were observed in an EAE mouse model, which could attenuate the pro-inflammatory cytokine profile of the Th1 and Th17 pathway [90]. This probiotic mixture reduced IL17, TNF-α and IFN-γ as a pro-inflammatory cytokines and induced the secretion of anti-inflammatory cytokines such as TGF-β1, IL10, and IL4 in EAE mice [90]. A reduction of some pro-inflammatory cytokines (IL17, IFN-γ, and IL6 along with T cell proliferation) was observed in EAE mice administered two-*lactobacillus* strains, especially when these were given as a mixture [120]. A study on the changes in intestinal microbial population showed that antibiotics could reduce *Lactobacillusmurinus* and *Bacteroides fragilis* and increase *Bacteroides thetaiotaomicron*, which led to suppression of EAE development by reducing iNKT cells, Th17 cells, and inflammatory cytokines, such as IFN-γ, TNF-α, IL-6, and IL-17 [134]. Probiotic administration could induce peripheral anti-inflammatory responses through decreased CD80 expression on monocytes, as well as decreased frequency of inflammatory monocytes, and HLA-DR expression on DCs [135]. *P.*

histicola could modulate the severity of inflammatory cytokines from the Th1 and Th17 pathway in EAE mice by effects on systemic immune responses [132]. In an in vivo study, antibiotic therapy in EAE mice altered the intestinal flora, which was associated with reduction of inflammatory cytokines such as IL17, IFN-γ, IL6, macrophage inflammatory protein (MIP) and monocyte chemoattractant protein (MCP), and increased IL-10 levels, as well as alleviation of the disease severity [136]. Administration of *Helveticus* in EAE mice significantly reduced pathogenic Th17 cells in the spinal cord. In addition, in a prophylactic mode of therapy, this *Lactobacillus* could reduce the ratio of Th17/$CD4^+$ T cells in inguinal lymph nodes before the onset of disease. Also, administration of *Lactobacillushelveticus* reduced production of IL-6 cytokines in in vitro therefore indirectly affected Th17 linage development [137]. Combination usage of *Bifidobacteriumanimalis* and *Lactobacillusplantarum* in a mouse model ameliorated severity of disease and clinical scores through inhibition in the development of Th1 and Th17 linage cells, via an increase in the frequency of $CD4^+CD25^+Foxp3^+$-T cells (Treg) in spleen and lymph nodes [120]. In MS, different inflammatory responses of T cells are responsible in the outcome and progression of the disease, but the roles of autoreactive B cells and autoantibodies are important in control of the time of onset and the disease severity. Induction of the Th2 linage could lead to secretion of cytokines that contribute to the expansion of autoantibody production and, however, could modulate the Th1/Th2 axis inversely. It was found that the effect of tryptophan on gut microbiota could reduce differentiation of Th2 and modulate immune responses [138]. Also, the effect of *Lactobacillus casei* in an autoimmune and allergic mouse model revealed that probiotic administration could prevent inflammation and modulate Th2 responses, and thereby decrease production of IgE and autoantibodies [139]. However, the effect of probiotics on modulation of Th2 immune responses in MS disease requires further study.

6.5 Concluding Remarks

MS is a complicated autoimmune disease with neuro-pathological and immuno-pathological responses that persistently destroy myelin, as an auto-antigen, in the spinal cord and CNS. Tissue damage in MS patients occurs mainly due to excessive activated T cell responses, especially Th1, Th17, and dysregulated Treg cells, and release of their related pro-inflammatory and inflammatory cytokines, such as IFN-ϒ, TNF-α, IL-6, IL-17, and low levels of IL-10 and TGF-β. Various studies have shown the role of probiotics on diverse aspects of innate and acquired immune cells. Tolerogenic probiotic restores the balance of gut–brain axis and the Th1/17–Treg axis through different known and unknown mechanisms. In fact, tolerogenic probiotics alter innate immune cells (especially DCs) and shift the direction of the response towards tolerance. Also, these probiotics could change the microbiota population, prevent the differentiation and expansion of autoreactive T cells (Th1 and Th17), and reduce the levels of IFN-ϒ, TNF-α, and IL-17. However, tolerogenic probiotics could help to maintain tolerance and hemostasis of immune response through restoring the Treg/Th1/Th17 balance. Increased numbers of $CD4^+$, $CD25^+$, $FOXP3^+$ regulatory T cells and elevated level of IL-10 and TGF-β were found to be associated with tolerogenic roles of probiotics, through decreased inflammatory cytokines and T cell branch expansion, leading to prevention of over-activated autoreactive T Cells in the CNS (Table 6.2). Due to the ease of use and the good safety and regulatory properties, tolerogenic probiotics can be used as a complimentary drug in suffering MS patients. However, these categories of probiotics must be studied in combination with cohort studies for more effective evaluation.

Conflicts of Interest The authors declare that there are no conflicts of interest.

Table 6.2 Results of different tolerogenic probiotics effects on modulation of immune responses in human and animal model

Probiotics strain	In vivo/in vitro model	Probiotic effect on				Description	Ref
		CNS	Th1	Th17	Treg		
L. plantarum DSM 15312, L. paracasei DSM 13434 and *DSM 15313*	EAE mice	*	*	*	*	– Reduction of CNS inflammation and clinical score – Indication of CD4 + CD25 + Foxp3+ regulatory T cells (Tregs), IL10 and TGF-β1 – Decrease of Th1 and Th17 pro-inflammatory cytokine profile (IL17, TNF-α, and IFN-γ)	[123]
L. plantarum A7 and *B.animalisPTCC 1631*	EAE mice	*	*	*	*	– Reduction in clinical score and infiltration of mononuclear cells into the CNS – Delay in the time of disease onset – Reduction of T cell proliferation – Decrease of IL17, IFN-γ, and IL6 – Increase of IL10 and TGF-β	[124]
Bifidobacterium animalis	EAE mice	*				– Delay in severity of EAE	[125]
P. Acidilactici R037	EAE mice	*			*	– Suppression of disease severity – Induction of FOXP3- IL10 – Producing Tr1 cells	[126]
Recombinant *Lactococcus lactis*	EAE mice	*		*	*	– Reduction of inflammatory cells infiltration and injury signs in the CNS – Increase of n-Treg, i-Treg, and CD4+ LAP+ Treg – Increase of IL10 and decease of IL17	[127]
Gut microbiota	MS patients, EAE mice	*				Change in disease course and susceptibility to MS	[128, 131]
Gut microbiota	MS patients	*				– Effect on TGF-α as inhibitory and VEGF-β as promoting factors of inflammation and cell damage in MS disease	[129]
Gut microbiota	Germ-free mice	*				– Regulation of blood-brain barrier (BBB) integrity	[130]
Bacteroides fragilis	EAE mice				*	– Induce differentiation of Foxp3+ Treg cell	[132]
MS patients gut microbiota	EAE mice				*	– Reestablish MS susceptibility – Decrease of IL10 and Treg	[133, 134]
B. fragilis	EAE mice				*	– Induction of Treg and IL10-producing cells	[135]
Prevotella histicola	Transgenic mice				*	– Increase the frequency of Treg (CD4 + FoxP3+) and tolerogenic cell (DC and macrophage)	[136]
Bifidobacterium, Lactobacillus, and *Streptococcus*	MS patients, healthy control			*	*	– In patients: Decrease the frequency of inflammatory monocytes, decrease of CD80 and HLA-DR expression on classical monocytes and DC – In healthy individual: Decrease expression of HLA-DQA1 and HLA. DPB1(MS risk allele)	[136]

(continued)

Table 6.2 (continued)

Probiotics strain	In vivo/in vitro model	Probiotic effect on				Description	Ref
		CNS	Th1	Th17	Treg		
Bacteroides thetaiotaomicron	EAE mice		*	*		– Decrease of Th17 cells and inflammatory cytokine such as IFN-γ, TNF-α, IL-6, and IL-17	[138]
Histicola	EAE mice		*	*		– Modulate the inflammatory cytokines of Th1 and Th17	[136]
Lactobacillus helveticus	EAE mice			*		– Reduction of pathogenic Th17 cells – Reduction the ratio of Th17/CD4+ T cells in inguinal lymph nodes	[137]

L Lactobacillus, *B Bifidobacterium*, *CNS* central nervous system, *EAE* experimental autoimmune encephalomyelitis, *MS* multiple sclerosis

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7 Multifaceted Roles of Long Non-coding RNAs in Head and Neck Cancer

Leslie Duncan, Chloe Shay, and Yong Teng

Abstract

The majority of RNA transcripts are non-coding RNA (ncRNA) transcripts with lengths exceeding 200 nucleotides that are not translated into protein. Unlike microRNAs (miRNAs), long ncRNAs (lncRNAs) are not confined to a single mechanism of action but have a large and diverse role in biological processes as they can function as transcription regulators, decoys, scaffolds, and enhancer RNAs. Currently, many lncRNA molecules are under investigation for their role in tumorigenesis, metastasis, and prognosis in different types of cancer. This review not only summarizes the characteristics and functions of lncRNAs but also discusses the therapeutic implications and applications of lncRNAs with roles associated with head and neck cancer. Our aim is to pinpoint the potential way to perturb specific lncRNAs for future therapeutic use.

Keywords

lncRNA · Roles and functions · Head and neck cancer

Abbreviations

BANCR	BRAF-activated non-coding ribonucleic acid
EMT	Epithelial-mesenchymal transitions
ERK	Extracellular signal-regulated kinase
H19	H19 imprinted maternally expressed transcript
HNC	Head and neck cancer
HNGA1	Head and neck squamous cell carcinoma glycolysis-associated 1
HNSCC	Head and neck squamous cell carcinoma
HOTAIR	HOX transcript antisense ribonucleic acid

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HuR	Human antigen R
lncRNA	Long non-coding RNA
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MAP	Mitogen-activated protein
MEK	Mitogen-activated protein kinase
miRNA	MicroRNA
mRNA	Messenger RNA
NAMA	Mitogen-activated protein kinase pathway and growth arrest
ncRNA	Non-coding RNA
NKILA	NF-κB interacting lncRNA
Raf	Rapidly accelerated fibrosarcoma
rRNA	Ribosomal RNA
sncRNA	Small non-coding RNA
tRNA	Transfer RNA

7.1 Introduction

The human body is a large and complex system that needs a precise set of directions to function properly. The genetic code that manages the body comes from DNA, and the process to make and replicate DNA is intense and complicated, and made up of many molecular stages. A key molecule used in this process is RNA, of which there are many different types. The three main types of RNA are messenger (mRNA), transfer (tRNA), and ribosomal RNA (rRNA). In a process known as transcription, DNA is transcribed into mRNA. The mRNA is then translated using tRNAs to make amino acid sequences known as peptide chains. Some genes, however, do not code for proteins. These genes result in a transcript known as a non-coding RNA (ncRNA). The ncRNAs are broken into two classes known as small ncRNAs (sncRNAs) and long ncRNAs (lncRNAs). Further subdivision of sncRNAs results in more classes of ncRNAs with one of the main types being the microRNAs (miRNAs). If the RNA is made up of 20–25 nucleotides, it is considered as miRNA. If the RNA is made up of 200 nucleotides or more, it is considered as a lncRNA [1–5]. In research, miRNAs are currently under intensive study as they are important regulators of the stability and expression of intracellular mRNAs. With many researchers examining miRNAs for possible therapeutic treatments for cancer, this has sparked research on lncRNAs. The function of lncRNAs is not fully understood, but these molecules are known to play a major role in inhibition and activation of many genes. Because previous research has exemplified the importance of ncRNAs, the lncRNA class is currently being studied in connection with human diseases, especially cancer.

7.2 Structure and Characteristics of lncRNAs

Generally, lncRNA is made up of 200 nucleotides or more and does not encode proteins. More than 98% of the genes transcribed are non-coding [1, 6–11]. While the exact function is unknown, evidence has shown the importance of lncRNA in splicing, imprinting, transcription, translation, cell cycle regulation, and apoptosis. These lncRNA are also suggested to play a part in cancer and other human diseases [12–14]. Structurally, an lncRNA molecule consists of a 3′ polyadenylated tail and a 5′ cap [1, 7]. Due to the abundance of lncRNA transcripts, a classification system has been created. Based on the location and context of the genome, the effect on DNA sequences, the mechanism of functioning, and the target mechanism, lncRNAs can be categorized [1–4, 15]. There are two classifications for the location of the gene called intergenic and intronic. Intergenic refers to an lncRNA that is between two coding regions. Intronic refers to an lncRNA that is transcribed from introns only. Similar to the location, the context also subdivides lncRNA into two categories known as sense and antisense. Sense lncRNAs are transcribed from the sense strand of protein encoding genes and contain exons. These may overlap with part or all of the protein encoding genes. Conversely, antisense lncRNA is transcribed from the antisense strand of protein encoding genes. It has been discovered that lncRNAs are involved in transcriptional regulation, and therefore they are also classified based on how they interact with DNA, known as *cis*- and *trans*-lncRNAs. The *cis*-

lncRNAs help regulate expression of genes that are close by. The *trans*-lncRNAs help regulate expression of genes that are further away. The mechanism of functioning for the lncRNA transcripts falls into three groups including transcriptional regulation, post-transcriptional regulation, and other forms of regulation [1, 16]. Both transcriptional and post- transcriptional regulation can be further subdivided based on how the regulation is occurring. The third classification for the mechanism of functioning is made up of unknowns. There are no subgroups, because there is not enough available information to classify these lncRNAs other than via the mechanisms of functioning. The last group of classification is target mechanisms, and there are four types known as signal, decoy, guide, and scaffold. Signal targeting mechanisms refer to specific expression based on cell type. Decoy mechanisms bind and move different protein targets as their only function. Proteins that are bound and directed to specific target regions are under control of guided target mechanisms. Lastly, proteins that are gathered are done so through scaffold target mechanisms which serve as a central platform. Further details regarding these classifications discussed above are given in Table 7.1. It is important to note that these classifications exist only through current knowledge of the subject and are expected to change as more information is gathered [17].

Table 7.1 Categorization and functions of lncRNAs

Category	Sub-category	Functions
Location and context of the genome	Intergenic lncRNA	Transcribed between coding regions
	Intronic lncRNA Sense lncRNA Antisense lncRNA	Transcribed from introns Transcribed from sense strand Transcribed from antisense strand
The effect on DNA sequences	Cis lncRNA	Regulates genes close
	Trans lncRNA	Regulates genes far
The mechanism of functioning	Transcriptional lncRNA	
	Post-transcriptional lncRNA	
	Other lncRNA	
The target mechanism	Signal lncRNA Decoy lncRNA Guide lncRNA Scaffold lncRNA	Regulates transcription in response to stimuli Binds and moves targets Directs proteins to targets Serves as central platform

7.3 lncRNAs Associated with Tumorigenesis and Development of Head and Neck Cancer (HNC)

With 98.5% of the human genome consisting of ncRNA, of which tens of thousands are of the lncRNA subtype, there are a multitude of studies being conducted on their potential roles. The new found discovery of lncRNA in the initiation and progression [18–22] of HNC has led to the idea of using them as a new treatment that may provide a better prognosis for patients. Due to the abundance of lncRNAs, there are numerous lncRNAs that are known to function in head and neck tumorigenesis, with more being discovered. There are many types of cancers that fall into the HNC category [23, 24]. The most prevalent subset of these is head and neck squamous cell carcinoma (HNSCC). This includes cancers found in the lining of the upper digestive tract including the throat, nose, and mouth regions. These cancers have been found to have a correlation with multiple lncRNAs including HOX transcript antisense RNA (HOTAIR), H19 imprinted maternally expressed transcript (H19), and HNSCC glycolysis-associated 1 (HNGA1) [25, 26]. HOTAIR was seen to be expressed at very high levels in HNSCC when compared to normal tissues [27–29]. It was discovered that a reduction in the HOTAIR levels leads to cell death and slow tumor development. H19 is also seen to be expressed highly in HNSCC and leads to a higher invasive capacity as well as a higher rate of tumor recurrence. HNGA1 is involved in

the process of glycolysis and increases cancer cell proliferation. Regulation of these three lncRNAs may offer a treatment for HNSCC via knockdown of the genes. It has been shown that knockdown of all three of these lncRNAs resulted in poor proliferation, migration, and invasion of HNSCC cells [18, 30–32]. Thyroid cancer has associated lncRNAs as well, partially overlapping with those of HNSCC. For example, HOTAIR has also been seen to function in thyroid cancer along with BRAF-activated ncRNA (BANCR) and non-protein coding RNA, associated with MAP kinase pathway and growth arrest (NAMA). BANCR has been shown to be upregulated in thyroid cancer cells in comparison with healthy tissue, and apoptosis was found to be induced by the reduction of BANCR expression, although there was no effect of this on cell migration [18]. Other evidence shows conflicting data suggesting unknown factors may be involved in the regulation of BANCR expression in thyroid cancer. NAMA, closely related to BANCR, has been found to be downregulated in thyroid cancer. In addition, NAMA expression was found to be stimulated by reduced BRAF expression, inactivation of the MAP pathway, or DNA damage. These changes lead to activation of the proto-oncogene serine/threonine-protein kinase/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway known to regulate cell growth and differentiation [18]. Therefore, the activation of this pathway is a potential target for future treatment of thyroid cancer, and further research in this area should help to confirm this.

7.4 lncRNAs Associated with Invasion and Metastasis of HNC

Metastasis is partially responsible for the relapse and poor prognosis [1] of HNC. Recently the discovery of the role of lncRNAs in mediating metastasis has led to possible future advances for treatment of HNC. Multiple lncRNAs have been studied specifically for their effects on metastasis in HNC including metastasis associated lung adenocarcinoma transcript 1 (MALAT1), HOTAIR, and NF-κB interacting lncRNA (NKILA) [33–36]. It has also been suggested that the lncRNAs could function by working in tandem with their corresponding mRNAs [1]. To understand how lncRNAs aid in the process of metastasis, the mechanisms underlying metastasis must first be understood. Epithelial mesenchymal transitions (EMT) are important for cancer cells to metastasize [37–43]. In this process, epithelial cells transition to resemble the mesenchymal phenotype. This allows the cancer cells to migrate, proliferate, and differentiate into specific tissues and organs since mesenchymal cells do not have a specialized function [41–48]. By traveling through the lymph nodes and bloodstream, these cells choose the best environment for proliferation based on the ability to obtain the best nutrition. MALAT1 has been studied for its role in metastasis based on its ability to aid HNC cells to undergo EMT and invade other tissues [1, 49]. It was seen that when MALAT1 was inhibited, the EMT of HNC cells was altered. One possibility for this change is thought to be the inactivation of β-catenin and NF-κB pathways. These pathways are regulators of EMT, so as they are inhibited, a chain reaction effect is seen in the modification of EMT [1, 33, 36, 49]. In this case, it was also seen that two EMT markers, N-cadherin and vimentin, in MALAT1 were knocked down in HNC cells [1, 33, 36, 49]. Several studies have provided evidence that the knockdown of MALAT1 in HNC could impair migration of the cancer. HOTAIR is another lncRNA that is being studied in its effects of cancer metastasis [1, 35, 50, 51]. Studies have shown several ways by which HOTAIR influences HNC metastasis. Recently it has been indicated that HOTAIR functions in EMT by decreasing E-cadherin levels. Other studies have shown that a knockdown in HOTAIR decreased the invasiveness of HNC significantly. Aside from other regulation responsibilities, HOTAIR has also been suggested to work in a regulatory loop progressing metastasis in correspondence with the RNA-binding protein Hu antigen R (HuR) [52]. HuR is an RNA binding protein that works to stabilize mRNA to better regulate gene expression. Unlike

Table 7.2 Known lncRNAs involved in HNC metastasis

lncRNA	HNC subtypes
HOTAIR	NPC, OSCC, LSCC
H19	NPC, LSCC
MALAT1	NPC
ANRIL	NPC
ROR	NPC
AFAP1-AS1	NPC
LET	NPC
LINC0086	NPC
LOC401317	NPC
PTENP1	OSCC
UCA1	OSCC
FOXCUT	OSCC
FTH1P3	OSCC
TUG1	OSCC
NEAT1	LSCC
PVT1	LSCC
LOC157273	LSCC

HNC head and neck cancer, *NPC* nasopharyngeal carcinoma, *OSCC* oral squamous cell carcinoma, *LSCC* laryngeal squamous cell carcinoma

MALAT1 and HOTAIR, NKILA suppresses metastasis. In addition, NKILA is being studied for its negative regulation of HNC and its function as a poor prognosis indicator [1]. It has been seen that the levels of NKILA are lower in HNC then in unaffected cells and normal tissue. The knockdown of NKILA actually resulted in the increased metastasis of HNC, confirming the negative correlation. These three lncRNAs are not alone in their regulation of metastasis. They are aided by multiple other lncRNAs shown in Table 7.2.

7.5 lncRNAs Associated with Treatment Resistance in HNC

While new therapies are being discovered as possible targets for HNC patients, there is also the concern of maintaining oral function [53]. In order to protect the functionality of patients, numerous therapies have been used in combination including chemotherapy and radiotherapy. The drugs frequently used in both chemotherapy and radiotherapy are known as antineoplastic drugs, which act to inhibit or stop the development of tumors. Combination therapies can lead to what is known as multiple drug resistance (MDR) leading to the resistance to non-structurally related drugs as well. Factors that have been linked to resistance include the ATP-binding cassette transporter (ABC) and cancer stem cells (CSCs). A gene known as *ABCB1*, or *MDR1*, produces P-glycoprotein (P-gp) which is known to be an ABC transporter associated with MDR. P-gp has been known to provide resistance in HNC. In one experiment, cell lines were treated with a drug known as doxorubicin. After treatment with this drug for 3 months, the cells were seen to be resistant. The overexpression of P-gp has been suggested to increase resistance more than 100 times greater than in normal cells [53–56]. While the process causing the expression of P-gp is unknown, recently it was demonstrated that P-gp is transferred between cells via micro-vesicles. However, this occurrence has yet to be seen specifically in HNC. Cancer stem cells aid tumors to maintain growth and recur in patients through renewing and preserving themselves. CSCs are recognized in HNSCC by the increased expression of the cell surface adhesion receptor, CD44. The ability of the cancer stem cells to alter their phenotype shows the relationship to EMT. It is seen that molecules important in the process of EMT are associated with poor prognosis. It has been theorized that the relationship between EMT and CSCs are a critical cause for the resistance to antineoplastic treatments.

7.6 Prognostic Role of lncRNAs in HNC

Some lncRNAs have been found to be associated with the prognosis of HNC. By analyzing a group of HNC patients and creating a model to identify specific lncRNAs, it has been suggested that five individual lncRNAs can be used to predict prognosis of HNSCC patients postoperatively [54]. This model also accounted for the co-expression of genes and lncRNAs, to serve as a final confirmation of prognosis. A risk score was calculated and this showed that as the mortality risk increased so did the risk score. Conversely, as the risk score

increased the expression of the five lncRNAs being studied decreased. The lncRNAs identified to be an indicator of prognosis are RP11-180M15.7, RP11-197N18.2, AC021188.4, RP11-474D1.3, and RP11-347C18.5. Not only are these lncRNAs closely associated with HNSCC but they are also involved in many cellular pathways associated with cancer proliferation.

Other studies showed similar results with more lncRNAs having prognostic value. For example, poor survival was seen in patients with over-expression of RP11-366H4.1, HOTTIP, RP11-865I6.2, and RP11-275N1.1 [55]. These lncRNAs differ from those previously mentioned, in that their over-expression correlates with a poor prognosis. While miRNAs have been studied in the past, they have not been studied in combination with lncRNAs. A risk score was developed for the performance of lncRNA, miRNA, and mRNA. Patients with a high risk score were also seen to have a worse survival than those with a low risk score. Success in predicting lncRNA, miRNA, and mRNA interactions may reveal much needed new information on HNSCC [55].

7.7 Conclusions

HNC is characterized by a poor prognosis due to the late-stage diagnosis and the aggressive nature of this form of cancer [57]. Improvements have been made in medical techniques, although about 50% of HNC diagnoses are still advanced cases. The treatments including surgical procedures, chemotherapy, and radiotherapy have all significantly improved as well. However, the 5-year survival rate of patients with advanced HNC does not reflect the most recent advances in detection and treatment. Increasing studies have demonstrated that lncRNAs play key roles in tumorigenesis and tumor progression, leading to abnormal signaling transduction, immune escape, and cellular metabolic rewiring. By better understanding lncRNAs and applying novel technologies (e.g., a new generation of gene-editing tools and effective tumor-seeking drug delivery systems) to target cancer-associated lncRNAs, there may be more hope for treatment of HNC patients. Currently, the research is still in its early stages and has large barriers to overcome, although great promise has been shown in using lncRNAs as both compelling indicators and targets for HNC.

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8

A Systematic Review on the Genotoxic Effects of Selective Serotonin Reuptake Inhibitors

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Abstract

Depression is a mental disorder and a major public health concern affecting millions of people worldwide. It is a common disorder that has been associated with several medical comorbidities often linked with aging, such as dementia, type II diabetes, cardiovascular and cerebrovascular diseases, as well as metabolic syndrome. There are a variety of medications available for depression treatment. Selective serotonin reuptake inhibitors (SSRIs) are one of the antidepressant drug classes that are most widely used to treat depressive disorders and depressive symptoms in other diseases. Due to many contradictory findings on the adverse effects and toxicities of SSRIs (especially genotoxicities), we reviewed the genotoxic

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effects of these drugs. Based on the guidelines proposed in the PRISMA statement, we performed a systematic review by searching international electronic databases including PubMed, Scopus, Embase, and Web of Science to find the published documents on SSRIs and their genotoxic effects from January 1990 to November 2019. After the removal of 203 duplicate articles, 385 articles were screened and 167 articles met the inclusion criteria and qualified for evaluation of their full texts. After this, 26 articles were appropriate for final review. This revealed that the proportion of genotoxicities was highest for citalopram and fluoxetine, with a smaller proportion for sertraline. Limited documentations showed genotoxic and partial genotoxic effects for paroxetine and escitalopram, respectively. Although a number of studies have found genotoxic effects of SSRIs, there are also some factors including doses, duration of exposure, model of experiments, and the type of technique assay that may affect the results.

Keywords

Antidepressants · SSRIs · DNA damage · Genotoxicity

8.1 Introduction

Depression, as a heterogeneous disorder, usually appears late in life and often co-occurs with serious comorbid medical conditions especially those seen more typically with advanced age such as cardiovascular and cerebrovascular diseases, type II diabetes, stroke, osteoporosis, and neurodegenerative diseases [1]. Selective serotonin reuptake inhibitors (SSRIs) are a class of antidepressant drugs, introduced in the late 1980s and prescribed in the treatment and management of different forms of psychiatric disorders [2]. The five SSRIs, including citalopram, fluvoxamine, fluoxetine, paroxetine, and sertraline, are currently marketed in many countries around the world. SSRIs increase the level of serotonin in the brain by inhibiting the uptake of this neurotransmitter into nerve terminals which relieves depression symptoms [3, 4]. The favorable safety profiles of these drugs support their widespread and long-term use [5]. Among the several adverse effects of SSRIs, genotoxic and carcinogenic reactions are among the most severe. Interactions between toxic agents and DNA can lead to gene mutation, recombination, chromosomal damage, or aneuploidy which, if not properly repaired, may cause different diseases including cancer, malignancies, cardiovascular diseases, and aging [6, 7], as well as alterations in heritable traits and impaired reproductive capacity [8].

For these reasons, it is vital to carefully evaluate the genotoxic risk of SSRI antidepressants [9, 10]. Some experimental studies on bacteria, molds, and mammalian cells have been conducted to evaluate substance-induced genetic damage and their interactions with DNA at low concentrations [11]. Due to widespread application of SSRIs and their controversial effects on genetic material, we designed this review to further assess the DNA toxicity of these compounds. To our knowledge, this is the first systematic review on this topic. Due to extensive consumption of these medications worldwide, the results of this review should receive widespread interest.

8.2 Methods

This review was conducted based on previous published articles on genotoxic effects of SSRIs according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [12].

8.2.1 Literature Search Strategy

Data for this systematic review were obtained through a comprehensive literature search of publications in the electronic databases consisting of PubMed, Scopus, Embase, and Web of Science from January 1990 until November 2019.

The keywords for our database search included "SSRI" and "genotoxicity" or "DNA damage."

8.2.2 Inclusion and Exclusion Criteria

All articles were evaluated by two independent investigators. Only studies written in English were reviewed. At first, the titles and abstracts were screened, and this was followed by full-text screening. The inclusion criteria were all published original articles with the abovementioned keywords, sufficient information, and studies with full-text articles. Additionally, studies with poor quality or without consistent, non-related articles, review articles, case reports, editorials, conference papers, letters to the editor, and non-related abstracts were excluded (Fig. 8.1).

8.2.3 Data Extraction

For a systematic review of literature, critical information including authors' names, models of experiments, technique assays, drug names, and outcomes were independently extracted from each study.

8.3 Results

8.3.1 Literature Search

The detailed explanation process of study selection is illustrated in Fig. 8.1. Initially, the searching of the above databases between 1990 and 2019 retrieved 755 records and 203 duplicate articles were removed. After screening all articles, 167 articles met the inclusion criteria and qualified for full-text evaluation. Subsequently, studies with missing data or those with inconsistent inclusion criteria were excluded. Finally, 26 of these articles were considered appropriate and reviewed in detail. A summary of the main characteristics of the qualified studies included in this review is given in Table 8.1.

8.3.2 Citalopram

In a study performed by Franco and colleagues, the possible genotoxic effects of citalopram in *Aspergillus nidulans* were examined. The results showed that nontoxic concentrations of citalopram (50, 75 and 100 μmol /L) could induce a recombinogenic effect in *A. nidulans* [13]. In the treated group, the homozygotization index (HI) rates were higher than 2.0 and significantly different compared to control ones. The authors claimed that the recombinogenic potential of citalopram may be related to the recombinational repair. In mice given 12 or 24 mg/kg of citalopram orally for 7 days, significant DNA strand breaking and micronuclei formation were observed. Moreover, the fluorescence in situ hybridization (FISH) analysis showed aneugenic and clastogenic effects on somatic cells [14]. Gürbüzel et al. employed the somatic mutation and recombination test (SMART) to observe the genotoxicity of citalopram and sertraline in two *Drosophila melanogaster* strains. SMART is a sensitive in vivo assay based on the loss of heterozygosity, which may happen through different mechanisms such as mitotic recombination. Although citalopram showed a genotoxic effect in *Drosophila*, sertraline did not show such an effect [15]. Attia and Bakheet tested the genotoxic effects of citalopram at multiple doses (6, 12, and 24 mg/kg/day, the recommended human doses) on germ cells of male mice. Their finding of increased sperm DNA strand breaks and the frequency of aberrant primary spermatocytes at the 12 and the 24 mg/kg/day dose suggested that citalopram is genotoxic after long-term treatment in germ cells of mice [9]. In addition, in 2017 Ilgin et al. showed a significant increase (44.8%) in sperm DNA damage in citalopram-administrated rats by using the single-cell gel electrophoresis comet assay® [16]. Magni and coworkers assessed the genotoxicity of two antidepressants frequently found in the aquatic

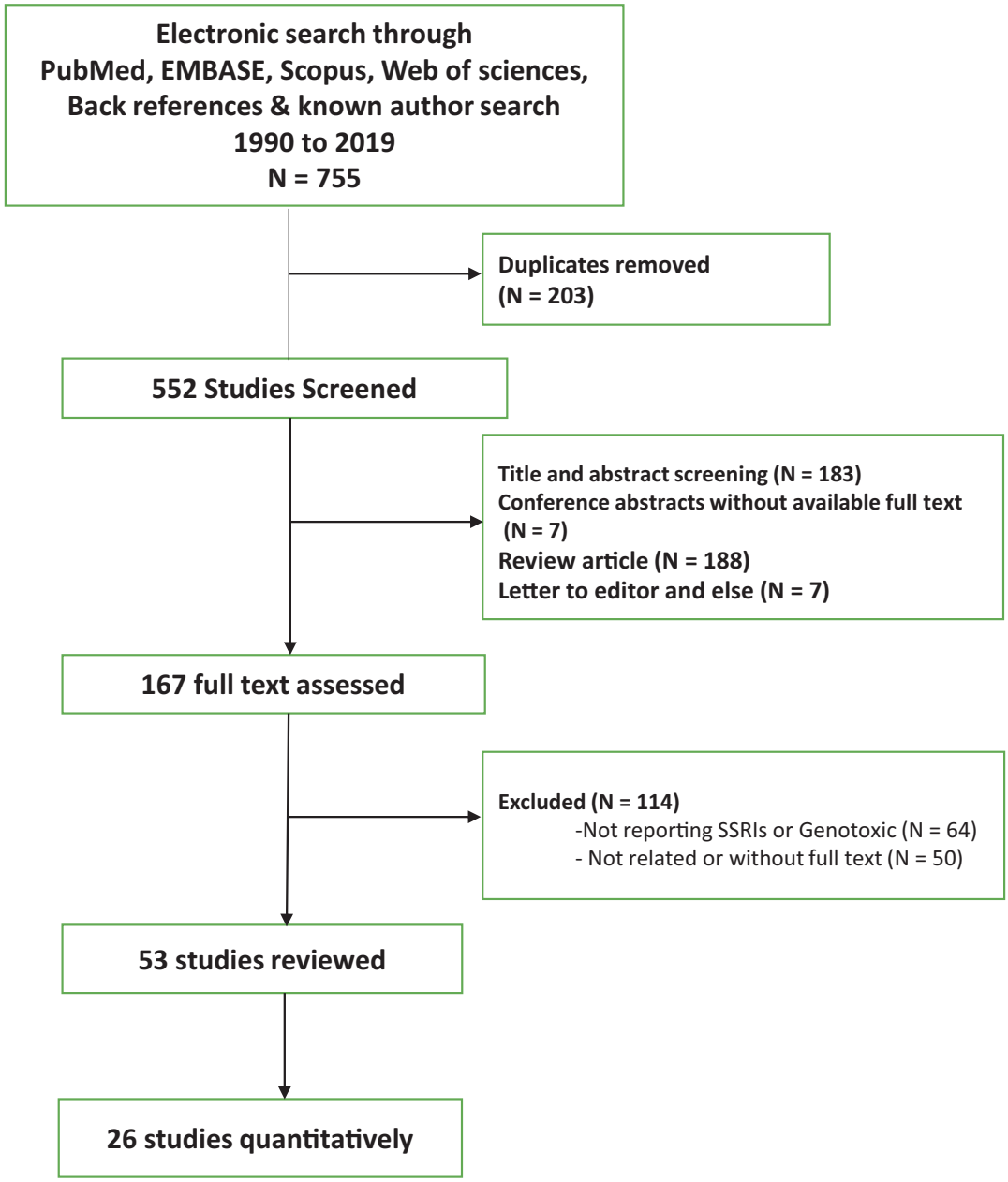

Fig. 8.1 Diagram showing the study selection protocol

environment (fluoxetine and citalopram) on *Dreissena polymorpha* using single-cell gel electrophoresis assay, DNA diffusion assay, and micronucleus (MN) testing. In this study, *polymorpha* specimens were exposed to fluoxetine and citalopram alone and in combination at the environmental concentration of 500 ng/L for 14 days. The obtained results revealed that fluoxetine, citalopram, and their combination did not cause evident damage to this organism [17].

Table 8.1 Details of included studies

Drugs names	Authors' names	Models	Technique assay	Outcomes
Citalopram	Franco et al. (2010) [49]	*Aspergillus nidulans*	Mitotic recombination	Genotoxic
	Gürbüzel et al. (2012) [50]	*Drosophila melanogaster*	Somatic mutation and recombination	Genotoxic (somatic mutagen)
	Magni et al. (2016) [17]	*Dreissena polymorpha*	Comet assay	Genotoxic
	Attia et al. (2013) [14]	In vivo	Comet assay	Genotoxic
	Attia et al. (2013) [9]	In vivo (germ cell-sperm)	Comet assay	Genotoxic
	Ilgin et al. (2017) [16]	Human (sperm)	Comet assay	Genotoxic
	Ahmadimanesh et al. (2019) [51]	Human	Comet assay	Non-genotoxic
Escitalopram	Cobanoglu et al. (2018) [18]	In vitro	SCE/comet assay/ micronucleus	Potentially genotoxic
Fluoxetine	Jin et al. (2018) [52]	E.coli	Ames	Mutagen
	Maranho et al. (2015) [53]	Ampelisca brevicornis	Alkaline precipitation assay	Genotoxic
	Magni et al. (2016) [17]	*Dreissena polymorpha*	Comet assay	Genotoxic
	Maranho et al. (2014) [54]	*Polychaete Hediste diversicolor*	DNA damage strand	Genotoxic
	Cortez et al. (2019) [55]	*Perna perna*	Comet assay	Genotoxic
	Ofoegbu et al. (2019) [23]	*Schmidtea mediterranea*	Comet assay	Genotoxic
	Slamon et al. (2001) [19]	In vitro	Comet assay	Genotoxic
	Lacaze et al. (2015) [28]	In vitro	Comet assay	Genotoxic
	Wieczerzak et al. (2018) [56]	In vitro	Comet assay	Genotoxic
	Djordjevic et al. (2011) [27]	In vivo	DNA fragment assay	Genotoxic
	Alzahrani et al. (2012) [20]	In vivo	SCE (bone marrow cells)	Genotoxic
	Dusman et al. (2014) [21]	In vivo/plant	Chromosome aberration	Non genotoxic or mutagen
	Elmorsy et al. (2017) [32]	In vivo	Comet assay	Genotoxic
	Safarinejad. (2008) [26]	Human (sperm)	SCSA	Genotoxic
Paroxetine	Lacaze et al. (2015) [28]	In vitro	Comet assay	Genotoxic
	Tanrikut et al. (2010) [25]	Human (sperm)	Tunel assay	Genotoxic
Sertraline	Davies et al. (1998) [57]	In vivo/In vitro	Ames	Non-genotoxic
	Gürbüzel et al. (2012) [50]	*Drosophila melanogaster*	Somatic mutation and recombination	Non-genotoxic
	Elmorsy et al. (2017) [32]	In vivo	Comet assay	Genotoxic
	Battal et al. (2013) [58]	In vivo	Micronucleus/comet assay	Non- genotoxic
	Atli et al. (2017) [59]	Human (sperm)	Comet assay	Genotoxic
	Bozkurt et al. (2004) [60]	Human	CA/SCE/HFC	Genotoxic
	Ahmadimanesh et al (2019) [51]	Human	Comet assay	Non- genotoxic

8.3.3 Escitalopram

Escitalopram, a member of selective serotonin reuptake inhibitors, increases significantly the SCE at 5 and 10 mg/mL. No statistically significant increase was reported by authors regarding DNA damage or MN formation [18].

8.3.4 Fluoxetine

Slamon et al. assessed the effects of acute exposure of fluoxetine on DNA damage in C6 glioma cells using an alkaline comet assay. They showed that the comet tail moment values increased in C6 cells with increasing concentrations of antidepressant drugs. Exposure to fluoxetine at 1 μM or 5 μM for 24 h showed the most DNA damage [19]. Alzahrani et al. assessed the effects of fluoxetine on genotoxic damage in somatic and germ cells. Sister chromatid exchanges (SCEs) and sperm abnormalities in mice were evaluated. The results indicated that oral administration of fluoxetine at 2.6, 7.8, and 13.0 mg/kg concentrations for 35 days increased the times of SCE and enhanced sperm abnormalities. Moreover, a dose-dependent reduction in sperm count and motility was observed. As a result, fluoxetine showed an in vivo genotoxic effect [20]. Using two model systems consisting of *Allium cepa* L. root meristem cells and Wistar rat bone marrow cells, Düsman et al. studied the cytotoxicity and mutagenicity of fluoxetine hydrochloride. They showed fluoxetine with or without concomitant vitamin A or C treatment was only cytotoxic to *A. cepa* cells. Wistar rats treated intraperitoneally or by oral gavage demonstrated no cytotoxic or mutagenic potential of the drug [21]. For the first time, the genotoxicity of fluoxetine in varying environmental conditions on the human adenocarcinoma cancer HT29 cell line was examined by Wieczerzak and colleagues. They found that environmental conditions such as low pH led to a synergistic increase in the DNA damage caused by fluoxetine [22]. The genotoxic effects of fluoxetine in the planarian *Schmidtea mediterranea* were tested by Ofoegbua et al., with the results confirming that fluoxetine caused DNA damage [23]. A recent study used an alkaline precipitation assay to examine the cytogenotoxic effects of fluoxetine in the tropical brown mussel *P. perna*. The authors concluded that fluoxetine causes DNA damage in *P. perna* mussels [24]. In another study, semen parameters of 74 fertile men diagnosed with depression and taking SSRIs were compared with those of healthy volunteers. In addition, physical examinations were performed on all participants. Lower sperm counts, lower motility, more abnormal sperm morphology, as well as more DNA damage were found in men taking SSRIs. Another study revealed abnormal DNA fragmentation in sperm in a significant proportion of human patients caused by paroxetine [25]. Safarinejad used a sperm chromatin structure technique and observed impairment in all semen parameters and enhanced sperm DNA damage in patients who were already receiving citalopram, escitalopram, fluoxetine, paroxetine, and sertraline for more than 6 months [26]. Djordjevic et al. reported augmented apoptotic signaling and DNA fragmentation in male rats after treatment with fluoxetine for 21 days. The authors investigated the possibility that DNA fragmentation under fluoxetine treatment may be due to a greater decrease in Bcl-2 expression than an increase in Bax [27]. In either case, this would lead to a shift in the balance toward increased apoptosis.

8.3.5 Fluvoxamine

No document is available for the genotoxic effect of fluvoxamine.

8.3.6 Paroxetine

Lacaze et al., using the comet assay, studied the effects of fluoxetine and paroxetine on blue mussel (*Mytilus edulis*) hemocytes. It was found that paroxetine and fluoxetine (at a dose of 15 and 10 mg/L, respectively) led to DNA damage genotoxicity, immunotoxicity, and cytotoxicity [28].

8.3.7 Sertraline

In 1998 Davies and Kluwe evaluated the toxic effects of sertraline in rats, mice, rabbits, and dogs using an extensive battery of tests for chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and frequency of SCEs (HFC). While genotoxic assays were negative in treated rats compared with non-treated controls, the number of benign liver tumors was slightly enhanced in sertraline-treated male mice. Consequently, the results of these studies supported the use of sertraline in humans with a low risk [29]. Bozkurt and colleagues studied the genotoxic effect of sertraline and did not detect a statistically significant alteration between SCE and CA frequency tests and the levels of HFC in studied peripheral lymphocytes of sertraline-treated (50 mg daily for 10 months to 1 year) and non-treated patient groups [30]. Battle et al. assessed the genotoxic potential of sertraline in peripheral blood lymphocytes (PBLs) using the alkaline comet assay and cytokinesis-block micronucleus (CBMN) assay. Male Wistar albino rats were exposed to sertraline at different doses (10, 40, and 80 mg/kg) under acute and chronic conditions. The results showed no significant difference between the sertraline-treated group and the control group. However, acute versus chronic administration of sertraline showed more DNA damage. Moreover, chronic and high-dose acute administration of sertraline to the rats revealed increased MN frequency in CBMN assay. Consequently, based on the findings using the CBMN assay, chronic administration of sertraline might affect some mechanisms of cell division [31]. Elmorsy and coworkers assessed the effects of fluoxetine and sertraline in a concentration range of 0.1–100 μM on primary endothelial cells of the blood-brain barrier in rats. Analysis of the data from the comet assays revealed that both drugs were genotoxic [32]. In another study, Atli and colleagues using the sperm comet assay clarified that consumption of sertraline (5, 10, and 20 mg/kg/day for 28 days) increased abnormal sperm morphology and DNA damage in male rats [33].

8.4 Discussion

Oxidative stress is considered as one of the main psychopathological mechanisms of depression [34–36]. This form of stress results from changes in the oxidant/antioxidant balance, which cause telomere instability, cell cycle arrest, and apoptosis. It has been documented that DNA or RNA damage resulting from oxidative stress is one of the earliest events in depression [37–40]. However, the results of some studies have led to the idea that antidepressants could have antioxidant properties through the suppression of mediators involved in oxidation reactions [41]. Also, Czarny et al. suggested that SSRIs have anti-inflammatory properties [42]. In agreement with these findings, Battal and coworkers revealed that sertraline treatment did not cause DNA damage and dose adjustment was important in the prevention of malignancy prognosis [31]. However, Lindqvist et al. suggested that upregulation of oxidative stress markers is associated with either no response or a poor response to antidepressant treatment. Since elevated levels of oxidative markers are correlated with increased DNA damage, treatment with SSRIs could possibly be associated with DNA damage or genotoxicity [43]. These effects are more prominent when these drugs are administered in pregnancy or for an extended period of time, since they are occasionally prescribed for more than 6 months or the therapeutic regimen is frequently repeated [44, 45]. Cardiovascular and sexual dysfunction, hyponatremia, mammary cancer and pheochromocytoma, as well as DNA damage are possible side effects of these drugs [46]. The present survey has been performed to evaluate the genotoxic potential of SSRI drugs. In the current review, we have provided comprehensive data to demonstrate a clearer outline of genotoxic-carcinogenic side effects of SSRIs. The list of experimental analyses on genotoxicity of SSRIs that were evaluated in this research are the bacterial mutation, alkaline comet, the SCE, HFC, CA, sperm chromatin structure, SMART, sperm DNA integrity, Comet-FISH studies, MN, CBMN, single-cell gel electrophoresis, and sperm DNA 8-hydroxy-

20-deoxyguanosine (8-OHdG) assays. Among those assays described in the following section, the most commonly used method for evaluation of genotoxicity of SSRIs is the comet assay.

The main mechanism involved in the genotoxicity induced by SSRI may be linked to instability in the oxidant/antioxidant balance. Increased reactive oxygen species (ROS) production and oxidative metabolism of lipids, decreasing antioxidant enzyme levels, and generation of antidepressant radicals are the most likely responsible factors in the genotoxic properties of SSRIs [16, 28, 47, 48]. According to the literature, the proportion of genotoxicity induced by citalopram and fluoxetine was 85.7% and 92.86%, respectively, while the proportion for sertraline was 42.8%. There have been only a small number of studies on paroxetine and escitalopram that revealed the genotoxic and semi-genotoxic effects of these drugs, respectively. Hence, more comprehensive studies on these medications are still needed. Since these studies are rarely performed on human subjects, an accurate genotoxic effect of these drugs is unclear. Additionally, the data available on sertraline-induced DNA damage is also controversial.

8.5 Conclusions

To the best of our knowledge, the current study is the first systematic review to examine the genotoxic effect of SSRI antidepressants. This study is of valuable interest since SSRI antidepressants are widely consumed. Due to limited data on human subjects or clinical trials and no available data on some SSRIs, the precise genotoxic effects of these medications are still equivocal. Although many studies investigated the genotoxic effects of SSRIs (fluoxetine and citalopram especially), many factors including doses, duration of exposure, model of experiments, and the type of technique assay might have affected the results.

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Conflict of Interests None.

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9 Toward a New Era for the Management of Circulating Tumor Cells

Marisol Miranda Galvis, Celeste Sánchez Romero, Thiago Oliveira Bueno, and Yong Teng

Abstract

Circulating tumor cells (CTCs) are malignant cells separate from primary tumors, which can migrate through the peripheral blood, colonize other tissues, and lead to the formation of metastases. The first description of CTCs dates back to 1869 when Thomas Ashworth recognized malignant cells similar to the ones of the primary tumor in the blood vessels of an autopsied patient with metastatic cancer. Currently, CTCs have been identified in various types of cancer and have been recognized for their clinical value in the prediction of prognosis, diagnosis of minimal residual diseases, assessment of tumor sensitivity to anticancer drugs, and personalization of therapies. However, research about these topics has several limitations, principally the rarity of CTCs in bloodstream and their heterogeneous characteristics, which makes detection and isolation difficult. As a result of these limitations, current studies are focused on improvement of isolation and characterization techniques to achieve better sensitivity in clinical applications. This review covers the methods of CTC isolation and detection and current research progression on CTC in different cancer types. The clinical applications, limitations, and perspectives of CTCs are also discussed.

Keywords

Circulating tumor cells · Cancer · Liquid biopsy · Micrometastases · Metastasis

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9.1 Introduction

Mechanisms of cancer progression and spreading have been a focus of surgeons and researchers for more than 100 years. These investigations have formed the basis that made possible the recent insights gained in these processes. Circulating tumor cells (CTCs) are malignant cells that disseminate from the primary tumor, circulate in the peripheral blood, and have the potential of colonizing other tissues, eventually leading to metastasis. Metastasis is the principal cause of cancer-related death, and it is accepted that it occurs when the microenvironment shows appropriate conditions for implantation and growth of CTCs in secondary sites, forming tumors in distant organs [1].

CTCs have been isolated from patients with various types of cancer and are recognized to be useful in increasing our understanding of tumor progression and metastasis, besides prognosis, monitoring of recurrences, therapeutic responses, and drug resistance mechanisms. Although clinical applications are limited by poor detection of CTCs using current isolation processes, frequently caused by the heterogeneous immunophenotypic features of CTCs, promising steps are being taken in the use of CTCs for personalized anticancer therapies [2].

9.2 Historical Background

The first morphological description of CTCs dates back to 1869 when the Australian physician Thomas Ashworth recognized malignant cells similar to the ones of the primary tumor in the blood vessels of an autopsied patient with metastatic cancer [3]. Later, the American surgeon William Halsted extended this theory to the lymphatic system and incorporated it into his practice by performing resections of axillary lymph nodes in breast cancer surgeries [4].

In 1889, the British surgeon Stephen Paget proposed the "seed and soil" theory of metastasis in which he compared selected tumoral cells with a seed that dislocates via the bloodstream and reaches specific distant organs that form the soils for sowing [5]. Paget examined more than 900 autopsy records of patients with several types of cancer. He observed discrepancies between the relative blood supply and the frequency of metastases in certain organs, and he also found that visceral and bone metastasis did not occur randomly, but followed distinct patterns. This discarded the belief of that time that metastasis was an outcome of fortuity, and he concluded that certain tumor cells (the "seeds") have specific affinity for the environment of certain organs (the "soil"), and metastases arise only when the seed and soil are compatible. Paget's observations still hold true today.

During the twentieth century, James Ewing suggested that mechanical factors prompted metastatic dissemination as a result of the anatomical disposition of the vascular system [6]. Subsequently, Weiss observed some differences between regional metastasis and organ distant metastases, as the clinical data were reviewed on site prevalence of metastases of diverse human malignancies [7]. The regional involvement could be related to anatomical or mechanical factors, such as the efferent venous circulation or lymphatic drainage to regional lymph nodes. In contrast, metastases to distant organs of several types of cancers were more site specific [8].

In 1980, Ian Hart and Isaiah Fidler supported the "seed and soil" hypothesis to explain the nonrandom pattern of cancer metastasis, when they documented the selective nature of metastasis in an assay of experimental metastasis of B16 melanoma in syngeneic mice [9]. The results showed that despite some occurrence of mechanical arrest of tumor cells in the capillary niche of distant organs, subsequent proliferation and growth into metastatic lesions were defined by specific organ cells (Fig. 9.1).

9.3 Detection Methods

The first methods for detection of CTCs in peripheral blood of patients with cancer were reported in the twentieth century, using filtration and sedimentation techniques. Using a filtration approach, Salgado et al. showed that tumor cells

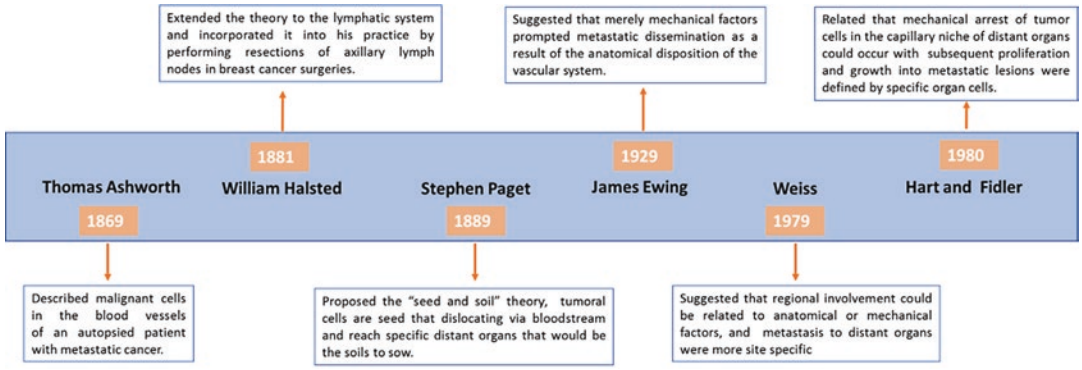

Fig. 9.1 Timeline of the historical discovery of CTCs

were commonly present in peripheral blood and in blood drained from tumor sites at surgery [10]. The protocol consisted of collecting blood samples in a syringe containing heparin followed by centrifugation. After this, the cells were incubated with a hemolyzing agent (streptolysin O) to destroy red blood cells and some white blood cells and then filtered through a Millipore filter. Finally, the cells were fixed and stained by the Papanicolaou technique. On the other hand, Alexander and Spriggs used a method to concentrate white cells by sedimentation followed by searching for the tumor cells [11]. In this technique, patient blood was mixed with a solution of dextran and heparin to sediment the erythrocytes and the fluid centrifuged for 10 min and then spread on slides to allow May-Grunwald-Giemsa staining. After this, the slides were examined under the microscope to detect unusual cells.

Today, almost a century later since the report of CTCs by Ashworth [3], numerous specific and sensitive technologies have emerged to detect CTCs in the bloodstream. However, the current methods used are an improvement over the first methods described. Detection techniques can be classified into two main groups according to the CTC properties based on biological or physical properties (Fig. 9.2).

9.3.1 Biological Properties

9.3.1.1 Density Gradient Centrifugation

The low density of CTCs (<1.077 g/mL) allows them to be distinguished from erythrocytes, leukocytes, and platelets. Centrifugation of blood samples allows separation in the pellet with normal blood cells and CTCs gathering at the interphase on differential step density gradients. Additional analyses can be performed in these CTCs by integrated cell culture (ICC) and polymerase chain reaction (PCR) [12].

9.3.1.2 Filtration

In 2000, Vona et al. [13] presented a new technique called "isolation by size of epithelial tumor cells *(*ISET)" in order to detect and count CTCs in peripheral blood samples of patients with carcinomas. In addition, this technique allows the immunocytological and molecular characterization of the cellular population. The method consists of diluting peripheral blood with a mixture of substances (saponin, paraformaldehyde, ethylenediamine tetraacetic acid (EDTA), and bovine serum albumin) to be subsequently filtered by gentle aspiration under vacuum, using a module of filtration and a calibrated polycarbonate mem-

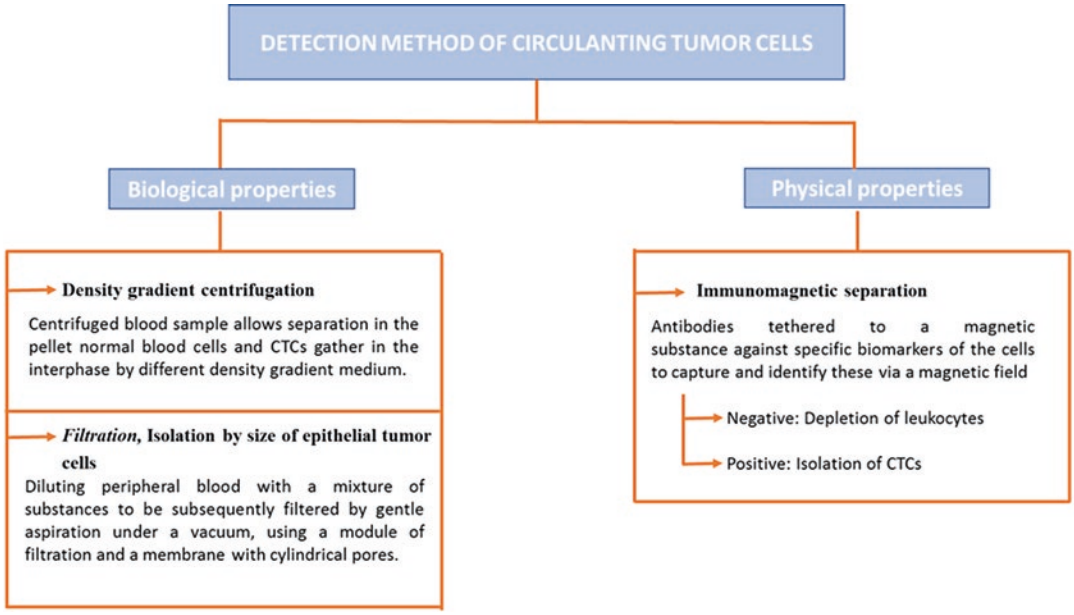

Fig. 9.2 Summary of detection methods of CTCs

brane with 8-μm-diameter cylindrical pores. Soon after, the isolated cells can be stained with hematoxylin and eosin (H&E) or May-Grunwald-Giemsa and studied by immunostaining, laser microdissection, and/or fluorescence in situ hybridization (FISH).

9.3.2 Physical Properties

The first immunomagnetic separation of CTCs was developed by Racila et al. [14]. At present, it is the most used technique due to its effectiveness for both detection and isolation. Also, it is the only method approved by the Food and Drug Administration (FDA) for this purpose. The assay combines immunomagnetic enrichment with flow cytometry and immunohistochemistry, using magnetic beads labeled with antibodies against specific target cell antigens, which can be used to identify the cells via a magnetic field. For the immunomagnetic separation phase, two approaches are available, although both selection procedures can be combined [15].

9.3.2.1 Negative Selection

This approach involves depletion of leukocytes using anti-CD45-labeled magnetic beads, with the CTCs being the non-selected cells.

9.3.2.2 Positive Selection

In this approach, the isolation of CTCs is achieved using magnetic beads labeled with antibodies against CTC surface proteins such as epithelial cell adhesion molecule (EpCAM), epithelial specific antigen (HEA), and anti-cytokeratin peptides.

In order to obtain more accurate results, modifications to this technique have been described by several authors; an example is the microchip technology developed by Nagrath et al. in the USA [16]. This method has the advantages of high-throughput processing, low shear, and efficient isolation with no requirement of prelabeling or processing of the samples [17]. This technology consists of the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions [16]. Additionally, Deng et al. showed

Table 9.1 Principal studies of CTCs in several types of cancer

Reference	Type of cancer	Method of CTC detection	*n* Groups[a]	Main conclusion
[22]	Metastatic, colorectal	CellSearch system	*n* = 109 ≤3 CTCs /7.5 mL >3 CTCs/7.5 mL	Patients with less CTCs had shorter free and overall survival
[23]	Resectable lung cancer	ISET method	*n* = 208	The number of CTCs was an independent prognostic factor for overall survival
[24]	Metastatic, prostate	CellSearch system	*n* = 231 >5 CTC/7.5 mL <5 CTC/7.5 mL	CTC count was the best predictor of prognosis; patients with more CTCs had shorter overall survival rates
[25]	Melanoma		*n* = 87	CTCs were identified in 29% of patients with primary melanoma and 62.5% with metastatic melanoma patients
[26]	Metastatic sarcoma	ISET method	*n* = 11	All patients showed CTCs
[27]	Locally advanced head and neck cancer	ISET method	*n* = 83 <6.5 CTC/mL ≥6.5 CTC/mL	CTCs were identified in 94% of patients and higher counts were strongly correlated with survival and response to treatment

CTCs circulating tumor cells, *ISET* isolation by size of epithelial tumor cells
[a]Some studies have categorized patients into high and low CTC count

that the use of anti-cytokeratin antibodies in combination with the anti-EpCAM antibodies enhances assay sensitivity significantly [18].

9.4 Research of Circulating Tumor Cells in Several Types of Cancers

Research in cancer is focused in the development of therapeutic targets and early identification of metastases to reduce mortality rates [19] In this case, the identification of CTCs was a big scientific breakthrough that has been studied in several types of cancer [2]. The principal studies performed are summarized in Table 9.1.

9.4.1 Breast Cancer

The first clinical study using the CellSearch System to identify and determine the prognostic significance of CTCs was performed in women with metastatic breast cancer (MBC). Patients ($n = 117$) were separated in two groups according to the number of CTCs before the chemotherapeutic treatment: (1) fewer than 5 CTCs/7.5 mL of whole blood and (2) higher than 5 CTCs/7.5 mL of whole blood. The results showed that the number of CTCs in patients with MBC is an independent survival predictor, due to subjects with higher counts of CTCs having shorter progression-free survival and overall survival rates than the patients with fewer CTCs [20].

9.4.2 Colorectal Cancer

Colorectal malignant neoplasms are the second cause of cancer-related deaths. Development of chemotherapeutic agents for specific targets has been investigated with the identification of CTCs [21]. Cohen et al. used EpCAM isolated magnetically to characterize CTCs from patients with metastatic colorectal cancer and later analyzed their prognostic significance [22]. The authors classified the patients into two groups based on CTC levels (≤3 CTCs /7.5 mL of blood and >3 CTCs/7.5 mL of blood) and showed that patients

with more than 3 CTCs had shorter median progression-free survival and overall survival, than those patients with lower CTC counts.

9.4.3 Lung Cancer

In 2011, the American Association for Cancer Research published a study that identified CTCs in approximately half of patients with resectable lung cancer ($n = 208$). This used the ISET method associated with cytologic analyses and correlated the presence and number of CTCs with clinicopathological features and survival. Patients showing 50 or more CTCs in blood had worse overall and disease-free-survival (DSF), independently of clinical stage, and presented higher risk of recurrence and cancer-related death. The number of CTCs was a significant and independent prognostic factor of overall survival, and it was proposed as a new prognostic biomarker [23].

9.4.4 Prostate Cancer

The relationship between CTCs and survival in patients affected by prostate cancer was studied initially by De Bono et al. in 2008 [24]. The prospective research employed the CellSearch System to detect and count CTCs in blood samples of subjects with progressive disease in different stages: (1) before treatment, (2) at the start of a new line of chemotherapy, and (3) monthly thereafter. The assay patients were categorized into either unfavorable or favorable groups according to the number of CTCs: >5 CTCs/7.5 mL or <5 CTCs/7.5 mL, respectively. The analysis showed that the CTC count was an accurate predictor of prognosis, given that patients with unfavorable counts before and after treatment had shorter overall survival rates than those with favorable counts.

9.4.5 Melanoma

In 2010, the first study of CTC identification was published concerning 87 patients with cutaneous melanoma by ISET method, followed by CTC characterization by immunohistochemistry (S-100, melanosome (HMB45), MART-1/Melan-A) and reverse transcription polymerase chain reaction (RT-PCR). The methodology included a control group of healthy volunteers with melanocytic nevi and non-melanoma skin lesions. CTCs were identified in 29% of patients with primary melanoma and in 62.5% of metastatic melanoma patients, while in the control group CTCs were not detected [25].

9.4.6 Sarcomas

Recently Chinen et al. performed for the first time the isolation, identification, and characterization of CTCs in sarcoma patients [26]. The study included blood samples from 11 patients with high-grade and metastatic sarcoma isolated by ISET. CTCs were identified in all of the cases by cytomorphology and characterized by double immunostaining with vimentin or pan-cytokeratin and CD45 antibodies. The number of CTCs identified varied from 2 to 48/8 mL of blood, with the highest number of cells found in the case of an epithelioid sarcoma and the lowest in an osteoblastic osteosarcoma. The ISET technique was limited to study epithelial malignancies, and thus this research showed the sensitivity of this method applied to sarcomas, a group of neoplasms with frequent metastasis and poor prognosis.

9.4.7 Head and Neck Cancer

A prospective study evaluating 83 patients affected by head and neck cancer demonstrated the prognostic role of CTCs in this malignancy through the ISET method. The study evaluated blood samples of patients diagnosed with non-metastatic locally advanced head and neck squamous cell carcinoma, treated with curative surgical resection plus adjuvant radiotherapy, or by a non-surgical strategy (radiotherapy and/or chemotherapy). Patients were sorted according to the count of CTCs at baseline (<6.5/mL versus

≥6.5/mL). CTCs were detected in 94% of the patients ($n = 78$) and significantly correlated with prognosis and response to treatment. The 2-year overall survival was 85.6% versus 22.9% (HR, 0.18; 95%CI, 0.06–0.49; $P < 0.0001$), revealing the prognostic potential of CTCs in head and neck cancer [27].

9.5 Clinical Applications and Limitations

Cancer-related death is usually provoked by dissemination, resulting in regional and distant metastases that could develop years after the removal of the primary tumor, despite the fact that tumor spread may not be evident at the time of the primary diagnosis. For example, many patients affected by breast cancer with negative axillary lymph nodes develop local or distant metastases. This could be explained by the presence of CTCs, which would represent the hematogenous phase of metastasis [28, 29].

Imaging has been the gold standard for disease monitoring in cancer. Combined with these traditional methods, CTC quantification must represent an alternative approach that could reveal micrometastases earlier than is currently possible, thereby improving the monitoring of disease status [30]. In this regard, Bud et al. compared CTC quantification to traditional radiologic assessment and suggested that CTC calculation is a reproducible tool that could be used earlier in the course of disease compared to imaging evaluation [31]. It has been proposed that CTCs play a crucial role for developing metastases. For this reason, monitoring CTCs may provide valuable information for treatment and, in the future, could be used as a real-time "liquid biopsy" [32, 33].

Until now, most of the studies of CTCs and prognosis using the CellSearch System (https://www.cellsearchctc.com/) for quantification have applied a cutoff value ≥5 CTCs/7.5 mL for categorization, as proposed by Cristofanilli et al. [20]. Prognostic correlations have been observed such that those few patients with very high levels of CTCs had a markedly short survival time. In one-third of MBC patients, CTCs were not detected, which constitutes a positive prognostic factor relative to patients with ≥1 CTCs/7.5 mL at baseline and during treatment [34]. In MBC, the prognostic properties of CTCs were shown to be robust during therapy by Hayes and colleagues [35].

Further than quantification, the evaluation of CTCs represents an accessible source of molecular information about the tumor, through the presence of treatment-relevant biomarkers (e.g., multidrug resistance proteins) [36].

Despite promising findings of several studies, CTC assessment still has not provided information on specific staging of disease, or in the guiding of adjuvant treatment. Pesta et al. proposed that analysis of the CTCs should provide information useful for the management of cancer patients, fulfilling the objectives of predictive, preventive, and personalized medicine (PPPM) [30]. However, the diagnostic value of CTC analysis is still not sufficient for clinical use. A three-step method to study CTCs was proposed to achieve specific uses for clinical practice. The first step is monitoring of treatment efficacy of cancer patients. The second one is to characterize the captured CTCs at the molecular level for the targeted treatment. The third stage is the culture of CTCs for use in a chemosensitivity assay. These steps would allow researchers to recognize and respond to changes in the phenotype of cancer cells during disease progression and introduce PPPM assisted by CTC analysis, into clinical practice.

While the clinical relevance of sequential CTC counts during treatment for use as an early response evaluation marker has been clearly demonstrated, the value of CTC characterization to guide treatment decisions in the clinic remains to be investigated [33].

There are three main limitations for CTC isolation. The first, and probably the most significant, is the rarity of CTCs in the bloodstream, since approximately ~1–100 CTCs per 10^9 blood cells are detected in cancer patients, and a significant quantity of normal hematological cells, such as erythrocytes and leukocytes, have to be eliminated to obtain pure CTCs. Secondly, there is an

apparent absence of CTCs in some patients. Finally, there are morphological and genetic heterogeneities of CTCs, even when they are disseminated from the same primary tumor. This latter property may make the isolation of CTCs difficult and the utility could be limited as the testing of drug response using such cells may differ from that of the primary tumor [2, 37–39].

It has been proposed that these limitations may be linked [40]. Studies have found that the lack or small number of CTCs in peripheral blood may actually be an issue of detection. It is known that most of the time, tumor cells require epithelial-to-mesenchymal transition (EMT) for major invasion and subsequent dissemination. When EMT occurs, CTCs suffer phenotypic changes, such as loss of expression of epithelial markers, and they acquire more mesenchymal-like phenotypes, which enables them to invade and survive in blood vessels and to invade other organs [41, 42].

Considering that CTCs are rare in peripheral blood and that CTCs with EMT may lose expression of EpCAM, this would result in the missing of EpCAM-negative CTCs in detection procedures based on use of the EpCAM antibody. In some cases, this could mean missing all of the CTCs in patients. This is an important limitation of the more extended and accepted technologies of CTC detection that are based in the presence of EpCAM in tumoral cells [2, 43, 44].

Following this hypothesis of EpCAM expression heterogeneity in CTCs, Hyun et al. [45] developed a model of EMT-induced MCF-7 breast cancer cells in order to study the physical and molecular characters of these cells. They showed that EMT-induced breast cancer cells have low levels of EpCAM expression. By RT-PCR and Western blotting, they observed that EpCAM mRNA was substantially reduced in MCF-7 cells, which indicates that EMT induction may result in decreased EpCAM expression levels. Also, they used a novel EpCAM-independent isolation system that demonstrated efficient isolation of CTCs regardless of heterogeneous EpCAM expression in breast cancer patient blood samples. This approach is called parallel multi-orifice flow fractionation (p-MOFF), which is a chip for high-throughput size-based CTC separation and was developed by the same research group in 2013 [46].

Hamilton et al. studied cell lines of primary, metastatic, and CTCs of small cell lung carcinoma (SCLC) and treated these in vitro with topotecan and epirubicin [37]. This showed that the CTC cell lines presented considerably more chemosensitivity than permanent SCLC cell lines, which suggests that response to second-line chemotherapy in SCLC patients may overestimate the effect on resident SCLC lesions and metastases. Chemosensitivity of CTCs compared to primary and metastatic tumors has been recently studied since, in some malignancies like SCLC, a decline in the CTC count during or after treatment could not reflect the response to chemotherapy of the permanent cells (from primary and metastatic tumors). Consequently, the detection of CTCs may facilitate a paradigm shift from treatment, based only on primary tumor features to a treatment that considers the molecular characteristics of CTCs [2]. Development of new technologies that overcome limitations of the more traditional techniques of CTC isolation will increase the understanding of CTC biology and association with prognosis and treatments, which is crucial in developing clinical applications. The principal clinical applications of circulating tumor cells are summarized in Table 9.2.

Table 9.2 Main clinical applications of detection and study of CTCs

• Monitoring disease status
• Detection of micrometastases
• Prognosis factor
• Pharmacological studies (e.g., drug resistance)
• Planning of personalized treatments

9.6 Conclusion

Evidence shows that CTCs have an important clinical value in early diagnosis of metastasis, as a predictor of prognosis, for monitoring of treatment and development of targeted treatment approaches. However, there are several challenges ahead, principally the rarity of CTCs in

bloodstream, their heterogeneous characteristics, and cell loss during isolation techniques. These factors make difficult the validation of a specific application of CTCs to improve survival rates in patients. Additional studies are needed to clarify the knowledge and to achieve better isolation techniques to pave the way for successful clinical applications in cancer patients.

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10 Telomerase: A Target for Therapeutic Effects of Curcumin in Cancer

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Abstract

Telomerases are attractive targets for development of new anticancer agents. Most *tumors* express the enzyme telomerase that maintains telomere length and thus ensures indefinite cell proliferation, a hallmark of cancer. Curcumin has been shown to be effective against several types of malignancies and has also been shown to have inhibitory effects on telomerase activity. Hence, the aim of this chapter is to review the available investigations of curcumin on telomerase activity. Based on the findings obtained from the different studies here, we conclude that the telomerase inhibitory effects of curcumin are integral to its anticancer activity, and thus curcumin may be useful therapeutically in the cancer field.

Keywords

Telomerase · Curcumin · Cancer · Herbal medicine

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10.1 Introduction

Proliferative capacity is one of the distinguishing differences between tumor and normal cells. Normal cells exhibit a limited lifespan and show replicative senescence, while *immortality* is a common characteristic of tumor cells [1]. Reactivation and upregulation of telomerase has been observed in almost 90% of the cancers, and this enzyme is responsible for tumor cell immortalization. Telomeres are the nucleoprotein complexes that are found at the ends of eukaryotic chromosomes. These structures protect the ends

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of chromosomes from exonucleolytic degradation and end-to-end fusions, thereby contributing to genomic stability [2]. The human telomerase enzyme is a DNA polymerase consisting of two subunits known as telomerase reverse transcriptase (hTERT, the catalytic subunit encoded by the *TERT* gene, located on chromosome 5p15.33) and telomerase RNA component (hTERC or hTR, encoded by the *TERC* gene positioned on chromosomal region 3q26) [2, 3]. This ribonucleoprotein complex is responsible for progressive synthesis of the telomeric DNA repeats. In addition, proteins like pontin, reptin, ribonucleoprotein complex subunit 1 (Gar1), H/ACA ribonucleoprotein complex subunit 2 (Nhp2), and telomerase Cajal body protein 1 are also associated with the telomerase core complex and necessary for proper telomerase assembly and recruitment to chromosomes [2].

Throughout history, plants and their constituents have been used in health management [4, 5]. Several studies in animal models, as well as clinical trials in humans, have reported pharmacological effects of medicinal plants, including antibacterial, antifungal, anticancer, antiviral, analgesia, and anti-inflammatory activities [6–9]. Over the past three decades, public interest in natural therapies has surged [10–13]. Notably, the high cost and side effects of many modern pharmaceuticals have encouraged the use of more affordable traditional medicines with potentially fewer side effects [8, 10, 14]. Telomerase inhibitors can be subdivided into those compounds that directly block the enzymatic activity of telomerase and those that downregulate the expression of the hTERT catalytic subunit [15].

Curcumin is a low-molecular-weight, lipophilic, bright yellow polyphenol, extracted from the rhizomes of *Curcuma longa* (turmeric), a member of the Zingiberaceae family. This plant is a perennial rhizomatous herb that is native to tropical Southern Asia but is now widely cultivated in both tropical and subtropical regions throughout the world [16]. *Curcuma longa* has been shown to possess a variety of pharmacological activities in both traditional and modern medicine. Curcumin is extensively used in Asian kitchens as well as for medicinal purposes. Vogel and Pelletier discovered curcumin in 1815 [17–20]. In 1910, Milobedzka and colleagues identified the chemical structure of curcumin as diferuloylmethane or 1,6-heptadiene-3,5-dione-1,7-bis (4-hydroxy-3-methoxyphenyl)-(1E,6E) (Fig. 10.1) [20, 21].

Curcumin possesses a wide array of pharmacological effects, with known anti-oxidant, anti-inflammatory, antimicrobial, immunoregulatory, epigenetic-modifying, anti-tumor, antiangiogenic and antimetastatic, chemo-sensitizing, analgesic, hepatoprotective, and anti-thrombotic properties [22–29]. Many of the pharmacological activities and experimental findings on the therapeutic effects of curcumin and curcuminoids have also been confirmed in clinical trials, although the need for further well-designed clinical studies has also been emphasized [30, 31].

In this review, we discuss the available studies relating to the telomerase inhibitory effects of curcumin. The efficacy of curcumin in targeting telomerase in different cancerous cells is also summarized in Table 10.1.

10.2 In Vitro Studies on Telomerase Inhibition

Several studies have documented the anticancer activity of curcumin in vitro. The capacity of curcumin on the regulation of telomerase activity and induction of apoptosis was investigated in the human leukemia cell line K-562. Induction of apoptosis by curcumin is initiated by the release of cytochrome c from mitochondria into the cytosol and is evidenced by an increase in DNA content in the sub-G1 region. Apoptosis was mediated by the activation of caspases 3 and 8, Bcl-2 downregulation, and Bax upregulation. Curcumin suppressed telomerase activity in a dose- and time-dependent manner, the inhibition being due to suppression of translocation of hTERT from the cytosol to the nucleus. Interestingly, the inhibition of telomerase activity by curcumin showed a positive correlation with several parameters of apoptosis [32]. In corroboration with these results, Hsin et al. found that reactive oxygen

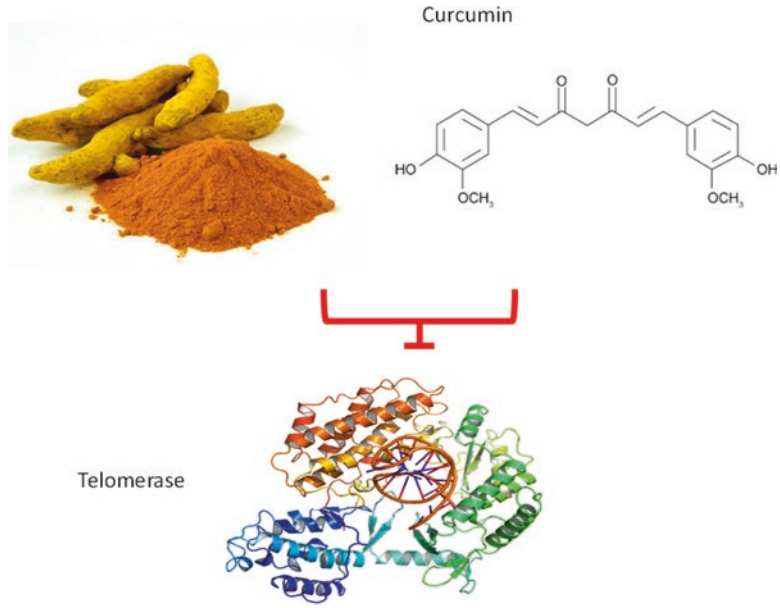

Fig. 10.1 Inhibitory effect of curcumin on telomerase

species (ROS) contribute to the curcumin-mediated telomerase activity inhibition and that Sp1 reduction contributes to hTERT downregulation by curcumin-induced ROS [33].

In a study conducted to investigate the potential of curcumin as an anticancer agent in different cell lines, the effects of curcumin were studied on cell growth and telomerase activity in three human cancer cell lines, Bel7402, HL60, and SGC7901. At concentrations of 1–32 μM, curcumin exhibited concentration-dependent antiproliferative effects on these cell lines in vitro. Anti-tumor effects were observed when curcumin (50–200 mg/kg) was orally administered to nude mice transplanted with the cancer cells. Exposure to 1 μM concentrations of curcumin led to apoptosis of cells as detected by acridine orange/ethidium bromide staining as well as flow cytometric analysis. Quantification of the polymerase chain reaction (PCR) products showed that suppression of telomerase activity in extracts of the cells treated with 1 μM of curcumin occurred in a time-dependent manner [34]. Ramachandran et al. also reported an inhibition of telomerase activity in MCF-7 breast cancer cells, due to downregulation of hTERT and mRNA expression of the viral oncogene c-myc [35]. Telomerase activity in MCF-7 cells was 6.9-fold higher than of the levels in human mammary epithelial cells, and a concentration-dependent decrease in telomerase activity following treatment with curcumin was observed. The authors suggested that the inhibition of telomerase activity in MCF-7 cells may have been due to downregulation of hTERT expression. Increasing concentrations of curcumin caused a steady reduction in the level of hTERT mRNA in MCF-7 cells, whereas there was no effect on hTERT and c-myc mRNA levels [35].

Khaw and Hande investigated the antiproliferative activity, DNA damage induction, and telomere-telomerase regulation in human glioblastoma and medulloblastoma cell lines following administration of curcumin. Curcumin treatment decreased cell viability in a dose-dependent manner in both brain tumor cell lines. The telomerase-positive cell lines showed higher sensitivity to curcumin in comparison to normal human fibroblasts. Curcumin LC_{50} concentrations were higher for normal cells (90 μM) in comparison to the telomerase-positive cancer cell lines (30–50 μM), and normal human fibroblasts exhibited greater DNA damage in comparison to the cancer cell lines. In addition, telomerase-positive cell lines displayed significant inhibition of telomerase activity following treatment with curcumin. A long period of treatment with curcumin also resulted in significant telomere short-

Table 10.1 The efficacy of curcumin in cancerous cells targeting telomerase

Curcumin type and dosage	Cancer cell type	Main findings	References
Curcumin-loaded NIPAAm-MAA nanoparticles (10–70 μmol/L)	Calu-6 lung cancer cell line	– Downregulation of telomerase gene expression – Inhibition of cell growth – Increment in the mRNA levels of pinX1 gene	[46]
Nanocapsulated curcumin (0–150 μM)	SW480 colorectal cancer cell line	– Reduction of hTERT gene expression	[48]
Curcumin (0–100 μM)	Human leukemia cell line K-562	– Suppression of telomerase activity – Induction of apoptosis	[32]
Curcumin (1-32 μM)	Human cancer cell lines Bel7402, HL60, and SGC7901	– Suppression of telomerase activity – Antiproliferative effect	[34]
β-Cyclodextrin -curcumin complex (5, 10 and 15 μM)	T47D breast cancer cell line	– Inhibition of telomerase expression – Induction of apoptosis	[50]
Curcumin (0–100 μM)	Human glioblastoma (A172, KNS60, and U251) and medulloblastoma cell line (ONS76)	– Reduction of cell viability – Inhibition of telomerase activity – Significant telomere shortening	[37]
Curcumin (0–100 mM)	Human glioblastoma multiforme cells A172 and medulloblastoma cells ONS76	– Increased cell death and DNA damage – Telomere shortening activity – Reduction in hTERT levels	[38]
Curcumin (3.5 μM)	P388D1 mouse lymphoma cells	– Over expression of TNF-α and IL-1β – Inhibition of the antiapoptotic Bcl-2 and human catalytic subunit hTERT	[56]
PAMAM encapsulating curcumin (0.5–60 μM)	T47D breast cancer cell line	– Antiproliferative effect – Inhibition of telomerase activity	[41]
Curcumin (0, 1, 10, 50 μM)	Human leukemia cell HL-60	– Induction of apoptosis – Inhibition of telomerase activity	[43]
Curcumin (5–100 μM)	T47D breast cancer cell line	– Antiproliferative effect – Inhibition of hTERT gene expression	[45]
Curcumin-loaded NIPAAm-MAA nanoparticles (10–70 μmol/L)	Calu-6 lung cancer cell line	– Elevation of PinX1 gene expression – Downregulation of the telomerase gene	[58]
Curcumin (0–100 mM)	Mammary epithelial (MCF-10A) and breast cancer (MCF-7) cells	– Downregulation of hTERT expression	[36]
β-Cyclodextrin-curcumin (5–100 μM)	T47D breast cancer cells	– Inhibition of telomerase expression	[52]

ening in cancer cell lines. Finally, curcumin reduced the upregulated signaling pathways in cancer cell lines [36].

A study by Khaw et al. showed that curcumin induced a significant increase in cell death and DNA damage in brain tumor cells. Curcumin treatment also resulted in significant telomere shortening in brain tumor cells which was consistent with a decrease in hTERT levels [37].

As telomerase is essential for the continued proliferation of primary and transformed cells, the activation of telomerase could be a definitive step in human carcinogenesis [38]. Crucially, the interaction of the molecular chaperone complex

Hsp90–p23 with the rate-limiting catalytic subunit of telomerase, hTERT, is critical for regulation of the nuclear localization of telomerase, and downregulation of hTERT by curcumin involves dissociating the binding of hTERT with p23 [39].

A study was undertaken to examine the capacity of curcumin to regulate telomerase activity in curcumin-induced apoptosis in P388D1 mouse lymphoma cells. Induction of apoptosis and telomerase activity by curcumin in the P388D1 lymphoma cells was confirmed by enumeration of apoptotic cells, measuring the percentage of DNA fragmentation and quantifying mRNA expression by PCR. The culture supernatant from curcumin-treated P388D1 lymphoma cells contained a higher level of nitric oxide. Furthermore, treatment of the cells with curcumin resulted in overexpression of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) and inhibition of the antiapoptotic Bcl-2 and hTERT when compared to untreated cells [40].

Polyamidoamine (PAMAM) encapsulation of curcumin increased its antiproliferative effect in a T47D cancer cell line. The authors suggested that curcumin exerts an effect on T47D cancer cells through inhibition of telomerase activity and, consequently, cancer cell proliferation was inhibited [1]. The polyamidoaminoid structure of PAMAM is biocompatible and enhanced curcumin uptake, thereby augmenting the cytotoxicity of the treatment [41].

In HL-60 cells treated with curcumin, apoptosis was induced as evidenced by the release of cytochrome c from mitochondria to the cytosol and an increase in the DNA content in the sub-G1 region, as observed in fluorescence-activated cell sorting (FACS) analysis. Upregulation of Bax and downregulation of Bcl-2 was followed by activation of caspases 3 and 8 and degradation of poly(ADP-ribose) polymerase (PARP), thereby mediating the apoptosis. Curcumin also inhibited telomerase activity in a concentration-dependent manner, suggesting that the telomerase inhibition by curcumin may be interpreted as an important event that leads to apoptosis [42]. In an elegant study, conducted by Pongsavee et al., a 744ins20 – ter240 BRCA1 frameshift mutation was found to drive oxidative stress. This mutation produced a DNA repair defect, and curcumin treatment could inhibit telomerase function and thereby reduce cancer cell growth [43].

Curcumin and silibinin exerted cytotoxic effects on T47D cells and inhibited telomerase gene expression in time- and dose-dependent manners. The mixture of curcumin and silibinin exhibited a relatively greater inhibitory effect on the growth of T47D cells and hTERT gene expression when compared with the effects of either agent alone [44].

Curcumin-loaded N-isopropylacrylamide-methacrylic acid (NIPAAm-MAA) nanoparticles inhibited the growth of the Calu-6 lung cancer cell line in both a time- and dose-dependent manner, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for measurement of cell metabolic activity. In addition, quantitative (Q)-PCR results demonstrated that the expression of the telomerase gene was downregulated as the concentration of curcumin-loaded NIPAAm-MAA nanoparticles increased. In parallel, a trend toward increasing mRNA levels of the PIN2 (TERF1)-interacting telomerase inhibitor 1 (*PINX1*) gene was apparent [45]. It was demonstrated that the NIPAAm-MAA nanoparticles could release the drug in a slightly acidic environment, such as occurs in inflammatory tissues, solid tumors, and intracellular endosomal compartments [46].

The results of the MTT assay showed that in the SW480 colorectal cancer cell line, nanocapsulated curcumin and chrysin (in comparison to free forms of these compounds) had a highly synergistic toxicity effect. Q-PCR analysis showed a significant reduction in hTERT gene expression in SW480 cells treated with nano-curcumin and nano-chrysin in comparison to the expression levels seen in untreated cells [47]. Of note, the telomerase gene has been shown to be overexpressed in cancer cells and is related to hTERT, and high expression has been reported in many colorectal cancer cell lines [47, 48].

The β-cyclodextrin-curcumin complex exerted a cytotoxic effect on the T47D breast cancer cell line, mediated by inhibition of telomerase expression and induction of apoptosis due to enhanced curcumin intake into the cells [49]. This is consis-

tent with reports showing that β-cyclodextrin enhances curcumin delivery via increasing its uptake into cells [50].

In the above-mentioned study by Pongsavee et al., the 744ins20 – ter240 BRCA1 frameshift mutation was found to produce oxidative stress and a DNA repair defect, and curcumin treatment led to inhibition of telomerase function and thereby reduced cancer cell growth [43].

Kazemi-Lomedasht et al. showed that β-cyclodextrin-curcumin resulted in higher cell toxicity in T47D breast cancer cells than did free curcumin. In breast cancer cells treated with cyclodextrin-curcumin, the level of telomerase gene expression was reduced as compared with that of cells treated with free curcumin [51]. Of note, the β-cyclodextrin-curcumin inclusion complex led to improvement in curcumin stability and solubility [52].

10.3 In Vivo Studies

Telomerase was found to be highly expressed in dimethylhydrazine dihydrochloride (DMH)-induced colorectal cancer in rats, and its high activity was associated with increased TERT expression. Telomerase activity was found to be absent, or present at lower levels, in normal tissue. PCR, Q-PCR, Western blot, and immunofluorescence analysis showed that CDK4, CDK2, cyclin D1, and cyclin E were highly expressed in the DMH treatment group. Following the administration of diclofenac and curcumin, telomerase activity was downregulated, and the expression of TERT, CDK4, CDK2, cyclin D1, and cyclin E was diminished. Inhibition of telomerase activity by diclofenac and curcumin was associated with upregulation of the tumor suppressor proteins p51, Rb, and p21, which are known to lead to cell cycle arrest and induction of apoptosis in colorectal cancer [53].

One study suggested that when curcumin is used in combination with cyclophosphamide or paclitaxel, it may have potentiated the anti-tumor effects of these drugs by inhibiting tumor markers like protein kinase C (PKC), telomerase, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and histone deacetylase (HDAC) both in in vitro and in in vivo models of breast cancer [54].

The expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 genes was significantly increased in the C57/B16 mouse model of melanoma following treatment with curcumin and chrysin, with the greatest effect being observed in the combination groups and the greatest observed increase being in the nano groups. Curcumin and chrysin treatment significantly decreased the expression of TERT, and mice in nano-treated groups showed a higher decrease in TERT levels compared to the mice in control treatment groups. Furthermore, the expression of matrix metalloproteinase (MMP)-2, MMP-9, and TERT genes was significantly reduced in the treatment groups [55].

10.4 Conclusions

Many studies have shown that curcumin possesses antiproliferative and anti-carcinogenic properties. Curcumin is therefore proving to be a promising anticancer agent. Moreover, telomerase activity has become a major target in anticancer research. Telomerase is the critical enzyme in overcoming growth limitations due to telomere dysfunction. The studies presented in the present review suggest that curcumin is a telomerase inhibitor, in addition to initiating apoptosis and promoting killing of cancerous cells, and is a good candidate for cancer therapy. Further investigations of these effects, with particular emphasis on in vivo experiments, are needed to verify the potential use of curcumin and related compounds in cancer treatment and prevention.

Conflict of Interest Muhammed Majeed is the Founder and Chairman of Sabinsa Corporation and Sami Labs Limited. Other authors declare no competing interests.

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11 Aspirin as a Potential Geroprotector: Experimental Data and Clinical Evidence

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Abstract

Aging is a biological process with effects at the molecular, cellular, tissue, organ, system, and organismal levels and is characterized by decline in physical function and higher risks of age-related diseases. The use of anti-aging drugs for disease prevention has become a high priority for science and is a new biomedicine trend. Geroprotectors are compounds which slow aging and increase lifespan of the organism in question. The common painkiller aspirin, a member of the non-steroidal anti-inflammatory drug (NSAID) family, is one of the potential geroprotective agents. Aspirin is often used in treatment of mild to moderate pain. It has anti-inflammatory and anti-pyretic properties and acts as an inhibitor of cyclooxygenase which results in inhibition of prostaglandin. Acetylsalicylic acid as an active compound of aspirin also inhibits platelet aggregation and is used in the prevention of arterial and venous thrombosis. Aspirin has shown life-extending effects in numerous model organisms. This chapter reviews the evidence for clinical efficacy of aspirin including cardiovascular disease prevention, anti-cancer effects, and improvement of cognitive function. However, there are some limitations of these therapies, including the risk of excessive bleeding. We have also summarized numerous experimental and analytical data that support health and longevity benefits of aspirin treatment by affecting pro-longevity pathways.

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Keywords

Aging · NSAID · Aspirin · Age-related diseases · Geroscience · Geroprotector

11.1 Introduction

Aging is a process of complex changes in an organism over the time with constant increasing of risks related to the development of pathologies. The slowdown of age-related diseases extends the lifespan and this might be determined at the developmental stage [1] and specific nutrient and nutrient-sensing pathways [2, 3]. Lifespan and age-associated pathologies can be pharmacologically treated with supplements and pharmaceuticals of artificial or natural origins [4–7]. Modern geriatric medicine and geroscience are mainly focused on the supplements and pharmaceuticals, which allow delaying the onset of all age-associated chronic disorders. Aspirin (acetylsalicylic acid, ASA) is a non-selective inhibitor of cyclooxygenase (COX), the first synthesized representative of the family of non-steroidal anti-inflammatory drugs (NSAIDs). Initially discovered as an anti-pyretic, analgesic, and anti-inflammatory drug, aspirin has later proven to have pleiotropic effects, both dependent and independent on its COX-inhibitor properties. Aspirin-like substances derived from salicylate-rich plants have been used for pain and fever relief for many centuries. Various species of willow (*Salix* spp.) were first mentioned in ancient medicinal texts, like the Ebers Papyrus (1550 BC) [8], and the active compound of willow bark was identified in 1832 by French chemist Charles Gerhardt, who attempted to neutralize salicylic acid by buffering it with sodium (sodium salicylate) and acetylchloride to create acetylsalicylic acid. Aspirin was patented on February 27, 1900, by Felix Hoffmann from the Bayer Company. As an anti-platelet agent, aspirin is the most prescribed agent for the prevention and treatment of thrombosis. Moreover, it may act as a calorie restriction mimetic and increase cellular stress responses. Despite its high overall safety profile and a massive body of evidence supporting clinical benefits of aspirin use, substantial risk of side effects, including gastrointestinal bleeding, exists for regular low-dose prescriptions.

11.2 Mechanisms of Action

In 1971 Vane proposed the mechanism of action of the aspirin-like drugs [9]. Anti-inflammatory and anti-platelet properties of aspirin are related to biosynthesis of prostaglandins (PGs). PGs and their derivatives thromboxanes (TXs) play homeostatic functions, such as in inflammatory responses [10]. PGs are synthesized from arachidonic acid via oxidation of fatty acids of membrane lipids. Phospholipases (PLAs) catalyze release of arachidonic acid from the plasma membrane. Arachidonic acid is further metabolized by cyclooxygenase (COX) to PGH2. Synthesis of PGs depends mainly on the activity of COX, which possesses both COX and peroxidase activity and has two isoforms COX1 and COX2 [11]. COX1 and COX2 are encoded by two different genes, although these enzymes share high similarity in amino-acid sequence [12]. COX1 and COX2 have the same enzymatic function participating in biosynthesis of PGH2. PGH2 is further transformed into PGE2, PGI2, PGD2, PGF2α, and TXA2 as a result of the inflammatory enzymatic cascade in the tissues (Fig. 11.1) [13]. Tissue-specific prostaglandins are involved in mediating physiological processes, including vasodilation or vasoconstriction, ovulation, bone metabolism, suppression of gastric acid secretion, inflammatory immune response, regulation of chemotaxis, and endocrine regulation.

The COX enzyme is a target of NSAIDs including aspirin. NSAIDs inhibit the COX-specific active site of the enzyme but have no effect on the peroxidase active site [14]. NSAIDs block the binding of arachidonic acid to the COX enzymes. It was demonstrated that aspirin irreversibly inhibits COX by acetylation of serine (Ser) residues, namely Ser-530 of COX1 and Ser-516 of COX2 (Fig. 11.1). COX1 is expressed in platelets, and acetylation of COX1 by aspirin leads to inhibition

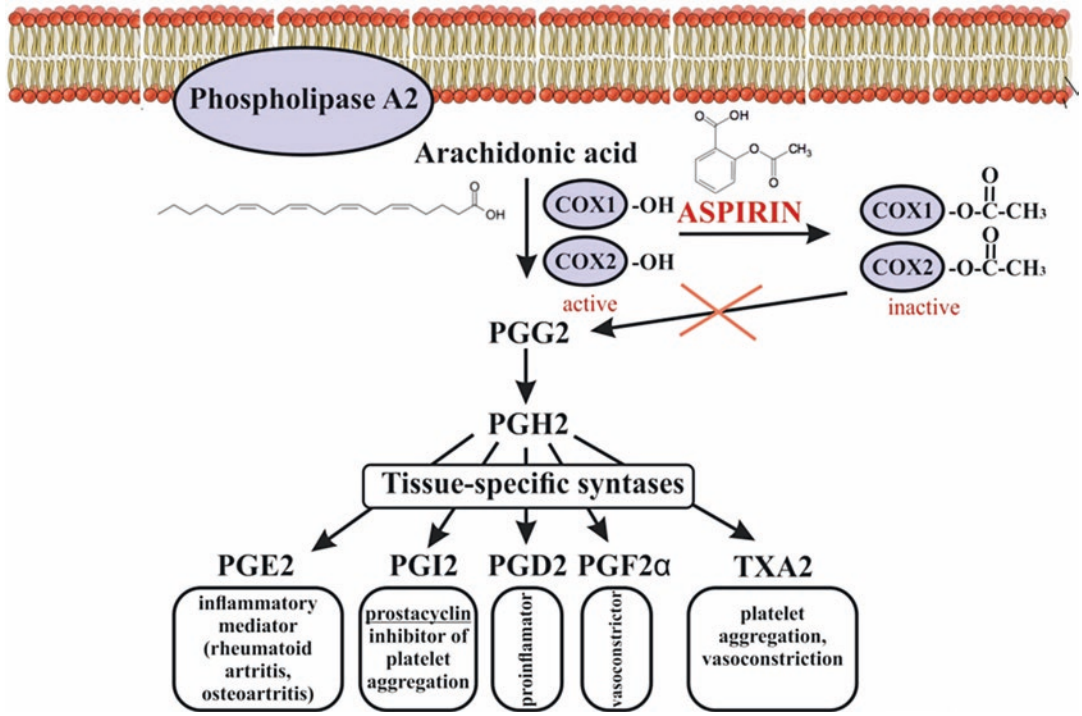

Fig. 11.1 Mechanism of action of aspirin. Aspirin blocks COX1 and COX2 by acetylation of serine residues. Acetylated COX is unable to synthesize PGG2 and subsequently its tissue-specific derivatives

of TXA2 synthesis. TXA2 acts as a vasoconstrictor and potent platelet activator; therefore, aspirin reduces platelet activation and aggregation reducing the risk of thrombosis [15]. COX1 is constitutively expressed in the endoplasmic reticulum of most cells [16]. COX2 is an inducible form, which is upregulated in response to pro-inflammatory stimuli. COX2 is rapidly expressed in several cell types in response to cytokines, growth factors, and pro-inflammatory molecules [17]. Aspirin totally blocks COX1 activity, while COX2 acetylated by aspirin can still convert arachidonic acid to 15-R-hydroxyeicosatetraenoic acid (15-R-HETE) [11]. Decreased production of prostaglandins and TXA2 under aspirin treatment is the basis of both the therapeutic effects and toxicities.

11.3 Lifespan Extension in Model Organisms

Aging is the physiological process that is characterized by loss of normal organ function caused by damage accumulation in cells and tissues [18]. Longevity can be modulated by alterations in age-related genes. Moreover, lifespan might be extended using some drugs. Discovering chemicals that can delay aging and extend lifespan is one of the most promising ways to improve the quality of life in older age. Today, many of the lifespan- and healthspan-extending drugs are effective at relatively low concentrations from 5 to 200 mg/kg of body weight [19]. However, it is important to know that every compound may possess some side effects. Furthermore, many drugs may extend lifespan by the so-called hormetic effect: the conditions in which relatively toxic substances may have beneficial effects.

There is a continuous need of optimal model systems to discover potential anti-aging approaches and evaluate the effects on healthspan. Ideally, a model system should maximally replicate the aging processes in humans, with high conservation of the relevant genes and signaling pathways. More often, researchers use simple model organisms such as nematodes, fruit flies, and rodents. Most studies aimed at investigating anti-aging drugs have been performed by

using invertebrate models, which are considered as useful for investigating human diseases and have been widely exploited for discovering potential anti-aging agents [20–22]. Pathways controlling lifespan and aging are partially conserved in a wide range of species, from yeast to humans [18, 23]. Previously, we have summarized the beneficial lifespan-extending effect of metformin as potential geroprotector for various animal models [7]. In this chapter, we have collected and summarized data obtained in invertebrate and rodent models regarding the anti-aging potential of aspirin.

11.3.1 *Caenorhabditis elegans*

Aspirin has been found to have many beneficial effects on physiological traits and is often used to treat pain and inflammation. Aspirin treatment extended the nematode lifespan and improved stress resistance [24, 25]. The data also suggested that aspirin may act in a dietary restriction-like manner [25]. Furthermore, it reduces the levels of reactive oxygen species (ROS) and activates expression of genes related to the main antioxidant enzymes including catalase, superoxide dismutase, and glutathione-S-transferase [24]. Recently, it was demonstrated that aspirin increases metabolism and regulates germline signaling to activate downstream DAF-12 and DAF-16 to extend lifespan [26]. Aspirin as a salicylic derivative extends the lifespan of *C. elegans* by activating autophagy and the mitochondrial unfolded protein response [27].

Conclusively, drugs and compounds of varied groups and origins have been shown to extend the lifespan of worms by affecting different pathways and mechanisms. However, there is a need to confirm the effects of these treatments in other organisms.

11.3.2 *Drosophila melanogaster*

Invertebrate model systems, including *Drosophila melanogaster*, are essential for better understanding of the genetic pathways that control aging. Furthermore, the fruit fly has proved to be valuable in testing chemical compounds that influence longevity. *Drosophila* possesses complex behavioral phenotypes and several good models of human age-related diseases are available. Furthermore, *Drosophila* experiments can be conducted in demographic cages, which allow researchers to investigate the effects of biodemographic on lifespan.

Aspirin has been found to suppress the aging process via interfering with oxidant production and cytokine response processes and by blocking glycooxidation reactions [28]. Prolonged *Drosophila* lifespan and improved healthspan have been observed under aspirin administration [29]. The lifespan of *D. melanogaster* females was longer and the duration of metamorphosis was prolonged in the experimental groups treated with acetylsalicylic acid, the active agent in aspirin, and acetaldehyde [30]. A recent study discovered the lifespan-extending effect of a set of NSAIDs, including aspirin [31]. These NSAIDs delay the age-dependent decline of locomotor activity and increase stress resistance. The effect of the lifespan increase was associated with decreased fecundity. Depending on the concentration, NSAIDs have demonstrated both anti- and pro-oxidant properties in *Drosophila* tissues [31]. The molecular mechanism of aspirin in this area is still unknown, but it influences the metabolism of amino acids, carbohydrates, and urea.

11.3.3 Rodents

The mouse has developed into the major mammalian model system for the research of compounds with potential lifespan-extending effects. The mouse possesses genetic and physiological similarities to humans, and its genome can be easily manipulated and analyzed. Furthermore, multiple tools, mutants, and inbred strains are available to simulate age-related diseases in the mouse with a high translational potential.

The National Institute on Aging Interventions Testing Program (ITP) tested the impact of some agents, which can potentially extend lifespan, including aspirin [32]. It was demonstrated that

aspirin administration led to increased lifespan of male but not female mice [32]. The detailed mechanism of the lifespan-extending effect in mice is still poorly understood. However, it is known that aspirin triggers protective autophagy in mice and acts as a calorie restriction mimetic [33].

In addition to mice, rats have been extensively used in studies related to aging. Although a study found no direct impact of aspirin supplementation on rat survival, it did normalize blood pressure in rats with a hypertension phenotype [34].

11.4 Health Benefits of Aspirin: Evidence from Clinical Trials

Aspirin was originally developed as an analgesic and anti-pyretic drug, although it is now predominantly used for primary and secondary cardiovascular prevention. However, many of its pleiotropic health effects regarding age-related diseases are still not completely understood [35]. Aspirin might be one of the most appropriate agents as an anti-aging drug due to low costs and simplicity of treatment and through its well-investigated multifaceted properties regarding cardiovascular disease (CVD) and cancer.

11.4.1 Aspirin and CVD Prevention in Elderly

CVDs represent an important health burden for modern society and are the leading cause of death in the older population [36]. In the USA, it has been reported that the percentage of people who have CVD among those aged 60–79 years is approximately 72% and reaches at least 80% in those aged more than 80 years [37, 38]. Because of these numbers and the increase in prevalence of other critical factors such as obesity and diabetes in the aging population, the number of deaths due to CVD in the future might dramatically increase [36, 37, 39].

A convincing body of evidence suggests that aspirin provides a beneficial reduction of CVD mortality and new CVD events [40]. Low-dose aspirin is currently routinely prescribed according to multiple evidence-based guidelines for management of acute vascular events and revascularization procedures as well as for secondary CVD prevention. Currently, nearly 20% of adults in the USA report taking aspirin daily, or every other day, with this number increasing to nearly 50% in those aged 65 years and older [41]. At low doses, aspirin is also widely used to prevent pregnancy-related vascular disorders, such as preeclampsia and intrauterine growth restriction [42].

The anti-platelet effect of aspirin is a result of acetylation of COX1 with subsequent inhibition of TXA2 production. Growing evidence suggests that aspirin-mediated acetylation might confer additional non-COX-dependent benefits in prevention of thrombosis, as well as anti-inflammatory and anti-tumor effects [43, 44].

Anti-platelet effect of ASA allows reduction of the risk of death from ischemic stroke and vascular complications for a wide range of patients, both in primary or secondary prevention settings [45, 46]. Stroke is a leading cause of mortality and disability worldwide [47]. Recently, two large randomized trials found a significant decrease of 7 recurrent ischemic strokes per 1000 patients and a nominally significant reduction of 4 deaths without further stroke per 1000 patients treated with aspirin [48, 49]. Overall, there was a net decrease of 9 per 1000 treated in the risk of further stroke or death in hospital indicating a benefit for acute initiation of aspirin after ischemic stroke [50].

In elderly patients with coronary heart disease (CHD), *angina pectoris* represents the most common cardiovascular disease. Studies have shown that the prevalence of angina rises with increasing age, with a mean age of onset of 62.3 years [51]. ASA is the preferred drug for CHD [52] and it can effectively inhibit vasoconstriction and platelet aggregation by preventing and controlling thrombosis in affected people [53, 54].

Along with *angina pectoris,* peripheral arterial disease (PAD) is another common disorder associated with a high risk of cardiovascular mortality [55]. The major risk factors for PAD are similar to those for coronary and cerebrovascular

disease [56]. The benefit of aspirin as a secondary prevention therapy in patients with atherosclerosis has been demonstrated in patients with prior ischemic stroke or acute myocardial infarction [57].

Clinical decision about prescription of low-dose ASA in primary prevention in elderly people remains complicated and requires careful individual evaluation of the risk-benefit ratio. A recent meta-analysis of nine trials of aspirin for the primary prevention of CVD demonstrated a significant reduction in the risk of myocardial infarction, ischemic stroke, and all-cause mortality in subjects allocated to long-term aspirin use. At the same time aspirin increased the risk of hemorrhagic complications, such as hemorrhagic stroke, major bleedings, and gastrointestinal bleeding. N. Raju et al. showed that this prevention strategy always requires evaluation of the balance between the potential benefit and harm of long-term ASA prescription [58]. High risk of bleeding, especially intracerebral hemorrhage, remains the major concern for different patient cohorts despite potential favorable cardiovascular outcomes and reduction of all-cause mortality. Selection of the correct dose and form of aspirin (coated or buffered), careful consideration of comorbidities and co-medications, and the possible twice-a-day dosing in patients with increased platelet turnover are among the variables that need to be taken into account for safe and effective prescription of aspirin [59, 60].

Implementing pharmacological strategies to decrease cardiovascular risks remains challenging for older patients because of the presence of declining physiological status compared to the younger population [36]. As reported in several papers, the presence of diabetes and obesity can have a strong negative effect on elderly people with CVDs because it can impair daily activity and reduce overall quality of life [37, 39].

11.4.2 Anti-Cancer Effects of Aspirin

A large body of evidence confirms that aspirin can improve overall survival in patients with diagnosed cancer, reducing the risk of cardiovascular events and, in some cases, influencing cancer-related survival and slowing down the rate of metastasis. A meta-analysis, involving 23 randomized controlled trials on low-dose aspirin and non-vascular deaths, reported a significant reduction in cancer deaths after 4 years of aspirin intake [61]. The results of meta-analyses have suggested that aspirin has possible protective effects toward breast [62, 63], prostate [64, 65], pancreatic [66], and gastric cancer [64, 67].

A recent cohort study performed by Loomans-Kropp et al. [39] on a total of 146,152 individuals aged 65 years and older found that aspirin use 3 or more times per week was associated with decreased risk of mortality from all causes (HR, 0.81; 95% CI, 0.80–0.83; $P < 0.001$), any cancer (HR, 0.85; 95% CI, 0.81–0.88; $P < 0.001$), gastrointestinal cancer (HR, 0.75; 95% CI, 0.66–0.84; $P < 0.001$), and colorectal cancer (HR, 0.71; 95% CI, 0.61–0.84; $P < 0.001$) [39].

Protective effects of low-dose ASA against certain cancer types seem to be variable depending on the population and cancer type. A meta-analysis by Hochmuth et al. demonstrated that aspirin can have a protective effect against non-small cell lung cancer [68].

Meta-analyses of trials regarding the potential survival benefits of aspirin in patients with diagnosed cancer often show conflicting results due to variability in research methodologies and the high degree of heterogeneity of the available studies [69, 70]. A meta-analysis of observational studies reported little or no effect of aspirin on breast cancer survival [63]. Another meta-analysis showed a reduction in breast cancer-specific mortality, all-cause mortality, and metastasis among aspirin and NSAID users [71]. However, a recent single retrospective analysis of 1113 women diagnosed with operable breast cancer between 1995 and 2015 showed that ASA use did not appear to alter breast cancer-related survival before their breast cancer diagnosis [70]. Another recently published paper showed that aspirin use was not strongly associated with mortality following breast cancer [72]. These results are in disagreement with a study claiming that improvement in breast cancer survival in aspirin-allocated women were observed only if treatment

was initiated after the cancer diagnosis, while pre-diagnosis treatment did not demonstrate clear benefits [73].

Aspirin intake was inversely related with prostate cancer-specific mortality, according to a meta-analysis by Liu et al. [65]. A systematic review and meta-analysis showed reduced mortality in colon cancer patients receiving aspirin as an adjuvant treatment [74]. It reduced mortality especially in cases of tumors expressing *PIK3CA* (p110α catalytic subunit of PI3K). Possible benefits were also demonstrated for patients with breast and prostate cancer.

In a more recent study, effects of aspirin on men with prostatic cancer (PC) were studied [75]. Daily aspirin use was inversely associated with prostate cancer mortality [HR, 0.59; 95% CI, 0.36–0.96] and case fatality (HR, 0.45; 95% CI, 0.22–0.94) in both populations without distinctions. Another study in men between 40 and 75 years of age concluded that regular aspirin use was associated with a lower risk of lethal PC and its post-diagnostic use was associated with better survival [76]. They used a proportional hazards regression to examine the association between current, past, or no regular use of aspirin (at least twice a week) in relation to lethal (metastatic or fatal) prostatic cancer and found that regular aspirin use was associated with a lower risk of lethal prostate cancer (HR, 0.80; 95% CI, 0.66–0.96). Moreover, post-diagnostic use of aspirin was associated with a lower risk of lethal PC (HR, 0.80; 95% CI, 0.64–1.00) and overall mortality (HR, 0.79; 95% CI, 0.69–0.90) [76].

Across epidemiological studies, the most significant reductions in risk have been observed in gastrointestinal cancer and particularly in case of colorectal cancer [77, 78]. Evidence of chemoprevention even inspired the US Preventive Services Task Force to recommend low-dose aspirin for primary prevention of CVD and colorectal cancer in adults aged 50 to 59 years, having greater than a 10% 10-year risk of CVD and no increased risk of bleeding [79]. Moreover, in the same study, an individualized treatment approach of low-dose aspirin use for the prevention of CVD and colorectal cancer in individuals aged 60 to 69 years was recommended [80]. Although the evidence for aspirin use among individuals 70 years and older remains insufficient [80], a new analysis found that older adults (> 65 years) who regularly took aspirin had a significant reduction in mortality from all causes of cancer compared with individuals who did not take aspirin [39].

A meta-analysis by Ye et al. showed that low-dose (75–325 mg daily), regular (2–7 times per week), long-term (more than 5 years) aspirin intake leads to significant reduction in colorectal cancer risk [81]. Evidence from studies, investigating CVD primary and secondary prevention, suggested that ASA administration reduces incidence of colorectal cancer and related mortality 10 years after treatment initiation [82]. Aspirin provides survival benefits for patients diagnosed with colorectal cancers, but only if administered after the diagnosis. Meta-analyses demonstrate that aspirin is beneficial as a post-diagnosis treatment especially in colorectal tumors overexpressing *COX2* and *PIK3CA* and reduces overall mortality in *PIK3CA* mutated cancers by 29% [83, 84].

Emillson et al. performed a network meta-analysis to explore low-dose aspirin as an alternative to traditional colorectal cancer screening methods (flexible sigmoidoscopy or fecal occult blood test) in reduction of colorectal cancer incidence and mortality [85]. Low-dose aspirin seemed to be as effective as the screening tools in colorectal cancer prevention, with effects more visible for malignancies localized in proximal colon [85].

Aspirin could be used for colorectal cancer prevention in especially vulnerable populations with high genetic risk of cancer, such as Lynch syndrome. This hereditary condition was addressed in the CAPP2 (Cancer Prevention Programme) randomized trial of 861 participants. Patients with Lynch syndrome received 600 mg/day of aspirin or placebo for up to 4 years. Allocation to aspirin resulted in a significant decrease of almost 60% in cancer incidence [86]. Also, aspirin effectively reduced risk of colorectal cancer among *MMR* gene mutation carriers in the Colon Cancer Family Registry [87]. According to the results of a meta-analysis of 15

randomized controlled trials, low-dose aspirin can also be used for secondary chemoprevention in patients with previous diagnosis of colorectal neoplasia [88].

However, not all studies reached same conclusions about post-diagnostic aspirin use and mortality in colorectal cancer. A study carried out on a large Scottish population-based cohort of patients with a diagnosis of colorectal cancer did not find any evidence of a reduction in cancer-specific mortality in aspirin users [89]. Also, in a clinical trial conducted by the Japanese Primary Prevention Project (JPPP) among patients aged 60 to 85 years and presenting with hypertension, dyslipidemia, or diabetes mellitus, low dose of aspirin failed to reduce colorectal cancer incidence or mortality. Surprisingly, this study found that the cancer incidence was significantly higher in the aspirin group than in the no-aspirin group and failed to show preventive effects of aspirin on cancer incidence or mortality during the average study period of 5 years [90]. Several studies (ASCOLT, ASPIRIN, US Aspirin for Breast Cancer (ABC) trial, PIK3CA-based trials, and the Add-Aspirin trial) aimed at exploring the potential role of aspirin as an adjuvant therapeutic agent for colorectal, breast, gastro-esophageal, and prostate cancer are now ongoing. The results of these trials should provide valuable insights about efficacy and safety of aspirin as a treatment in cancer.

Patients with certain types of myeloproliferative neoplasms, like polycythemia vera or essential thrombocythemia, might be at high risk of thrombotic events due to high rate of platelet turnover and rapid emergence of new platelets with unacetylated COX enzymes. Such patients might require tailored, more frequent dosing for efficient prevention of cardiovascular events [91, 92].

Data regarding the anti-cancer effect of aspirin in older adults has been conflicting and uncertain. A multicenter double-blind randomized controlled trial allocated elderly patients, without prior CVD, cognitive deficit, or established disability, to ASA 100 mg/day or placebo and found an increased rate of all-cause mortality in the aspirin group (5.9% vs. 5.2%, $P < 0.05$). This was attributed to the increased risk of cancer in the aspirin-allocated individuals (3.1% vs. 2.3%, $P < 0.05$) [93]. Researchers hypothesize that aspirin might make cancer symptomatic via increased risk of bleeding that is especially pronounced in elderly individuals [91]. A recent meta-analysis combining data from 13 randomized controlled trials and a total of 164,225 participants found no significant difference in cancer incidence between ASA-allocated patients and patients receiving placebo [94].

Anti-cancer effects of ASA are multimodal including COX1 and COX2 inhibition and anti-inflammatory properties, along with mechanisms not related directly to COX inhibition. Rectal mucosal COX2 inhibition is considered crucial in prevention of colorectal neoplasia [79]. Possible targets and pathways implicated in the anti-carcinogenous properties of aspirin include inhibition of IκB kinase β, preventing activation of NF-κB, and inhibition of extracellular-signal-regulated kinase (ERK) and Wnt/β-catenin signaling [95]. Aspirin can also act as a 5′ AMP-activated protein kinase (AMPK)-activator, inhibiting downstream activity of the mammalian target of rapamycin complex 1 (mTORC1) [96].

11.4.3 Anti-inflammatory Properties of Aspirin

The anti-inflammatory properties of ASA occur by direct COX-inhibition-mediated mechanisms and indirect modulation of NF-κB pathway, along with inhibition of interleukin (IL)-6 pathways [43]. Limited evidence exists about the role of aspirin in prevention of sepsis, a life-threatening condition that often affects the elderly. A meta-analysis with propensity matching showed 7–12% mortality risk reduction in sepsis patients taking aspirin prior to sepsis onset [97]. Another randomized controlled trial investigating effects of ASA in sepsis patients showed that it was able to induce pro-inflammatory effects in septic monocytes, signifying that patients suffering from sepsis-induced immune deficiency might benefit from aspirin treatment [98].

Inhibition of NF-κB by aspirin might play a significant role in bone health. Inhibition of NF-κB signaling and reduction in the expression of receptor activator of NF-κB ligand can suppress the formation of osteoclasts and potentially prevent bone loss [99]. A recent meta-analysis of observational trials suggests that aspirin use is associated with 17% lower risk of bone fractures and has a modest positive effect on bone mineral density [100].

11.4.4 Aspirin and Cognitive Function

Anti-platelet effects of aspirin are potentially beneficial for neuroprotection via reduction of neuroinflammation and small vascular lesions. The impact of ASA on white matter lesions (WMLs) was evaluated in patients from the Women's Health Initiative Memory Study of Magnetic Resonance Imaging (MRI). There was no significant difference between the MRI volumes of WMLs among aspirin users and non-users [101]. In patients with Alzheimer's disease, aspirin did not provide any additional therapeutic benefit and conversely increased risk of intracranial bleeding, putting patients at risk of additional cognitive loss [102]. A recent meta-analysis by Veronese et al. polling data from 36,196 patients did not confirm a protective effect of aspirin against cognitive decline in older age [103]. Low-dose aspirin was neither associated with significantly better global cognition nor delayed the onset of dementia or cognitive impairment. The results of ASPREE (ASPirin in Reducing Events in the Elderly) trial, assessing role of aspirin in maintenance of disability-free and dementia-free life in a healthy population of elderly people, did not show favorable effects of aspirin if administered at an earlier age or continued for a longer period of time [93]. In particular, the study showed that primary end points of death, dementia, or physical disability occurred in 921 participants in the aspirin group (21.5 events per 1000 person-years) and in 914 individuals in the placebo group (21.2 events per 1000 person-years). The between-group difference was not significant (HR, 1.01; 95% CI, 0.92–1.11; $P = 0.79$).

11.5 Adverse Effects of Aspirin

Despite a promising portfolio of pleiotropic effects, long-term use of aspirin is associated with certain risks. The aim of this section is to give an update about the critical use of aspirin in association with the appearance of adverse effects in vulnerable populations of patients including the elderly, patients with comorbidities, and those taking multiple medications. Several conditions associated with aspirin hypersensitivity including Reye's syndrome and aspirin-exacerbated respiratory disease are rare and difficult to diagnose and manage at the clinical level [104]. Aspirin poisoning is another overlooked problem, and this is associated with substantial morbidity and mortality. In the USA, about 20,000 patients per year are admitted with salicylate poisoning [105].

11.5.1 Aspirin and Risk of Bleedings

Data from both observational studies and randomized controlled trials are important to assess risks associated with administration of low-dose aspirin and to identify the primary factors associated with their reported outcomes [106]. Evidence shows that the risk for gastrointestinal bleeding with and without aspirin use increases with age [107, 108]. NSAID therapy combined with aspirin approximately quadruples the risk for serious gastrointestinal bleeding compared to the risk with aspirin alone [60, 109].

A recent review of randomized controlled trials and observational studies investigating the bleeding risk with aspirin therapy identified age and *Helicobacter pylori* infection as factors that may increase the risk of gastrointestinal bleeding [110]. The incidence of major bleeding events and the absolute risk of bleeding in individuals receiving low-dose aspirin increased with age [111]. These results are in agreement with a meta-analysis of randomized trials by the Antithrombotic Treatment Trialists' (ATT) collaboration that identified age as an important predictor of the risk of bleeding associated with low-dose aspirin, with an approximate doubling of the absolute risk of bleeding with low-dose aspirin for every 10-year increase in age [112].

Moreover, in elderly people, higher doses of aspirin do not appear to confer additional benefits but increase toxicities [108]. Overall, the rates of gastrointestinal complications increase steeply beyond the age of 70 years and fatality rates show a similar trend [113]. Since a limited number of elderly persons have been included in previous primary prevention trials, the risk-benefit balance of aspirin on increased risk of bleeding and CVDs in this age group is still uncertain [114, 115]. A confirmation that in elderly populations the risk of CVD and increased risk of bleeding are higher than in younger populations [116] comes from a meta-analysis review published by McNeill et al. [93]. In their primary analysis of the "Aspirin in Reducing Events in the Elderly" (ASPREE) trial, they investigated the use of aspirin in older people without history of CVD to see whether or not the health benefits outweighed the risks. They first showed that daily use of low-dose aspirin did not prolong disability free-survival among the elderly and, secondly, it did not prevent the effect of aspirin on CVDs and bleeding. Indeed, there was no significant difference between groups in the rate of disability-free survival (HR, 1.01; 95% CI, 0.92–1.11), but when they analyzed the effects on CVDs, aspirin increased the risk of major hemorrhage [117]. They found 8.6 events per 1000 people per year in the aspirin group versus 6.2 per 1000 in the placebo group (HR, 1.38; 95% CI, 1.18–1.62). Researchers concluded that for elderly people without known CVD, regular low-dose aspirin does not only prolong disability-free survival or reduce the risk of CVD but puts them at higher risk of having a major hemorrhage and higher mortality from other causes [118]. In conclusion, the use of low-dose aspirin as a primary prevention strategy in older adults resulted in a significantly higher risk of major hemorrhage and did not result in a significantly lower risk of CVD than placebo [117].

11.5.2 Low-Dose Aspirin on Intracranial Hemorrhage (ICH) and Cerebral Microbleeds

Hemorrhagic stroke, although rare, is one of the most serious and potentially fatal aspirin side effects. Estimates suggest a relative increase of 32–36% in hemorrhagic strokes in aspirin users from a baseline rate of 0.03% per year [107]. A meta-analysis of a randomized clinical trial of a group with a dose of aspirin of 270 mg per day calculated an average absolute risk increase in ICH of 12 events per 10,000 persons [119]. In subjects with ICH who had been taking regularly moderate doses of aspirin immediately before the onset of stroke, one study observed poor short-term outcomes and increased mortality, probably attributable to rapid enlargement of intracerebral hematomas [120]. The study showed that a 3-month mortality of the 208 identified subjects with ICH was 33% with a risk factor for death at the onset of ICH of 2.5 (relative risks [RR], 2.5; 95% CI, 1.3–4.6; $P = 0.004$) [120]. In comparison, the estimated absolute risk reduction in myocardial infarction was 137 events per 10,000 persons and 39 events per 10,000 persons in ischemic stroke [119].

In agreement with previous results, Garcia-Rodriguez et al. reported increased ICH risks in patients taking daily aspirin [121]. They showed that the overall risk of ICH was increased by approximately 40% with long-term low-dose aspirin, which is similar to the estimates from randomized trials, although not consistently reported in all studies [117]. Interestingly, they also found that in low-dose aspirin users, the absolute risk of bleeding, but not the RR for bleeding compared with non-users, increased with age [60].

Aspirin has been linked to an increased risk of cerebral microbleeds in the form of dark hole patches in an MRI study [122]. This study was conducted in a population-based sample of 1062

persons from a non-dementia longitudinal cohort, with an age range of 60 years and older. Compared with placebo, cerebral microbleeds were more prevalent among users of platelet aggregation inhibitors (adjusted odds ratio [OR], 1.71; 95% CI, 1.21–2.41) while lobar microbleeds were more prevalent among aspirin users (adjusted OR compared with non-users, 2.70; 95% CI, 1.45–5.04) [121].

11.5.3 Adverse Effects of Aspirin in Elderly Patients Having Surgical Procedures

Elderly patients have the highest postoperative mortality and morbidity rate in the adult surgical population. The use of low-dose aspirin before surgeries can cause prolonged bleeding after operations for up to 10 days [123]. However, clinical decisions to stop aspirin therapy before surgery are challenging and considered a key risk factor in patients with coronary stents [124, 125]. Patients might face either the risk of cardiovascular thromboembolic complications in case of therapy cessation or the risk of hemorrhagic surgical complication in case of the therapy continuation [126–128].

To make these decisions even more difficult, mixed results have been found in studies of elderly patients undergoing surgical procedures after use of aspirin. In high-risk patients aged over 70 years, a controlled trial showed that low-dose aspirin reduced the risk of major cardiac events without increasing bleeding complications [129].

Conversely, a study of 6499 people undergoing elective surgery found that 30 patients required reoperations to control bleeding [124]. Among these patients, 20 had diffuse bleeding while 10 had bleeding from a specific site. Diffuse bleeding was associated with the preoperative use of aspirin alone or in combination with other NSAIDs in 19 of the 20 patients with diffuse bleeding.

The adverse effects of aspirin in patients having surgical procedures were also reviewed in two meta-analyses by Kwok and Loke [130]. The hemorrhagic adverse effects of aspirin therapy in patients were first evaluated during surgical revascularization of coronary artery disease [131] and then with use of aspirin in cutaneous surgery. Meta-analysis results showed that bleeding was increased in groups treated with aspirin (RR, 2.32; 95% CI, 1.31–4.08; $P < 0.01$). The authors concluded that patients who receive aspirin within 7 days of surgery were at higher risk of blood loss [129].

Regarding the risk of postoperative bleeding and complications in dermatological surgery when aspirin was in use, studies also reported mixed results [132–134]. A meta-analysis of 4 prospective studies and 2 retrospective studies with a total of 1373 patients reported that aspirin or NSAIDs were associated with increased risk of moderate to severe complications compared to controls (OR, 2.0; 95% CI, 0.97–4.13; $P = 0.06$) and this risk was greater with warfarin than aspirin [132–134].

11.5.4 Aspirin and Potential Drug–Drug Interactions

Aspirin can interact with other medications, potentially altering their effects or increasing the risk of adverse events [135–137]. A total of 315 drugs are known to interact with aspirin but the most common of these are other NSAIDs, such as diclofenac, ibuprofen, indomethacin, and naproxen. Co-administration of these medications with aspirin leads to increased risk of gastrointestinal bleeding [137].

Aspirin competes for cytochrome P450 metabolism with other agents metabolized via this system. Methotrexate, an immunosuppressant and anti-proliferative agent, is widely used in chronic autoimmune diseases and cancer [137–139]. Aspirin acting on cytochrome P450 can reduce hepatic clearance of methotrexate, resulting in high circulating plasma levels of methotrexate and potential toxicity [137, 138].

The combination of selective serotonin reuptake inhibitors (SSRIs) and aspirin has been associated with increased risk of bleeding [137, 140]. Yuet et al. advised that patients on SSRIs

are 40% more likely to develop severe gastrointestinal bleeding, especially if they are taking NSAIDs such as aspirin [141]. Another study showed that SSRIs interact with other NSAIDs such as ibuprofen and naproxen, as well as vitamin K antagonist anticoagulants such as warfarin and the anti-platelet medication clopidogrel [142]. If aspirin is taken with warfarin, it can reduce the anticoagulant effects of the latter and increase the risk of bleeding [137, 141, 142].

11.6 Conclusions and Future Directions

Aspirin remains a cornerstone of primary cardiovascular prevention in high-risk individuals and as anti-platelet treatment of acute vascular events and secondary prevention of CVDs. Apart from thrombosis prevention, beneficial effects of aspirin may include reduction of all-cause mortality, risks of cancer, and bone fractures. As a classical non-selective COX inhibitor, aspirin exerts anti-inflammatory, analgesic, and anti-pyretic effects. Inhibition of COX in platelets reduces aggregative properties of platelets and thereby prevents vascular thrombosis. Apart from COX-mediated effects, aspirin possesses the properties of a calorie restriction mimetic, which has been widely studied in model organisms. In model animals, aspirin significantly extended lifespan in *C. elegans*, fruit fly, and mice. Anti-cancer effects of aspirin are mediated through inhibition of IκB kinase β, ERK, and reduction of NF-κB and Wnt/β-catenin signaling. Aspirin also acts as AMPK activator, contributing to inhibition of mTORC1 signaling. A few ongoing trials are currently in progress to evaluate the potential role of aspirin as an adjuvant treatment for colorectal, breast, gastro-esophageal, and prostate cancer.

All these effects should be carefully weighed with substantial risk of bleedings, caused by the non-selective COX-inhibitory activity of aspirin. Patients of older age, particularly smokers and patients with poorly controlled blood pressure, are at high risk of major extracranial bleedings and hemorrhagic stroke. The majority of clinical recommendations for primary prevention do not include routine prescription of aspirin to patients older than 70 years and to patients of any age with high bleeding risk. Factors, substantially increasing the bleeding risk and precluding the use of aspirin, include previous history of gastrointestinal bleeding or peptic ulcer disease, bleeding from other sites, thrombocytopenia, coagulopathy, and chronic kidney disease. Combination of aspirin with other NSAIDs and corticosteroids also contributes to increased risk of hemorrhagic complications, while concurrent use of proton-pump inhibitors and statins may provide a protective effect. Given both these potential positive and negative effects of aspirin therapeutics, further research is required to assess both the risks and benefits in specific disease cases and guided by individualized medicine principles.

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Targeting Stem Cells in Chronic Inflammatory Diseases

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Abstract

Mesenchymal stem cell (MSC) dysfunction is a serious complication in ageing and age-related inflammatory diseases such as type 2 diabetes mellitus. Inflammation and oxidative stress-induced cellular senescence alter the immunomodulatory ability of MSCs and hamper their pro-regenerative function, which in turn leads to an increase in disease severity, maladaptive tissue damage and the development of comorbidities. Targeting stem/progenitor cells to restore their function and/or protect them against impairment could thus improve healing outcomes and significantly enhance the quality of life for diabetic patients. This review discusses the dysregulation of MSCs' immunomodulatory capacity in the context of diabetes mellitus and focuses on intervention strategies aimed at MSC rejuvenation. Research pertaining to the potential therapeutic use of either pharmacological agents (NFκB antagonists), natural products (phytomedicine) or biological agents (exosomes, probiotics) to improve MSC function is discussed and an overview of the most pertinent methodological considerations given. Based on in vitro studies, numerous anti-inflammatory agents, antioxidants and biological agents show tremendous potential to revitalise MSCs. An integrated systems approach and a thorough understanding of complete disease pathology are however required to identify feasible candidates for in vivo targeting of MSCs.

Keywords

Mesenchymal stem cells · Type 2 diabetes · Accelerated ageing · Probiotic · Antioxidant · Exosome · Extracellular vesicles

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12.1 Introduction

Ageing is often associated with the clinical onset of a plethora of different chronic diseases, such as cardiovascular disease, neurodegenerative disease, cancer and type 2 diabetes mellitus (DM). Although unique in terms of disease-specific triggers, the progression of these conditions is significantly exacerbated by chronic systemic inflammation and oxidative stress-induced cellular dysfunction [1, 2]. These two underlying conditions are prominent in both normal ageing and the accelerated ageing commonly reported to result from chronic psychological stress and/or metabolic dysregulation [3, 4].

Given the multi-faceted nature of many ageing-associated chronic diseases and the complexity involved in their management, addressing the common characteristic of inflammation may contribute significantly towards treatment—either preventatively or therapeutically—for many of these diseases. Considering the inherent nature of mesenchymal stem cells (MSCs) to respond to their environment and to counter dysregulation in order to restore homeostasis [5], an assessment of the feasibility to target MSCs in the treatment of age-related chronic inflammatory disorders is warranted. Although a large body of evidence exists in support of anti-inflammatory or antioxidant supplementation, especially in the phytomedicine sphere, significant limitations, such as poor transfer of preclinical results into human models [6–8] and risk of toxicity [9], are slowing the progress towards a generally applicable anti-inflammatory strategy.

In this review, we will focus on DM as chronic inflammatory disease and discuss the most relevant stem cell pathology in the context of DM-associated inflammation. We will provide an overview of methodological considerations and discuss research pertaining to therapeutic strategies targeting MSCs.

12.2 Mesenchymal Stem Cells

MSC dysfunction is a severe and often overlooked complication of numerous chronic degenerative, metabolic and inflammatory disorders [10–12]. MSCs mainly reside in bone marrow and have very specific characteristics which researchers use as criteria for identification. These include the expression of cluster of differentiation (CD) markers ($CD90^+$, $CD105^+$, $CD73^+$, $CD45^-$, $CD34^-$, $CD14^-$, $CD11b^-$, $CD79alpha^-$, $CD19^-$, $HLA\text{-}DR^-$), fibroblast-like morphology, multilineage differentiation capacity (osteogenic, adipogenic, chondrogenic) and ex vivo plastic adherence [13]. Despite sharing these characteristics, there can be various MSC subtypes with distinct functional preferences (immunomodulatory, growth promoting, pro-angiogenic, pro-regenerative) within a single population of cells residing in the same niche [14]. In addition to the bone marrow-resident MSCs, various stem/progenitor cells with mesenchymal properties have also been identified in perivascular and avascular niches (skin, pancreas, heart, lungs, kidneys, adipose tissue, muscle, cartilage, tendon, teeth) throughout the body [15]. Given the heterogeneous nature of MSCs, other than their tissue of origin, consistent distinction between various subtypes within a single population of cells remains challenging.

Under normal/healthy circumstances, MSCs play an important role in immune surveillance and the maintenance of tissue homeostasis [5]. Their self-renewal capacity and ability to mount a paracrine response upon injury (stimulation by microenvironmental cues) form part of the body's innate repair system. The tissue of origin and subtype influence the multifunctional properties of these cells, with bone marrow MSCs having the greatest capacity for immunomodulation [16–18]. Physico-chemical and structural changes within the niche microenvironment activate MSCs and trigger immunomodulatory and pro-regenerative paracrine signalling [19–21]. This is mediated through direct cell-to-cell contact (mitochondrial transfer), cytokine/chemokine/growth factor secretion and/or microvesicle/exosome (containing lipids, miRNAs and RNA as cargo) release [22].

However, due to their longevity, MSCs (as other stem/progenitor cells) are vulnerable to the accumulation of cellular and DNA damage, leading to stem cell depletion and/or senescence [23–25]. The evidence in support of allostatic stem

cell depletion with age is undeniable and this ultimately results in failure of endogenous repair mechanisms [26, 27]. The age-related replicative senescence of MSCs mainly involves genomic instability and telomere attrition [11, 28–30]. Independent of chronological ageing, in vitro studies suggest that persistent inflammation and oxidative stress (characteristic of chronic disease) can accelerate telomere shortening (without repeated cell cycles being a confounding factor) and cause cellular defects that lead to premature senescence [11, 30–32]. This has been confirmed in MSCs derived from DM animals, in which DNA damage, mitochondrial fragmentation and hyperactivation of the nuclear factor kappa B (NFκB) inflammatory signalling pathway are prominent [12]. These cellular defects alter MSCs' immunomodulatory ability and hamper their pro-regenerative function, which in turn leads to an increase in disease severity and the development of comorbidities [12]. For example, Xu et al. demonstrated that intraperitoneal transplantation of (radiation-induced) senescent adipose-tissue-derived MSCs into healthy young mice resulted in physical dysfunction and significantly shortened the life-span and overall health of these animals [33], demonstrating the cardinal importance of MSC health. Furthermore, in line with an exacerbating effect of accelerated ageing, the authors further indicated that these negative effects of transplanted senescent cells were exacerbated in mice with underlying metabolic disturbances [33]. These effects were not related to tumour formation, and the study provided proof-of-concept evidence that oral administration of senolytic agents could alleviate some of the age-related diseases that developed in recipient animals, as evident in the selective elimination of senescent cells in tissue explants and a 65% reduction in mortality rate [33]. Targeting stem/progenitor cells to restore their function and/or protect them against ageing-associated impairment should thus be part of therapeutic strategies in inflammatory conditions with underlying metabolic disturbances, such as DM.

Currently, approximately 9.3% (463 million people) of the world's population is estimated to have DM, of which up to 25% suffer from debilitating comorbidities such as retinopathy and non-healing ulcers [34, 35]. Rejuvenating MSCs in these patients could thus improve healing outcomes and significantly enhance their quality of life. In order to design treatment strategies targeting MSCs, it is necessary to fully understand the dysregulatory effects of disease aetiology on stem cell function.

12.3 Dysregulation of MSCs' Immunomodulatory Properties

Inflammation is the body's first defence mechanism against invading pathogens, and phagocytic inflammatory cells (neutrophils, macrophages) play an essential role in removing cellular debris to prepare injured tissues for repair/regeneration. Unresolved inflammation can however cause autoimmunity, accelerate ageing and hamper the progression of healing.

Depending on the activating signal, the functional plasticity of MSCs allows them to take on either a pro-inflammatory (MSC1) or anti-inflammatory (MSC2) phenotype [5, 36]. Danger response signals such as Toll-like receptor (TLR)-4 ligands or bacterial lipopolysaccharides (LPS) that are usually present following an infection have been shown to induce a pro-inflammatory phenotype (MSC1) in MSCs under standard culture conditions [36]. Co-culture experiments further demonstrate that MSC1s release chemokines (macrophage inflammatory protein (MIP)-1α, MIP1β, regulated upon activation normal T cell expressed and presumably secreted (RANTES), chemokine C-X-C motif ligand (CXCL)-9 and CXCL10) to recruit immune cells (T-lymphocytes, neutrophils, macrophages) or to induce T-cell effector responses through antigen presentation [5, 36, 37].

On the other hand, in the presence of an inflammatory microenvironment (high levels of tumour necrosis factor alpha (TNFα), interferon gamma (IFNγ) and interleukin (IL)6), MSCs take on an anti-inflammatory phenotype (MSC2) to protect tissues against maladaptive damage and promote regeneration. MSC2s have been shown

to suppress effector T-cell activation/proliferation through the release of soluble factors such as indolamine 2,3 dioxygenase (IDO), prostaglandin E2 (PGE2), nitric oxide (NO), transforming growth factor (TGFβ) and hepatocyte growth factor (HGF) or by modulating the function of regulatory T cells [5, 37, 38]. In addition to suppressing activation of Th1, Th2 and Th17 cells, MSCs also inhibit the release of IFN-γ and IL17 from these effector cells whilst promoting the release of anti-inflammatory IL10 from regulatory T cells [37]. MSC2s furthermore promote the resolution of inflammation by suppressing the respiratory burst of neutrophils [39–42] and by inducing a phenotype switch in macrophages from phagocytic (M1) towards pro-regenerative (M2) [43–48]. Thus, in order to mediate repair, the sensitivity and responsiveness of MSCs to changes in their microenvironment is pivotal (Fig. 12.1a).

Indeed, the literature provides significant evidence of maladaptive MSC regulation in the context of ageing and metabolic dysregulation. Upon pro-inflammatory activation, these dysfunctional/senescent MSCs deviate from their counter-responsive nature and amplify inflammatory signals instead of exerting anti-inflammatory immunosuppressive effects [12, 49, 50] (Fig. 12.1b). The repressive effect of MSCs specifically on neutrophils and macrophages is thought to be dependent on functional IL6 signalling [46, 51, 52]. Phillips et al. [46] recently demonstrated that in the presence of pro-inflammatory signals (IFN-γ and IL1β), MSCs released high levels of NO, IL6 and PGE2 that in turn upregulated the expression of M2 macrophage-related genes, promoted IL10 release and suppressed TNFα secretion in macrophages. The authors further demonstrated that co-culture of MSCs with IL6Rα-deficient macrophages was unable to repress the M1 macrophage phenotype in the presence of inflammatory cytokines, suggesting that IL6 signalling is an essential trigger of MSC-mediated M2 macrophage polarisation. This interpretation was supported by Yin et al. [51], who indicated that in vitro blocking of IL6 signalling using siRNA abrogated allogeneic MSCs' ability to suppress M1 macrophage activation in both in vivo (T2DM mice with β-cell dysfunction) and in vitro co-culture (THP-1 cells) models [51]. It is thus not surprising that IL6 deficiency and subsequent dysregulation of IL6/signal transducer and activator of transcription (STAT)-3 signalling on molecular level in dysfunctional diabetic MSCs resulted in the skewing of the immunomodulatory properties of these cells [49]. Previous studies done by our group have demonstrated an increased pro-inflammatory gene expression (MIP1α, MIP1β, macrophage chemotactic protein (MCP)-5, CD40, IL23, IκBα) profile at baseline [49] and excessive TNFα release upon stimulation with wound fluid [50] in diabetic MSCs when compared to healthy control counterparts.

In addition to an altered secretome, diabetic MSCs also have an impaired proliferation capacity [53, 54]. This is consistent with the literature indicating that senescence-associated low-grade inflammation (SALI) is a prominent characteristic of cellular senescence and that the senescence-associated secretory phenotype (SASP) of stem cells impacts the biological processes of surrounding tissues [31, 55]. In the context of chronic disease-associated accelerated ageing, the composition of the SASP will however also likely be dependent on the specific stem cell type affected and the disease-specific causes that had induced premature senescence in these cells. Preventative measures and strategies to rejuvenate senescent MSCs should thus take the underlying pathogenesis into account.

12.4 Methodological Considerations in the Interpretation of Intervention Strategy Outcomes

The most relevant studies that have investigated the efficacy of various therapeutic agents to either protect or rejuvenate MSCs in the context of DM are presented in Table 12.1. However, to be able to accurately interpret the outcome of these studies, the following methodological considerations should be noted.

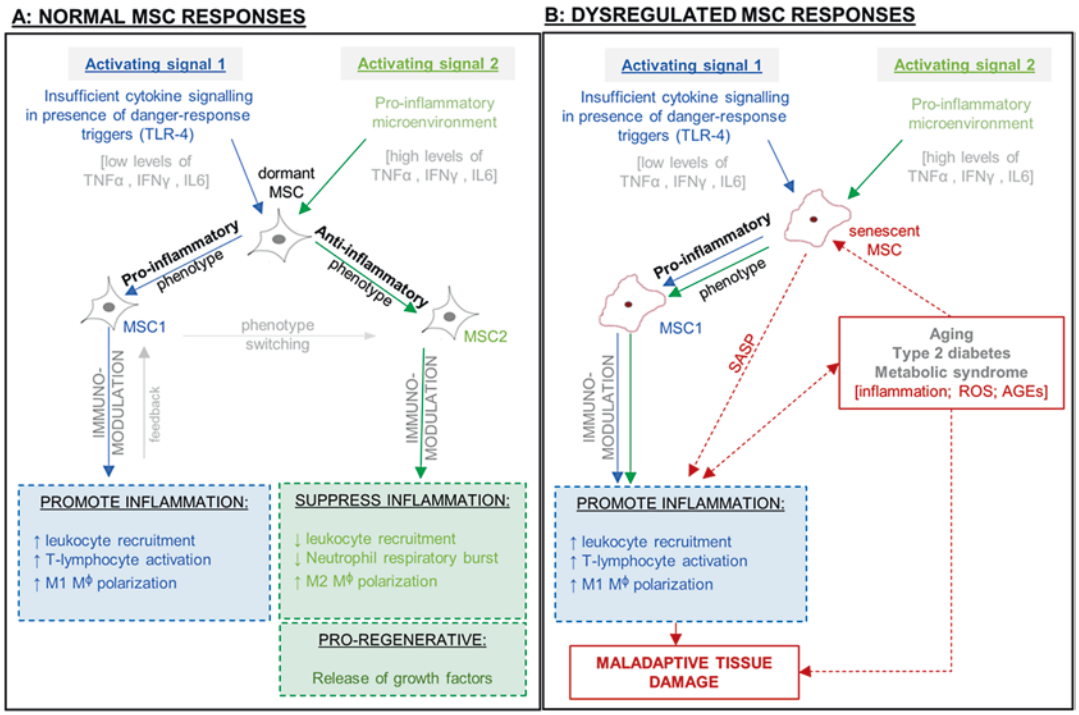

Fig. 12.1 Overview of immunomodulatory responses of (**a**) healthy MSCs under normal circumstances and (**b**) senescent MSCs in age-related type 2 diabetes mellitus. (**a**) Under normal circumstances, in the absence of pro-inflammatory cytokines, healthy MSCs respond to danger triggers such as TLR4 by taking on a pro-inflammatory phenotype (MSC1). These pro-inflammatory MSC1s promote inflammation by increasing leucocyte recruitment, T-lymphocyte activation and polarisation of macrophages to a pro-inflammatory/phagocytic phenotype (M1) (*activating signal 1, blue arrows*). As the microenvironment becomes more pro-inflammatory, the high levels of cytokines trigger the MSC1s to switch to an anti-inflammatory phenotype (MSC2) to restore homeostasis. In the presence of a pro-inflammatory microenvironment (*activating signal 2, green arrows*), healthy MSCs take on an anti-inflammatory phenotype (MSC2) that suppress inflammation by downregulating leucocyte recruitment, inhibiting the respiratory burst of neutrophils and promoting polarisation of macrophages to an anti-inflammatory/pro-regenerative phenotype (M2). MSC2s also release various growth factors to promote regeneration. (**b**) Senescent diabetic MSCs are desensitised to pro-inflammatory signals (dysregulated feedback). Both activating signals (*blue and green*) induce MSCs to take on a pro-inflammatory phenotype (MSC1). The senescent MSCs furthermore have a senescence-associated secretory phenotype (SASP) and, together with the pathogenesis of diabetes (oxidative stress (ROS), advanced glycation end products (AGEs)) further amplify inflammation that ultimately leads to maladaptive tissue damage (*red arrows*)

To date very few randomised controlled trials have been performed that focus on the long-term beneficial effects of supplementation to prevent the development of comorbidities and microvascular complications in DM patients. The evidence supporting the potential beneficial effects of supplements is mainly based on animal studies with various methods for inducing DM: high-fat diet (prediabetes), genetic mutation (ob/ob and db/db strains) and streptozotocin administration (type 1 DM)—each of which has its own advantages and disadvantages. Nonetheless, these ex vivo studies demonstrate that preconditioning or pre-treatment of diabetic MSCs with various agents can be used to optimise/improve cellular function prior to their use in cell therapy (Table 12.1). Whilst several of these studies report positive data, their focus has been mainly on mechanisms of MSC rejuvenation. However, there is a paucity of information available on the effectiveness of supplements to prevent the functional decline of MSCs over a prolonged period

Table 12.1 Overview of studies: preconditioning as strategy to improve MSC function in the context of DM

Stem cell type	Disease model (stem cell pathogenesis)	Intervention	Study design	Outcome: MSC function	Outcome: post-transplantation	Ref
NFκB blocking						
Bone marrow MSCs	Mouse: Non-obese diabetic (NOD)	SN50 (p65 inhibitor)	*Ex vivo:* Viability, proliferation, apoptosis with and without blocking of NFκB signalling	*NFκB inhibition:* ↑ proliferation, ↑ colony forming, ↓ apoptosis **REJUVENATE**	N/A	[69]
Bone marrow MSCs	Rats: Diabetic (Goto-Kakizaki) vs. wild-type (Wistar)	NFκB suppression through BMAL1 overexpression	*Ex vivo:* Osteogenesis, viability, colony-forming, senescence with and without BMAL1 overexpression or inhibition	*NFκB suppression:* ↑ osteogenesis, ↓ osteoclastic induction *BMAL1 overexpression:* Restore homeostasis of bone metabolism **REJUVENATE**	N/A	[70]
Antioxidants & anti-inflammatory agents						
Bone marrow MSCs	Mouse: Obesity-type 2 DM (ob/ob) vs. wild-type (C57BL/6 J)	NAC + AAP	*Ex vivo:* Paracrine response upon stimulation with wound fluid with and without preconditioning	*Preconditioning:* ↓ excessive TNFα release and ↑ IL10 in the presence of wound fluid **REJUVENATE**	*Application:* Wound healing (not transplanted)	[50]
Bone marrow MSCs	Mouse: Obesity-type 2 DM (ob/ob) vs. wild-type (C57BL/6 J)	NAC + AAP	*Ex vivo:* Viability, proliferation, migration with and without preconditioning Stimulation with wound fluid	*Preconditioning:*↑ ex vivo viability and protected MSCs against the toxicity of chronic wound fluid Could not fully restore proliferation capacity **REJUVENATE**	*Application:* Wound healing (not transplanted)	[54]
Bone marrow MSCs	Mouse: Diabetes (STZ-induced type 1 DM) vs. wild-type (C57BL/6 J)	NAC	**Hydrogen peroxide**-induced in vitro injury	*Preconditioning:* ↑ upregulate pro-survival genes, ↓ apoptosis, ↑ viability, ↓ senescence, ↓ oxidative stress **PROTECT**	N/A	[56]
ADSCs (human)	N/A	NAC + AAP	**Hydrogen peroxide**-induced in vitro injury	*Preconditioning:*↓ ROS generation, ↑ stabilise mitochondrial membrane potential, ↓ mitochondrial fragmentation, ↓ apoptosis **PROTECT**	N/A	[73]

Bone marrow MSCs (rat)	N/A	NAC	**Hydrogen peroxide**-induced in vitro injury	*Preconditioning:* ↑ GSH levels, ↓ROS, ↓ apoptosis, ↓ senescence **PROTECT**	*Application*: Rat femur defects – collagen-sponge + pre-treated MSCs, ↑ bone formation	[74]
Bone marrow MSCs	Mouse: Diabetic (db/db) vs. wild-type (C57BL/6 J)	NAC Nox4 siRNA	*In vivo:* Transplantation of diabetic MSCs (db/db) into wild-type C57BL/6 mice with hindlimb ischaemic insult *Ex vivo*: Pre-treatment of MSCs from db/db mice with NAC or NOX4 siRNA	*Preconditioning:* ↓ oxidative stress, ↓ adipogenic differentiation, ↑ endothelial differentiation **REJUVENATE**	*Application*: Post-ischaemic neovascularisation (hindlimb ischaemia)	[113]
Bone marrow MSCs (human)	N/A	Astragaloside IV (AS-IV)	**High-glucose** (25 nM) culture conditions	*Preconditioning:*↑ proliferation, ↓ TLR4 expression, ↓ NFκB p65 translocation, ↑ MMP2 expression **PROTECT**	N/A	[79]
ADSCs	Rat: Wistar (STZ-induced type 1 DM)	RSV	*Ex vivo*: Viability with and without preconditioning *In vivo*: Autologous ADSC transplantation with and without preconditioning	*Preconditioning*: ↑ expression of p-Akt, ↑ viability **REJUVENATE**	*Application:* Pancreatic damage (↑ pancreas function, ↓ blood glucose levels)	[77]
ADSCs	Rat: Wistar (STZ-induced type 1 DM)	RSV	*Ex vivo*: Viability under **high-glucose** culture conditions with and without preconditioning *In vivo*: Autologous ADSC transplantation with and without preconditioning	*Preconditioning:* ↑ viability, ↑ IGF secretion **REJUVENATE**	*Application*: DM liver damage (↓ fibrosis)	[114]
Bone marrow MSCs (wild-type mouse)	N/A	Phytocannabinoids: THCV, CBD, CBG, CBDA, CBGA	**Palmitate** treatment to induce insulin resistance	*Preconditioning:* ↑ colony-forming capacity, ↑ viability (CBD, CBDA,CBGA,THCV), ↑ adipogenesis (CBG, CBD) **PROTECT**	N/A	[78]

(continued)

Table 12.1 (continued)

Stem cell type	Disease model (stem cell pathogenesis)	Intervention	Study design	Outcome: MSC function	Outcome: post-transplantation	Ref
Bone marrow MSCs	N/A	EGF + Curcumin	*Ex vivo*: Culture MSCs in the presence or absence of curcumin + EGF polymeric matrix *In vivo:* MSCs + curcumin + EGF bandage used to treat full thickness diabetic skin wound	*Preconditioning:* ↑ expression of Oct3/4, Sox2, and Nanog, ↑ viability **OPTIMISE FUNCTION**	*Application*: Wound healing (↑ wound closure, ↑ granulation tissue formation)	[83]
Bone marrow MSCs	Rats: Sprague Dawley (high-fat diet vs. normal diet, STZ-induced type 1DM)	Curcumin	*Ex vivo:* Culture MSCs in the presence of diabetic serum with or without preconditioning *In vivo:* Scaffold with curcumin-releasing microspheres + MSCs	*Preconditioning:* ↑ viability, ↑ migration, ↓ ROS, ↑ osteogenic differentiation **REJUVENATE**	Application: Bone defects (↑ bone formation)	[87]
Bone marrow MSCs	Rats: Sprague Dawley (STZ-induced type 1 DM) vs. wild-type	Oxytocin or curcumin or carvedilol or rosuvastatin	*Ex vivo*: Viability, proliferation, angiogenic capacity with and without preconditioning *In vivo:* Transplantation of preconditioned MSCs into myocardial infarction	*Oxytocin preconditioning*: ↑ proliferation, ↑ tube formation, ↑ KLF2 expression, ↑ angiogenesis **REJUVENATE**	*Application*: Myocardial infarction (↑ cardiac function, ↓ fibrosis)	[115]
MSC (cell line)	N/A	Salidroside	*In vitro:* **High-glucose** (25 nM) cultures with or without preconditioning *In vivo:* Transplantation of preconditioned MSCs into full thickness diabetic skin wounds	*Preconditioning:* ↑ survival, ↓ ROS, ↑ HO-1, ↑ FGF2, ↑ HGF, ↑ migration **PROTECT/OPTIMISE FUNCTION**	*Application:* Type 1 DM wounds in mice (↑ healing)	[116]
Other factors						
Bone marrow MSCs	Mouse: C57BL6/J (STZ-induced type 1 DM)	IGF1 + FGF2	*Ex vivo:* Anoxic and **high-glucose** culture (to mimic diabetic heart microenvironment) with and without preconditioning	*Preconditioning:* ↑ SOD activity, ↓ apoptosis, ↑ angiogenic potential, ↑ chemotactic mobility **REJUVENATE**	N/A	[117]
ADSCs (human)	N/A	Hyaluronic acid + butyric acid + retinoic acid	*Ex vivo:* Angiogenesis, angiogenic gene expression *In vivo:* Transplantation of co-cultured ADSCs + pancreatic islets	*Preconditioning:* ↑ VEGF, ↑KDR, ↑HGF **OPTIMISE FUNCTION**	*Application:* Type 1 DM rats (↑ engraftment, ↑ glycaemic control, ↑ vascularisation)	[118]
Bone marrow MSCs (mouse)	N/A	SDF1α	*Ex vivo:* Viability, migration with and without preconditioning *In vivo:* Transplantation of preconditioned MSCs into type 1 DM mice	*Preconditioning:* ↑ proliferation, ↑ migration **OPTIMISE FUNCTION**	*Application:* Type 1 DM rats (↑ homing to pancreas, ↑ pancreatic islet structure)	[119]

ADSCs (human)	N/A	SDF1α	*Ex vivo:* Hypoxic and serum-depleted culture conditions *In vivo:* Transplantation of preconditions ADSCs into type 1 DM mouse wounds	*Preconditioning:* ↓ apoptosis, ↑ viability **PROTECT/ OPTIMISE FUNCTION**	*Application:* Type 1 DM mouse wounds (↑ healing)	[120]
Bone marrow MSCs (rat)	N/A	Fluoxetine	*Ex vivo*: Viability, angiogenesis, neural marker expression *In vivo*: Transplantation of preconditioned MSCs into type DM rats with neuropathy	*Preconditioning:* ↑ viability, ↑ paracrine activity (↑ BDNF, ↑ VEGF), ↑ immunomodulation (↑IL10, ↓ IL1β) **OPTIMISE FUNCTION**	*Application*: Type 1 DM rats diabetic neuropathy (↑ neuronal histomorphology)	[121]
Bone marrow MSCs	N/A	Melatonin	*Ex vivo:* Proliferation with and without preconditioning *In vivo:* Transplantation of preconditioned MSCs into type 1 DM rats	*Preconditioning:* ↑ proliferation, ↑ SOD enzyme, ↑ IL10, ↑ beclin 1 **OPTIMISE FUNCTION**	*Application:* Type 1 DM rats nephropathy (↑ kidney function)	[122]
MSCs (cell line)	N/A	EPO Rapamycin	*In vitro:* **High-glucose** (25 nM) cultures with and without preconditioning *In vivo:* Transplantation of preconditioned MSCs into type 1 DM mice	*EPO Preconditioning:* ↑ VEGF, ↑ proliferation, ↑ migration, ↓ TNFα **OPTIMISE FUNCTION**	*Application:* Type 1 DM mouse wounds (↑ angiogenesis)	[123]
ADSCs (cell line)	N/A	Metformin	*In vivo:* Transplantation of preconditioned ADSCs into high-fat diet induced mice	N/A **OPTIMISE FUNCTION**	*Application:* High-fat diet induced mice (↑ glucose metabolism, ↓ dyslipidaemia)	[124]
Bone marrow MSCs	N/A	Deferoxamine	*In vitro:* Chemotaxis with and without preconditioning *In vivo:* Transplantation of preconditioned MSCs into type 1 DM rats	*Preconditioning*: ↑ HIF1α, ↑ CXCR4, ↑ MMP2/9, ↑ migration **OPTIMISE FUNCTION**	*Application*: Type 1 DM rat (↑ homing to pancreas)	[125]

The table includes studies that have used preconditioning with a specific pharmacological or natural agent (not biologicals) to either restore the function of impaired diabetic MSCs (rejuvenate) or to improve the function of MSCs in the context of DM. **Abbreviations:** AAP, ascorbic acid 2-phosphate; ADSCs, adipose tissue-derived stem cells; BDNF, brain-derived neurotrophic factor; BMAL1, brain and muscle ARNT-like 1; DM, diabetes mellitus; EGF, epidermal growth factor; EPO, erythropoietin; FGF, fibroblast growth factor; GSH, glutathione; HGF, hepatocyte growth factor; HIF1α, hypoxia-inducible factor 1 alpha; HO-1, heme oxygenase-1; IGF, insulin-like growth factor; IL, interleukin; KLF2, Krüppel-like factor 2; MSCs, mesenchymal stem cells; MMP, metalloprotease; N/A, not applicable; NAC, N-acetylcysteine; NFκB, nuclear factor kappa B; NOD, non-obese diabetic; ROS, reactive oxygen species; RSV, resveratrol; SDF1α, stromal-derived factor 1 alpha; SOD, superoxide dismutase; STZ, streptozotocin; TLR4, Toll-like receptor 4; TNFα, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor

and demonstrated using in vivo models. For the most part, the evidence supporting the protective effects of supplementation is based on in vitro models with acute supraphysiological onslaughts using various MSC types, but without the presence of any underlying disease.

DM is a complex disorder, the pathogenesis of which involves the accumulation of advanced glycation end products (AGEs), generation of excessive reactive oxygen species (ROS) and persistent inflammation. The combination of these leads to DNA damage, mitochondrial fragmentation, defects in cellular membrane repair and senescence in bone marrow MSCs [12, 56–58]. Intervention strategies (other than proper glycaemic control) should thus focus on counteracting all of these elements. It is however very difficult to simulate the DM microenvironment in culture to investigate the potential protective effects of therapeutic agents.

High-glucose (25 mM) culture conditions are often used to simulate hyperglycaemia and have been shown to effectively induce oxidative stress and pro-inflammatory signalling in various MSC cultures [59–61]. The responses of different MSC types to these conditions have been inconsistent and do not always represent the endogenous characteristics of diabetic MSCs. Adipose tissue-derived MSCs (also known as ADSCs) seem to retain their characteristics and stemness under high-glucose culture conditions [60, 62], whereas DM patient-derived ADSCs have impaired viability, proliferation and an altered secretome [58]. Bone marrow-derived MSCs are however more sensitive to glucose toxicity. Despite an improved growth rate, a reduction in stemness, increased apoptosis/senescence and impaired multilineage differentiation capacity are evident in bone marrow and nucleus pulposus (vertebral disc)-derived MSCs in hyperglycaemic cultures [59, 63, 64]. In contrast, other studies show that despite limiting the osteogenic differentiation capacity, high-glucose culture conditions have a limited impact on proliferation and stemness of compact bone-derived MSCs [65]. It is thus important to note the MSCs' tissue of origin when interpreting data, especially since the various MSC subtypes have different propensities for immunomodulation. It is also noteworthy that these high-glucose culture models involve acute hyperglycaemia exposure in isolation, whilst failing to consider the dyslipidaemia that is prominent in the metabolic dysfunctional DM patient.

The phytomedicine studies that do focus on metabolic dysregulation have mainly been on targeting ADSCs for the purpose of developing anti-obesity treatments. In these studies, researchers aim to reduce the proliferation of ADSCs and limit adipogenic differentiation [8, 66–68]. This strategy is however not without risk, since interfering with the body's natural fat storage capacity can lead to ectopic lipid accumulation in a variety of tissues. The aim should rather be on preventing senescence and SASP (the excessive release of release of pro-inflammatory cytokines) to limit adipocyte hypertrophy and M1 macrophage accumulation in adipose tissue. Very few studies however focus on strategies to improve the immunomodulatory capacity of endogenous ADSCs.

The following sections will discuss the limited data available on the most promising potential intervention strategies targeting MSCs.

12.5 Interventions

12.5.1 Pharmacological Blocking of NFκB Signalling

Various in vitro studies have demonstrated the potential benefit of different NFκB antagonists in the rejuvenation of diabetic MSCs by counteracting the hyperactivation of the NFκB signalling pathway. Gu et al. [69] indicated that the proliferation and colony-forming capacity of impaired bone marrow MSCs derived from non-obese diabetic mice could be restored by treating the cells with a NFκB p65 inhibitor to attenuate the aberrant activation of p65 and downstream p53/p21 signalling. In the context of impaired bone healing in DM, inhibition of NFκB has been shown to restore the osteogenic differentiation potential of MSCs [70] and to reinstate their ability to resolve inflammation through TGFβ release and subsequent M2-macrophage polarisation (tamoxifen-

induced inhibition of NFκB) [71]. Given the important role of normal NFκB signalling in maintaining tissue homeostasis, permanent inhibition thereof in vivo might cause serious adverse effects, as illustrated in a study by Zhang et al. [72]. The authors attempted to protect mice against age-associated metabolic dysregulation by blocking NFκB signalling in skeletal muscle (IκBα overexpression), and although it protected aged animals against insulin resistance, it was detrimental to muscle function. There was however no underlying pathology associated with excessive NFκB activation in these animals, and it is unclear if similar unwanted effects will be evident with tissue-specific NFκB inhibition in DM mice.

Given the risk of adverse effects, anti-inflammatory agents that suppress NFκB signalling—but do not completely block its activity—might be a safer and more feasible option. To date the most promising agents in this category seem to be potent antioxidants which also have anti-inflammatory properties. Numerous in vitro studies performed on MSCs have indicated the protective effects of antioxidants such as N-acetylcysteine (NAC) and ascorbic acid-2-phosphate (AAP) against acute hydrogen peroxide-induced oxidative damage [56, 73, 74]. In support of these findings, our group demonstrated that a combination of NAC/AAP treatment can improve the ex vivo viability of diabetic bone marrow-derived MSCs and the release of anti-inflammatory cytokines, although it could not restore the proliferation capacity of severely impaired MSCs [50, 54]. This suggests that although these antioxidants can partially rejuvenate diabetic MSCs, they hold more promise as protective agents (preventative supplements) against the functional decline of MSCs in patients to limit disease progression—a potential benefit which warrants further investigation. Similarly, there are numerous natural antioxidants and anti-inflammatory agents in the phytomedicine sphere that hold therapeutic promise.

12.5.2 Natural Antioxidants and/or Anti-inflammatory Agents

Various natural products are commonly used as daily antioxidant supplements, some of which (such as the polyphenol anthocyanidins, resveratrol and phytocannabinoids) have been shown to modulate the functional properties of MSCs [75–78]. Similarly, traditional Chinese phytomedicines (specifically astragaloside IV—the active ingredient in the leguminous herb *Astragalus membranaceus*) have been reported to restore the proliferative capacity of DM patient-derived MSCs under hyperglycaemic culture conditions by decreasing TLR4 expression upstream of NFκB signalling [79]. Unfortunately, despite these positive results, very few of these products have been subjected to in-depth (or in vivo) investigation to elucidate specific mechanisms of action involved, and even less information is available in the context of age-associated inflammatory conditions. A comprehensive review of each of these products is not within the scope of this review. Rather, we will discuss only one natural antioxidant (the polyphenol curcumin)—one of the very few which have been extensively reported on—to illustrate the potential benefits of natural antioxidants specifically on MSC function.

In the context of obesity-associated inflammation, curcumin was recently reported to inhibit adipogenic differentiation of human bone marrow MSCs *via* downregulation of peroxisome proliferator-activated receptor (PPAR)-γ signalling [80]. PPAR-γ is known to direct differentiation of cells towards pro-inflammatory phenotypes [81], suggesting that curcumin may facilitate a relatively more anti-inflammatory phenotype in MSCs. In line with this and being highly relevant given our discussion in the previous section, curcumin was also reported to block NFκB signalling in gastric cancer-derived MSCs [82]. These mechanistic studies are supported by demonstrated in vivo benefit. For example, very recently, enhanced diabetic wound healing was

reported after topical application of a bandage containing bone marrow MSCs, epidermal growth factor (EGF) and curcumin in a DM rat model [83]. This is in line with a recent review of human clinical studies in the context of curcumin supplementation, where it was concluded that curcumin may have beneficial action in the context of obesity, metabolic syndrome, DM as well as other chronic inflammatory conditions [84]. Together, these studies suggest that at least some of the beneficial outcomes ascribed to curcumin may be *via* its facilitation of an anti-inflammatory phenotype in MSCs.

A limiting factor when it comes to translating these pre-clinical findings into human models may at least in part be due to the poor absorption and bioavailability of plant products such as curcumin [85, 86]. However, with the advances being made in nanoscience and controlled drug delivery systems, these limitations may soon be overcome. A relatively recent study demonstrated superior MSC-dependent bone repair in DM rats when the repair scaffold was loaded with curcumin, with even more enhanced repair when curcumin was delivered using PLGA microspheres to optimise delivery. Most relevant to the current topic, curcumin delivered in this way was shown to exert protective effects on bone marrow MSCs under DM conditions *via* upregulation of the endogenous Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid-2-related factor-2 (Nrf2)/heme oxygenase-1 (HO-1) antioxidant signalling pathway and inhibition of ROS secretion by MSCs [87].

The importance of using suitable delivery systems when targeting stem cells with natural antioxidants is clear from the literature. Several groups have however described a risk for pro-oxidant toxicity [9, 88–90] after treatment with high doses of antioxidants to account for low bioavailability. In the context of stem cells specifically, the well-published antioxidant polyphenol resveratrol was recently shown to affect human bone marrow MSC differentiation at low concentrations by mimicking insulin activity in differentiation media—however, high doses of resveratrol resulted in lipid accumulation in both osteogenic and adipogenic media [91]. Furthermore, undifferentiated neuronal stem/progenitor cells were illustrated to have increased sensitivity to antioxidant (ascorbic acid)-driven DNA damage than differentiated cells [92]. This suggests that the risk for pro-oxidant effect is also an important consideration at the level of MSCs. Taken together it is thus clear that although phytomedicines hold tremendous promise as supplements, their use is not without risk, especially since there is limited data available on their mechanisms of action and long-term safety.

12.5.3 Biological Agents

12.5.3.1 Microvesicles and Exosomes

MSC-derived extracellular vesicles—which include both microvesicles (size: 100–1000 nm) and exosomes (size: 30–100 nm)—are thought to play a crucial role in the paracrine signalling of MSCs over long distances [93]. Although the study of exosomes in the context of regenerative medicine and immunomodulation is a relatively young research niche, a fair number of reports exist and have contributed to our understanding of how MSCs may remotely affect regenerative processes. To date the majority of research in this area has been focused on the potential use of allogeneic exosome preparations for transplantation purposes. The use of exosomes instead of intact allogeneic MSCs for therapeutic application holds numerous advantages such as high stability, standardisation and ease of in vivo delivery, as well as decreased risk of unanticipated effects.

In a recent review, Casado-Diaz et al. [94] concluded that transplantation of MSC-derived exosomes resulted in faster and more optimal healing of diabetic and age-associated skin ulcers by modulating inflammation (most notably *via* suppression of IL1β and TNFα) and reducing scar formation (suppression of TGFβ) [94]. The cargoes within these extracellular vesicles are however crucial to ensure optimal target effects. In-depth sequencing studies are attempting to elucidate specific exosome constituents of importance whilst others focus on engineering exosome content for a greater therapeutic effect. For example, ADSC-derived exosomes containing

high concentrations of the circular RNA (circRNA), *mmu_circ_0000250*, were recently shown to promote wound healing in diabetic mice to a greater extent than exosomes with a low concentration of this specific circRNA. The mechanisms of action were thought to be related to upregulation of sirtuin-1 expression and absorption of miR-128-3p to promote angiogenesis and suppress apoptosis [95, 96]. Similarly, injecting exosomes containing the long noncoding RNA H19 (*lncRNA H19*) into diabetic foot ulcers in a mouse model limited inflammation *via* upregulation of the phosphatase and tensin homolog (PTEN) protein and, by doing so, promoted healing [97]. Of specific interest, Liu et al. [98] demonstrated that highly purified extracellular vesicles derived from human induced pluripotent stem cells (iPSCs) were able to alleviate the ageing phenotype of senescent MSCs by reducing intracellular ROS production through delivery of peroxiredoxin antioxidant enzymes. Together, these reports highlight the potential of exosome therapy in the context of age-related chronic inflammatory diseases.

The choice of stem/progenitor cells from which to prepare exosomes for therapeutic intervention is however an important consideration since ageing- or disease-linked maladaptation can extend to the extracellular vesicles and alter their content. A comparison of bone marrow MSC-exosome preparations derived from either healthy or type 1 DM rats indicated that diabetic MSC-exosomes have an impaired ability to promote bone repair [99]. Similarly, pancreas-derived MSCs were implicated in the secretion of exosomes carrying the auto-immune antigen/trigger causing diabetes in non-obese diabetic (NOD) mice [100]. These exosomes could transfer the diabetogenic phenotype to healthy mice, which elucidates the caution that should be exercised in the choice or preparation of MSC-derived exosomes. On a more positive note, preconditioning of MSCs with LPS resulted in exosomes with a greater capacity to induce an anti-inflammatory macrophage phenotype (M2) and upregulated the expression of anti-inflammatory cytokines [101], confirming that exosome manipulation for therapeutic benefit may indeed be possible.

12.5.3.2 Probiotics: An Emerging Therapeutic Hope?

A very novel area of research with potential therapeutic application is MSC-microbe interactions. It was recently shown that canine ADSCs assume a pro-inflammatory phenotype in response to direct contact (either cell surface binding or host cell invasion) with the pathogen *Salmonella typhimurium* as well as the commonly known probiotic *Lactobacillus acidophilus* [102]. More specifically, microbial exposure upregulated gene expression of inflammatory mediators, IL6, IL8 and cyclooxygenase 2 (COX2) and increased secretion of IL6, IL8 and PGE2, without affecting markers indicative of humoral immune activation (major histocompatibility complex (MHC)-II, CD80, CD86, CD1). The study further demonstrated that chemotactic migration of ADSCs in a Matrigel-transwell system was inhibited by the pathogen, but not the probiotic. This chapter provided mechanistic evidence for the pro-inflammatory role of MSCs in situations where systemic inflammation is insufficient (as discussed and illustrated in this review), as well as qualifying the claim for "immune-boosting" effects often made for probiotics. The same study further reported that it was possible to tolerise the ADSCs by pre-exposure to these microbes, to result in a dampened inflammatory response to subsequent microbial exposure. Importantly, neither the pathogen nor the probiotic resulted in either cell death, degeneration or compromised proliferation in the ADSCs [102]. In support of this study, other reports are emerging that provide indirect proof of the potential low-risk therapeutic benefits of probiotics at the level of MSCs. Preconditioning of gingival MSCs with the *Lactobacillus reuteri* secretome (rich in the specific active reuterin) has been shown to improve their migration, proliferative and osteogenic differentiation capacity, which in turn resulted in significantly faster wound healing (full thickness gingival wounds). A parallel in vitro experiment on these gingival MSCs revealed that the probiotic exerted its effect *via* activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway to increase β-catenin-dependent upregulation of TGFβ and metalloprotease (MMP)-1 expression

[103]. The PI3K/Akt pathway is central to chronic inflammatory disease pathology, but the significance of these findings remains to be evaluated in suitable models of inflammatory pathology.

Similar to the findings above but more relevant to DM, murine skin-derived precursor cells (SKPs) (which have the ability to differentiate into the mesenchymal lineages in vivo) [104] were very recently reported to exhibit improved proliferation and self-renewal capacity after exposure to the secretome of the commensal lactic acid bacterium *Enterococcus faecium* L-15 [105]. Of interest to the topic of DM, the survival of this specific probiotic bacterium in the gut is disrupted *via* competitive inhibition by *Enterococcus faecalis* [106], which grows relatively more abundantly as part of the gut dysbiosis characterising diabetes [107] and is commonly implicated in wound infection in diabetic foot ulcers [108, 109]. Current probiotic treatment in DM is however focused on the genera *Lactobaccillus* and *Bifidobacteria*, as *E. faecium* does not affect glucose metabolism (although it has positive effects on lipid profile) [110]. This relative unavailability of *E. faecium* in DM may, at least in part, contribute to the poorer MSC performance seen in diabetic wound healing—and may be corrected with probiotic conditioning of diabetic MSCs. More research in this context is clearly warranted.

The studies discussed up to this point, although encouraging, have been limited to experimental models and, importantly, have been conducted in the absence of inflammatory or stem cell pathology. To our knowledge, reports of direct benefits of probiotics on MSCs in the context of chronic inflammatory disease do not yet exist. However, in the context of tenofovir-associated osteoporosis (mouse model), bone-derived MSC proliferation and osteogenic capacity were rescued after supplementation with the probiotic *Lactobacillus rhamnosus* GG [111], providing some support for successful translation of these data to conditions of pathology.

12.6 Conclusion

From our search of the literature, it is clear that targeted stem cell therapy using either pharmacological agents, natural products or biological agents, at least in the context of chronic inflammatory disease, is still in its infancy. The above sections do however clearly suggest potential for the use of supplements and/or biologicals to target MSCs in the context of chronic inflammatory disease such as DM. It also highlights the importance of an integrated systems approach and thorough understanding of complete disease pathology, in order to identify feasible candidates. Finally, in terms of patient safety, recent advances in technology—such as MSC extracellular vesicle isolation, or the synthesis and purification of probiotic peptides *via* homologous expression systems [112]—will enable (low-risk) cell-free treatments specifically designed to address very specific targets on pathological MSCs.

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13 Therapeutic Strategies and Nano-Drug Delivery Applications in Management of Aging Alzheimer's Disease

Thuy Trang Nguyen, Tuong Kha Vo, and Giau Van Vo

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder in which the death of brain cells causes memory loss and cognitive decline. Existing drugs only suppress symptoms or delay further deterioration but do not address the cause of the disease. In spite of screening numerous drug candidates against various molecular targets of AD, only a few candidates, such as acetylcholinesterase inhibitors, are currently utilized as an effective clinical therapy. Currently, nano-based therapies can make a difference, providing new therapeutic options by helping drugs to cross the blood-brain barrier and enter the brain more effectively. The main aim of this review was to highlight advances in research on the development of nano-based therapeutics for improved treatment of AD.

Keywords

Alzheimer's disease · Nanotherapeutic · Molecular targets · Oxidative stress · CNS

13.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that involves loss of memory, thinking, and reasoning and several behavioral characteristics in elderly people. Approximately 47 million people suffer from dementia worldwide. This figure is anticipated to increase to more than 131 million by 2050 [1]. A complex of insoluble accumulations of beta amyloid protein (Aβ) plaques and tau neurofibrillary tangles in the brain suggests that these play a role in the pathology of AD [2–5]. However, the precise mechanism of how Aβ potentiates pathogenesis in AD is still not completely understood [6–9], while a vast number of studies on the

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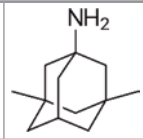
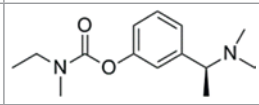
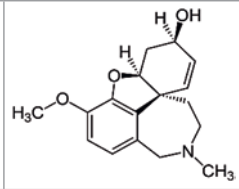
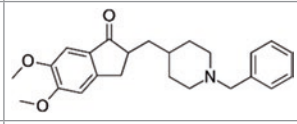
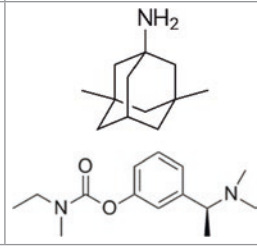
presence of crosstalk between Aβ and many molecular signaling pathways have been done. Therefore, strategies for early detection as well as treatment of AD are among the most challenging and timely areas in modern medicine.

Currently, the most common strategies aim to treat AD through cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. So far, memantine, donepezil, rivastigmine, and galantamine are the only drugs in use which have obtained Food and Drug Administration (FDA) approval for treatment of AD (Table 13.1). However, these drugs only have marginal effects on reducing disease symptoms or in delaying further deterioration, as they do not target the underlying pathology [10].

In addition, the blood-brain barrier (BBB) is a formidable gatekeeper which prevents materials from the blood from entering the brain. However, the BBB is semi-permeable as it allows some materials to cross, but prevents others from traversing it. The development of nanotechnology aims to overcome this problem through tethering of AD drugs to surface-modified nanoparticles (NPs). Nanomedical approaches provide new therapeutic options by helping drugs to enter the brain, thereby facilitating targeting of the direct cause of the disease. Crossing the brain-cerebrospinal fluid barrier requires specific targeting ligands, which should be smaller and more stable than conventional approaches.

To cross the BBB and deliver a drug into the brain, various alternative strategies are required to effectively target Aβ production, aggregation, and clearance, as well as tau phosphorylation and assembly into neurofibrillary tangles [11, 12]. These include lipidic, polymeric, inorganic, and other types of NPs. Nanoparticle development marks a crucial step in development of nano-based drug delivery systems for direct targeting of pathological processes in the relevant tissues. In order to raise awareness for

Table 13.1 FDA-approved drugs for AD treatment

Generic name	Chemical structure	Brand name	Stage of AD approved	Principally targeted
Memantine		Namenda	Moderate to severe	NMDA receptor antagonist
Rivastigmine		Exelon	Mild to moderate	Acetylcholinesterase and butyrylcholinesterase inhibitor
Galantamine		Razadyne	Mild to moderate	
Donepezil		Aricept	All stages	Selective acetylcholinesterase inhibitor
Memantine + donepezil		Namzaric	Moderate to severe	Combined action

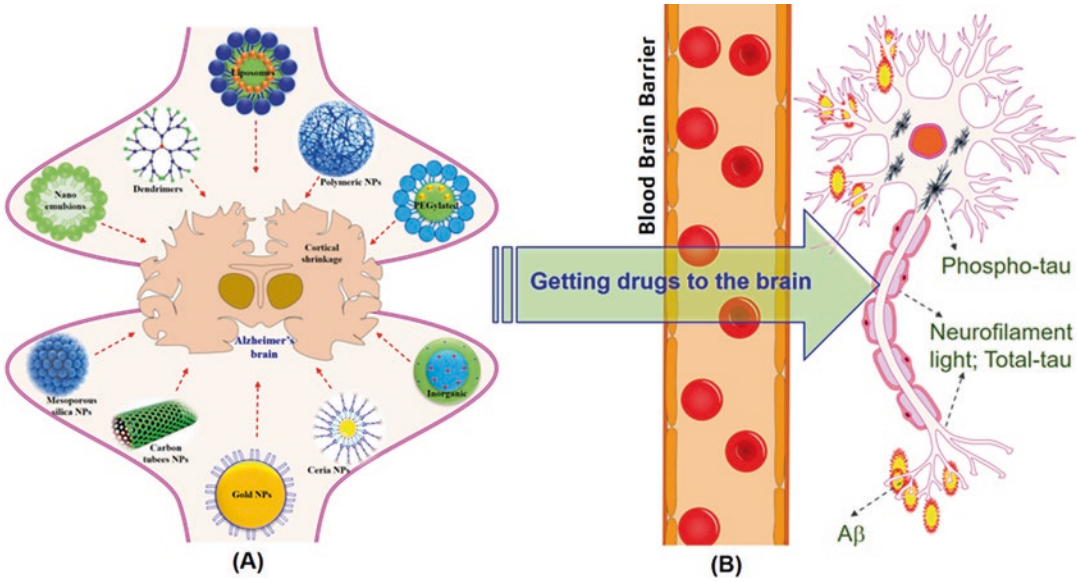


Fig. 13.1 Current approach to the treatment of Alzheimer's disease using nanocarriers

the potential of nanomedicine in combating AD, the current review highlights the most recent advances on production and testing of nano-based strategies for AD treatment (Fig. 13.1a).

13.2 Targeting the Amyloid Cascade

Aβ fibrillar formation and plaque clearance are considered to be the primary target in the treatment of AD pathogenesis based on the amyloid cascade hypothesis. Due to high binding affinity toward the $A\beta_{1-42}$ peptide (the "sticky" form of Aβ which leads to plaque formation), several lipid-based NPs have been developed as potential treatments. Gobbi et al. developed a liposome functionalized with phosphatidic acid and cardiolipin to effectively target the $A\beta_{1-42}$ form of Aβ with high affinity [13]. Intraperitoneal injection of this molecule resulted in decreased levels of Aβ in the brains of the APP/PS1 transgenic mice, a widely used model of AD [14]. Another study used curcumin-loaded nanospheres and found decreased Aβ load in the brains and an amelioration of the memory deficits in an AD mouse model [15]. Recently, a dopamine-melatonin nanocomposite (DM-NC) was developed that could be activated to release the melatonin cargo by a near-infrared and photothermal effect [16]. This led to inhibition of Aβ nucleation, self-seeding, and propagation as well as disruption of preformed Aβ fibers in neuroblastoma cells and in the brains of an AD mouse model. In addition, Karthivashan et al. reported on the development of NP-functionalized monoclonal antibodies that could target Aβ fibril formation [17]. Another study used an antibody-coated NP to effectively target cerebrovascular Aβ in the Tg2576 mouse model of AD [18]. In addition, several approaches using organic molecules to coat inorganic nanoparticles have been used to target Aβ cascades in AD. Another study managed to enhance drug delivery across the BBB using a molecular Trojan horse such as a transferrin receptor-MAb [19], and lipoprotein bodies have also been developed which can deliver biomaterials to targeted sites within the brain for diseases such as AD [20].

13.3 Nanomaterials Against Amyloid Diseases

Amyloid fibrils have good affinity for nanoparticles (NPs) through residue coordination. Therefore, drug-based NPs have an intrinsic propensity for binding to amyloid proteins. This effect is controlled by competitive protein-protein versus protein-NP interactions. If protein-NP interaction dominates, then Aβ fibrillation is either accelerated or inhibited [21, 22]. For example, gold (Au)-NPs are a typical nanomaterial that interacts with amyloid proteins based on the nature of their surface ligands. Citrate- and polyethylene glycol (PEG)-conjugated Au-NPs accelerated the fibrillation of Aβ, resulting from the conformational changes in pancreatic islet amyloid polypeptide (IAPP) induced by the Au-NPs, from random coils to α-helices and then to β-sheets [23]. However, when the size of PEG was increased to 3000 Da, the distance between the Au-NP surface and IAPP was increased, thus diminishing the acceleration effect [21]. Similarly, changing the concentration of NPs can induce different effects on amyloid fibrillization. Lago et al. demonstrated that cationic polystyrene NPs accelerated the Aβ fibrillization at lower concentrations but inhibited the fibrillization at higher concentrations [24]. This effect was explained on the basis of a balance between fibrillization of free peptides in the solution versus surface-assisted nucleation and then fibrillization. In addition, curcumin-capped Au-NPs were found to inhibit Aβ fibrillization [25] and cadmium telluride (CdTe)-NPs interacted with Aβ via multiple binding sites to inhibit the peptide fibrillization [26]. Another study showed that the hydrophobic surface of single-walled carbon nanotubes (CNTs) induced deposition of Aβ on their surface in a non-amyloid form [27].

In further studies of carbon-based nanomaterials, graphene quantum dots (GQDs) have been tested in amyloidosis due to their small size, hydrophobic nature, and strong binding with amyloid proteins [28]. Through a combination of hydrophobic interaction, H-bonding, salt bridging, and π-stacking, GQDs were found to inhibit Aβ formation in in vitro and in vivo models due to its conversion from α-helices and β-hairpins to random coils. A number of in vivo studies have tested nanomaterials in amyloid disease. These include polymeric NPs ligated with the lysine-leucine-valine-phenylalanine-phenylalanine (KLVFF) peptide that targeted Aβ and cleared the latter from the brain of an AD mouse model [29]. This amino acid sequence corresponds to the Aβ16-20 region which plays a critical role in generation of Aβ fibrils. In addition, lipoprotein-based NPs crafted from apolipoprotein E3 demonstrated cross-talk ability with Aβ and bound and cleared Aβ monomers from transgenic AD mice [30]. All of these approaches faced the challenge of overcoming the complexity of in vivo environments that could render NPs ineffective against amyloidosis. Another major challenge is the lack of suitable animal model systems with translational potential. Therapeutic modalities that show efficacy in mouse models have largely failed to show similar results in human clinical trials [31]. The pathology of amyloid diseases is related to aging and therefore involves multiple pathophysiological pathways. It remains a challenge to establish such translational animal models reflecting the variety of pathophysiological pathways involved in the onset and progression of amyloid-related diseases [32]. NPs offer an opportunity to overcome such problems with their ability to transport bioactive molecules across the BBB thereby reducing toxicity and improving therapeutic efficacy through targeting of the relevant tissues (Fig. 13.1b) [33, 34].

13.4 Nanotechnologies for AD Treatment

13.4.1 Targeting Androgen and Estrogen NPs

The gonadal steroids including estrogens and androgens play a critical role in central nervous system (CNS) development and function [35]. To decrease the risk of AD, estrogen treatment can promote the growth and survival of cholinergic neurons and significantly reduce cerebral amy-

loid deposition [36]. Estradiol-encapsulated PLGA NPs have been used as an alternative approach to prevent AD syndrome [37, 38]. Also, an active anti-progesterone compound, mifepristone (11β-[4-dimethylamino]phenyl-17β-hydroxy-17[propynyl]estra-4,9-dien-3-one), was found to slow down the progression of cognitive decline in AD patients because of its mechanism related to P-glycoprotein (P-gp) transporter-mediated efflux of Aβ [39]. In addition, encapsulation of mifepristone within poly(ethylene glycol)-block-poly(lactide) (PEG-b-PLA) NPs enabled evaluation of the increase in drug bioavailability after oral administration [40]. The use of nano-delivery systems offers a means of overcoming high-dosage requirements that are normally due to poor pharmacokinetics associated with the conventional drug delivery systems [41]. With advancements in technology, the carrier techniques offer additional functionalities such as specific molecular targeting enabling higher efficiencies. For these reasons, the use of NPs as drug delivery systems has been studied in different diseases [42].

Localized drug release has received considerable attention, due to the non-invasive character, reduction of side effects, and improved control over bio-distribution [43]. This has been achieved due to the smaller size of nanomaterials (typically ranging from one to several hundred nanometers) compared with other delivery systems, as well as appropriate charge and a higher surface-volume ratio [43, 44]. In the case of the neuronal system, the BBB is one of the major challenges due to its highly selective semipermeable border that separates the circulating blood from the brain and extracellular fluid in the CNS [45, 46]. With molecular weights less than 400 Da and sizes below 100 nm, the particulates lipophilic nature allows them to pass easily through the BBB through diffusion mechanisms [47]. In addition, the various nanotechnology-based approaches meet this demand by improving efficacy and sustained release of the entrapped drug. Such effects have now been demonstrated in many studies involving use of nanomaterials as drug delivery systems [38, 48–61] (Table 13.2).

13.4.2 Polymeric Nanoparticles

Encapsulation of active compounds, such as perfumes, in polymeric microcapsules is the current method of choice for protection from aggressive environments and sustained release in consumer goods. Although polymeric shells provide robust encapsulation, they do present some drawbacks, like a poor deposition on targeted substrates and a release mechanism restricted to mechanical force [62, 63]. The lactic-co-glycolic acid (PLGA) form of NPs is an example of this for pharmaceutical applications [63]. In addition, a PLGA-block-PEG has been conjugated with triphenylphosphonium (TPP) to form PLGA-b-PEG-TPP in targeting inflammation as an AD treatment [64, 65]. Biodegradable PLGA NPs have also been found to have neuroprotective effects in treatment of AD [66], and coenzyme Q10 (CoQ10)-loaded PLGA NPs have been employed to minimize the cytotoxicity of Aβ and rescued memory in an AD mouse model [67]. In addition, HDL-associated α-tocopherol treated with lipophilic compounds has been shown to be efficiently and selectively taken up in the brain in AD studies [68], and enhanced delivery of plasma apolipoprotein A-I via manipulation of HDL transcytosis has been developed as a treatment for brain disorders [69–71]. Also, specific ligands and antibodies have been conjugated into solid lipid NPs and demonstrated in both in vitro and in vivo studies to reduce Aβ aggregation [72–76].

13.4.3 Inorganic Nanoparticles

Considering the rapidly aging population and the resulting increase in the incidence of dementia, there is an urgent need to address the risk presented by this disease. One way is through the use of NPs that can effectively deliver their therapeutic cargo directly to the brain. Once in the brain, NPs can have major effects, such as amelioration of harmful reactive oxygen species (ROS) activity and reduction of Aβ aggregation behavior as occurs in AD pathophysiology [77]. Some biocompatible NPs have been developed

Table 13.2 Representative NPs for Alzheimer's disease studies

Nanoparticle form	Size (nm)	Structure	Properties	AD targeted	Example
Liposomal	10–100	Spherical vesicles	Biodegradability	Aβ, cholinergic dysfunction	mApoE [48] H102 [49] XO4 [50]
Chitosan	<70	Modified polysaccharides	Biocompatibility Biodegradability	ACh	Tacrine [51] GH [52] Piperine [53]
Synthetic polymeric	100–300	PLGA, PEG	Solubility Permeability	Aβ Tau Estrogen	Estradiol [38] RVG29 [54] D-peptide [55] siRNA [56] shRNA [54]
Gold	1–100	Metal-NPs	Low cytotoxicity	Aβ Tau ACh	Tau-mab [57]
Magnetic	<70	Metal-NPs	Metal-ion chelators	Aβ Tau ROS	TPP [58]
Carbon nanotubes	1–100	Allotropes of carbon	Advanced thermal conductivity and cell penetration	Aβ ACh ROS	Ach [59]
Carbon dots	1–10	Tunable zero-dimension	Photoluminescence Biocompatibility Nontoxicity	Aβ ACh	Transferrin [60]
Curcumin	<100	Natural	Polyphenolic antioxidant, ROS scavenger	Aβ Tau	[61]

and suggested to have therapeutic potential, such as biopolymers, chitosan, gelatin, polymers, and metal-NPs [78]. Many novel agents for minimizing Aβ aggregation and delaying Aβ fibrillation have been developed as monomers [79], gold carriers [80], a magnetic core [81], carbon-based NPs [60, 82, 83], and graphene oxide sheets [82]. For example, a report showed that graphene oxide (GO)/Au-NPs disrupted Aβ aggregation and cytotoxicity in vitro [84] and nano-metallo-supramolecular complexes were found to suppress Aβ-induced biosynthesis of heme and iron uptake in PC12 cells [85]. Quantum dots (QDs), which are colloidal fluorescent semiconductor nanocrystals with a diameter of 3–30 nm, have been used to treat AD by targeting mitochondrial dysfunction [86]. The inhibitor dihydrolipoic acid (DHLA) has been conjugated with QDs which inhibited $A\beta_{1-42}$ fibrillation with rapid kinetics [87].

A novel approach using molybdenum disulfide (MoS2)-NPs was developed and applied to prevent Aβ aggregation and destabilize Aβ fibrils in vivo [88]. In addition, ceric oxide (CeO_2)-NPs have been considered as recyclable ROS scavengers due to their shuttling capacity between Ce^{3+} and Ce^{4+} oxidation states [89, 90]. Therefore, these CeO_2-NPs could be a potential therapeutic candidate for treating mitochondrial oxidative-stress-induced damage in AD. Another study used nasal application of titanium oxide (TiO_2)-NPs as a model of neurotoxicity in mice and found decreased activities of glutathione peroxidase, catalase, and superoxide dismutase [91]. This latter system could be used as a model for the screening of compounds that can delay or ameliorate oxidative damage in the brain. Table 13.3 shows a number of potential NP-based delivery systems to treat various pathophysiological effects observed in Alzheimer's disease [92–104].

Table 13.3 Potential nanoparticle-based delivery systems to treat Alzheimer's disease

Nanoparticle material	Targeting ligand	Bioactive molecule	Particle size (nm)	Zeta potential (mV)	Model	Pharmacological effect	Reference
Nano-LP (DSPC + CH)	TrF-mAb + PEG	Curcumin	110 ± 6		Cell	Retardation of Aβ aggregation	[92]
LP (SPG + CH)	Phosphatidic acid/ cardiolipin	Modulate tau phosphorylation and glycogen synthase kinase 3 activities	102 ± 2	−25.06 ± 1.5	APP/PS1 transgenic mice injection over 3 weeks	Reduced Aβ peptide	[14]
Retro-inverso peptide inhibitor nanoparticles	RI-OR2-TAT + maleimide-PEG	Inhibitors of aggregation of the Alzheimer's Aβ peptide	131 ± 43		APP_{SWE} transgenic mice	Inhibited formation of Aβ oligomers and fibrils in vitro	[93]
Iron oxide	DSPE-PEG-NHS + Congo Red	H_2O_2-responsive therapy of Alzheimer's disease	250–350		APPswe/PS1dE9 transgenic mice	Interfered with Aβ aggregation and neurotoxicity	[94]
Chitosan	Transferrin receptor antibody PEG	Z-DEVD-FMK (caspase-3 inhibitor)	650 ± 2	20 ± 4	Mouse 2-h MCAO and 24-h reperfusion	Decreased infarct volume, neurological deficit, and caspase-3 activity	[95]
Chitosan	Transferrin receptor antibody	Z-DEVD-FMK and bFGF	747 ± 42		Mouse 2-h MCAO and 24-h reperfusion	Decreased infarct volume, increased motor function deficit scores, 3-h therapeutic window	[96]
Cationic bovine serum albumin	PEG	Tanshinone IIA	114 ± 14	−17.8 ± 1.6	Rat 2-h MCAO and 24-h reperfusion	Decreased infarct volume, neurological deficit, neutrophil infiltration, and neuronal apoptosis	[97, 98]
Lipidic (squalene)		Adenosine	120	−25 ± 4	Mouse 2-h MCAO and 22-h reperfusion 24 h of permanent MCAO	Decreased infarct volume, increased neurological deficit scores	[99]
PLA	B6 peptide (transferrin substitute PEG)	NAPVSIPQ (NAP: neuroprotective peptide)	118.3 ± 7.8	−22.65 ± 0.5	Mice injected with aggregated $AB_{1\text{-}40}$	Enhanced drug uptake in brain, ameliorated learning impairments, cholinergic disruption, and loss of hippocampal neurons	[100]
PBCA	Polysorbate 80	NGF	250 ± 30		Rats with an acute scopolamine-induced amnesia	Reversed scopolamine-induced amnesia and improved recognition and memory	[101]

(continued)

Table 13.3 (continued)

Nanoparticle material	Targeting ligand	Bioactive molecule	Particle size (nm)	Zeta potential (mV)	Model	Pharmacological effect	Reference
PLGA	Trimethylated chitosan	Coenzyme-Q10	150	20	APP/PS1 transgenic mice	Enhanced uptake to brain area (cortex, paracele, third ventricle, and choroid plexus epithelium	[102]
BCA	Polysorbate 80	Amyloid affinity drug ^{125}I clioquinol	50 ± 5		APP/PS1 transgenic mice model, mice injected with aggregated $A\beta_{42}$ peptide	High specificity for Aβ plaques both in intro and in vivo, more located at brain region and uptake of NPs in AD mouse models, in addition to promising delivery vehicle in vivo for SPECT or PET imaging	[103]
Carboxyl-conjugated Au-NPs (negative charged)		Aβ fibrillization and alter preformed Aβ fibrils	~30	−38		Disrupted Aβ fibrillation and fragmented the fibrils already formed	[104]

DSPC 1,2-distearoyl-sn-glycero-3-phosphocholine, *CH* cholesterol, *TrF-mAb* anti-transferrin monoclonal antibody, *LP* lyposome, *DSPE-PEG-NHS* 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol)]-hydroxy succinamide, *bFGF* basic fibroblast growth factor, *DVD-FMK* N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone, *MCAO* middle cerebral artery occlusion, *BCA* n-butyl-2-cyanoacrylate, *PEG* poly(ethylene glycol), *NGF* nerve growth factor, *PBCA* poly(n-butyl cyanoacrylate); *PET* photon emission tomography, *PLA* poly(lactic acid), *PLGA* poly(lactic-co-glycolic acid), *SPECT* single photo emission computerized tomography

13.4.4 Mitochondrial Targeting

Mitochondria are vital organelles involved in cell survival and maintenance, which play a central role in energy production and important processes such as apoptosis [105]. Therefore, they have been recognized as a potential therapeutic target in multiple disorders, such as AD. Mitochondrial dysfunction has been shown to trigger AD pathology due to formation of Aβ plaques and neurofibrillary tangles [106]. The findings of other studies have suggested that mitochondrial dysfunction contributed to synaptic abnormalities and neuronal degeneration in AD [107, 108]. Additionally, mitochondrial dysfunction is known to be one of the earliest events in AD [108–110]. In addition, post-mortem brain studies have shown that Aβ accumulates in the mitochondria of AD patients [111–113].

Notably there are likely common mechanisms that link mitochondria and autophagy dysfunction in AD such as aging, immune dysfunction, and others [114–117]. Among common antioxidants, the mitochondrial respiratory chain molecule CoQ10 is known as a potential treatment against excessive ROS production and has been suggested as a potential treatment in AD in both in vitro and in vivo studies [118–120]. Although the molecule is relatively safe and well-tolerated, there have been no published clinical trials of CoQ10 in AD [119]. Studies have shown that the benzoquinone idebenone, which targets mitochondria, inhibited Aβ-induced neurotoxicity in both in vitro and in vivo studies [121, 122]. In addition, other antioxidants such as lipoic acid, vitamins C and E, and glutathione (GSH) have also been investigated in clinical studies of mild cognitive impairment and AD [123–125]. Thus far, lipoic acid has been shown to prevent the decline of cognitive processes in AD patients [126, 127].

Another possibility is to use lipophilic cations such as triphenylphosphonium (TPP) to target mitochondria directly [128] (Fig. 13.2). One study conjugated TEMPOL to the TPP cation to produce MitoTEMPOL. This accumulated in energized mitochondria where it was reduced by direct reaction with mitochondrial ubiquinol. The authors described this as a way of using mitochondria-targeted compounds to modulate the mitochondrial CoQ10 pool in vivo [129].

13.5 Targets for Future Drugs

Aβ is the chief component of plaques, one hallmark of Alzheimer's disease. We now have a detailed understanding of how this protein fragment is cleaved from the amyloid precursor protein (APP) and how the toxic $A\beta_{1\text{-}42}$ fragment is generated. Researchers have therefore attempted to develop new medications aimed at almost every point in the APP processing pathway, including blocking activity of the processing enzymes β- and γ-secretase, blockade of plaque formation, and use of antibodies against Aβ plaques to clear these from the brain [130].

Other treatments are aimed at preventing the tau protein from collapsing and twisting into tangles that destroy neuronal signaling [131, 132]. AADvac1 is a vaccine that stimulates the body's immune system to attack the toxic form of the tau protein [133]. Initial results have shown that 98.2% of participants who were given the vaccine generated antibodies to the tau protein and there were no differences in adverse events between the treatment and control groups. Furthermore, several biomarkers known to be altered in AD showed trends [134], which suggested that AADvac1 may slow disease progression. In line with this, AADvac1 treatment led to positive changes in some cognitive endpoints. Other new treatments target inflammation which appears to be a root cause of the disease [135].

13.6 Conclusion, Challenges, and Future Perspectives

Current pharmacologic research in AD focuses principally on the development of disease-modifying drugs that can slow or prevent AD progression [136]. Advances in the appropriate design and fabrication of NPs can effectively overcome conventional neurotherapeutic hurdles such as oral and gastric barriers, as well as that of

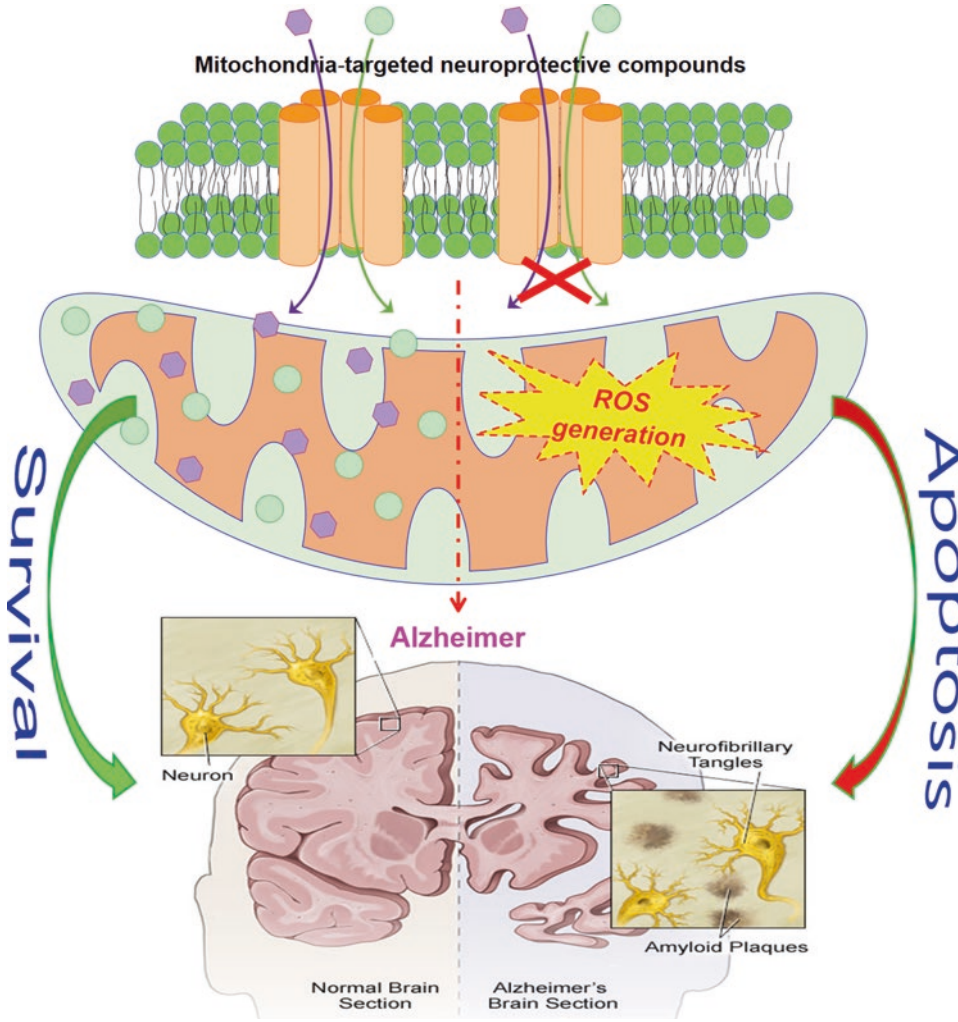


Fig. 13.2 Targeting mitochondrial metabolism

the BBB in the delivery of drug candidates to their intended site of action in biological systems. Alternatively, several studies have revealed that NPs can effectively cross the BBB and exert inhibitory effects. Other polymeric NPs have also been found to have enhanced targeting and efficacy at specific pH values and temperatures [137]. This requires suitability of size, shape, and charge, as well as the correct surface properties of the appropriate biocompatible nanocarrier in targetting the appropriate site or mechanism of action.

Another potenital avenue involves the use of mitochondria-targeted therapeutic interventions that could be translated from in vitro and in vivo studies to human clinical trials. Potential treatments in the future include the use of devices to infuse neurotrophic factors, such as growth factors, to ameliorate AD symptoms and disease progression. It is now clear that Aβ deposition, tau neurofibrillary tangles, and neuroinflammation are involved in the pathophysiology of AD and the generation of toxic forms of Aβ and tau oligomers appear to be precipitating steps in the disease process [138]. Therefore targeting targeting these forms of the molecules with NP-functionalized monoclonal antibodies might lead to greater success in clinical studies. Finally,

more studies are required on the pharmacokinetic and pharmaco-dynamic profiles of the released drugs prior to translation into clinical studies. Hence, an evaluation of the safety and efficacy of suitable NPs through human clinical trials should be performed to identify the most promising cost-effective AD therapeutics for future use. This work should provide the required systematic knowledge to develop optimal NPs targeting specific AD pathologies and pave the way to improved therapeutic options for individuals suffering from this debilitating neurodegenerative disoder.

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Conflict of Interest The authors declare that there is no conflict of interest.

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14

Psychometric Evaluation of Stress in 17,414 Critical Care Unit Nurses: Effects of Age, Gender, and Working Conditions

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Abstract

Recent events regarding the COVID-19 pandemic have demonstrated the importance of healthcare workers around the world and the stressful working conditions that are often associated with their profession. The severity of stress can be influenced by a number of factors such as age, seniority gender, family status, and position in the wards. Thus, it is important to monitor signs of stress and other psychiatric symptoms in order to understand the mediating factors and guide appropriate interventions. Here, we describe a cross-sectional study of 17,414 nurses from 31 Iranian cities carried out from 2011 to 2015, using a 22-item tool of work stressors. The tool examined interactive, managerial, and situational domains and the main objective was to identify the main background variables associated with the stress of nurses in critical care settings.

Keywords

Psychometric · Stress measure · Nurse · Age · Gender · Iran

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14.1 Introduction

Nursing is a job with a high level of stress, especially in critical care units (CCUs), including intensive care units [1, 2]. Occupational stress in the healthcare area is associated with specific situations, such as problems with co-workers, conflicts, double shifts, pressure from superiors according to the individual's perception, changes in occupation, and coping with emergency situations. Among the healthcare professionals, nurses often suffer from the consequences of occupational stress, showing problems such as dissatisfaction with work, burnout syndrome, and absenteeism [2–4].

This situation has come more into the public eye with the COVID-19 outbreak, which has now spread to more than 200 countries and territories worldwide [5]. Healthcare workers who are directly involved in the diagnosis, treatment, and care of patients with this deadly virus are at high risk of both physical and mental harm. The widespread media coverage and increasing number of cases and deaths, along with the overwhelming workload, inadequate supply of personal protective equipment, and current lack of effective treatments, can contribute to the mental health effects on these key workers. Previous studies on the severe acute respiratory syndrome (SARS) outbreak in 2002–2003 reported detrimental psychological effects in healthcare workers on the front lines [6–10]. Similar effects on healthcare workers were reported during and in the aftermath of the Middle East respiratory syndrome (MERS) outbreak which began in 2012 [11–14].

One factor that can affect stress and anxiety levels is age. Epidemiologic surveys of the general population have found that anxiety disorders occur more frequently in younger adults compared with older individuals [15, 16]. Conversely, depression occurs more often in older adults compared to the younger population [17, 18]. Working in CCUs has also been positively associated with greater stress levels. The crucial responsibilities of nurses in critical and intensive care include the operation of sophisticated technologies and fast decision-making, which can be affected by excessive workload, different management styles and skills, professional disagreements, and the emotions involved in caring [19–22]. Such characteristics and conditions can lead to both emotional and mental stress for nurses working in CCUs, and this can lead to an inability to cope and cessation of work [1]. Therefore, it is important to develop and apply tools to aid in assessing the stress levels of CCU nurses.

To understand the stressors and the means of their resolution, several studies have been conducted. Although different tools have been developed for this purpose, a major point that has been omitted in most of these is social and environmental differences in different hospitals and different social and cultural environments [19–21]. An instrument to adequately measure stress and which incorporates these factors is of prime interest in public health research.

In order to investigate the stressors of nurses in special sectors in Iran, we carried out a comprehensive study across Iran to assess the impact of culture, facilities, access to services, and types of patients in relation to nurses' stress levels. We used a partial least square (PLS) approach for psychometric evaluation of a stress scale among 17,414 nurses across 31 Iranian cities. Our main objective was to provide a system for assessing the mental health of nurses and other healthcare workers during the continuing COVID-19 crisis and future pandemics.

14.2 Methods

14.2.1 Design, Setting, and Procedures

The details on methodology of this study have been reported elsewhere [1]. Briefly, a cross-sectional study was conducted in 31 Iranian cities during the period between January 1, 2011, and December 1, 2015. A multistage cluster random sampling scheme was used to collect all data. In a second stage, ten hospitals were selected randomly as clusters in each city. In the final stage, 5 hospitals with more than 100 working nurses in CCUs were selected through a cluster random

sampling scheme. In 5 of the cities, the hospitals had fewer than 100 critical care nurses, in which case all 10 hospitals were selected. The participants who were (1) aged >18 years, (2) registered nurses, (3) working in the intensive care unit (ICU), and (4) willing to participate in the study were included and those who were not available to complete the questionnaire were excluded. The eligible and consenting nurses completed the two-part survey including the demographic and work stressor variables, which typically took place over 10–15 min.

14.2.2 Ethical Considerations

The study was approved by the institutional review board at Baqiyatallah University of Medical Sciences (Tehran, Iran). The ethical issues were reviewed and approved by ethical committee of each hospital. Since the research presented no more than minimal risk of harm to participants and involved no procedures for which written consent is normally required outside the research context, the principle of implied consent was adopted. This meant that by completion of the survey instrument, the participant demonstrated their willful consent to participate after the purpose of the study was explained to them. The participants were free to take part, to refuse, or to withdraw from the study at any time, and confidentiality of personal data was guaranteed to them. Although Iranian medical ethics laws (http://mehr.tums.ac.ir/Codes.aspx) do not specifically address this topic, it is in accordance with other international ethics codes and laws including the US Federal Code of Regulations (45 CFR 46.117c). All parts of the study were reviewed and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology statement [23].

14.2.3 Sample Size

The sample size determination process was reported elsewhere [1, 22], considering 95% confidence level and 90% power and taking into account the main outcome of relations between background variables and stress. A total of 17,414 nurses took part in the study. This sample size was higher than the minimal requirement sample size to conduct PLS confirmatory factor analysis (PLS-CFA) that assumes ten times the largest number of structural paths directed at a particular construct in the structural model [24].

14.2.4 Measurements

The measurements used in this study consisted of demographic/background variables and work stressor items. In the first part, the demographic/background variables involved age, gender, marital status, number of children, body mass index (BMI), education level, years of critical care nursing experience, shift schedule, frequency of working holidays, and ratio of nurses to patients and hospital. A 22-item tool of work stressors was administered (Table 14.1) as reported previously [1, 22]. This list was

Table 14.1 Stressful situations scale with 22 items

1. Dealing with patient's pain and suffering
2. Family presence
3. Heavy workload
4. Relatives' reaction
5. Time pressure
6. Communicating bad news
7. The necessity of having continual readiness for emergency procedures
8. Death & dying
9. Staff shortage
10. Non-nursing tasks
11. Patients' reactions
12. Physician not available
13. Instability of patient's clinical condition
14. Lack of resources
15. Working extra hours
16. Physicians' demands
17. Decision-making
18. Unpleasant tasks
19. Shift rotation
20. Poor cooperation in dialysis, CCU, and ICU
21. Poor cooperation & communication in other depts.
22. Disproportionate between salary and job hardness

CCU critical care unit, *ICU* intensive care unit

extracted in nursing working environments in CCU wards, categorized and prioritized qualitatively and quantitatively. The items were quantitatively prioritized by three panels of experts with the output from each step used as the input for the next step, to arrive at a final list. A qualitative analysis consisted of unstructured interviews administered utilizing content analysis, and the categories of nursing job stressors were extracted. Finally, the items in the quantitative and qualitative parts were merged, and the scale was derived and validated in a 3-classic-round Delphi technique [25].

The expert panel consisted of five psychiatric nurses, one psychologist, one psychiatrist, five ICU nurses, five CCU nurses, five dialysis unit nurses, three intensivists, three cardiologists, three nephrologists, and five ICU administrators. After the sessions, the Kendall's k agreement coefficient test was 0.89 which indicated a good agreement [26].

The final scale consisted of three subscales: (1) interactive and communicative (items 1, 2, 4, 11, 20, and 21), (2) managerial and administrative (items 3, 9, 10, 12, 14, 15, 16, 19, and 22), and (3) exclusive and situational (items 5, 6, 7, 8, 13, 17, and 18). Items assessing work stressors were rated on a five-point Likert-type scale ranging from "1: causes me no stress" to "5: causes me extreme stress." The total score of the scale was calculated by sum over the items and the scores on subscales were calculated by sum over the items on that subscale. The total scores ranged from 22 to 110 with higher scores indicating higher stress.

The content validity of the scale was assessed both quantitatively and qualitatively by the expert panel. In the quantitative part, the content validity index (CVI) and content validity ratio (CVR) were calculated based on a designed form consisting of questions relating to the relatedness, simplicity, and clarity using a four-point ranking scale. In the qualitative part, the experts had some recommendations on modification of some words, sentences, and/or structure of the items which were implemented and the scale was finalized accordingly.

Based on the results of a pilot study [1, 22], the threshold for significant stress was set at 67, with higher values being indicative of the highest stress levels. This cutoff was derived using both qualitative and quantitative assessments, with the latter conducted by receiver operating characteristic curve analysis (results not shown).

14.2.5 Statistical Analyses

Statistical analysis was conducted using STATA (ver.13) (StataCorp LLC, College Station, TX, USA) and SmartPLS (ver. 3.2.8) (https://www.smartpls.com) software and *P*-values <0.05 were considered as significant in all analyses. Normality of the numeric variables was checked by the Kolmogorov-Smirnov test and data were expressed as mean (SD) and median (min-max) for the numeric normal and non-normal variables, respectively, and frequency (%) for categorical variables.

PLS-CFA was used to assess the construct validity of the scale, by PLS structural equation modeling (PLS-SEM). A second order PLS-CFA was fitted to the data. In the first step of the model, three subscales of interactive, managerial, and situational stress comprised the items, and, in the second step, the stress scale comprised the three subscales. PLS-SEM lacks a fitting index of the chi-square-based model to assess the theoretical model adjustment with collected data, unlike *covariance*-based SEM (CV-SEM), which depends on the *predictive nature* of *PLS*. Therefore, fitting the indices in this approach was associated with assessment of *model adequacy in prediction of dependent variables* [27]. To modify this and reaching an adequate model, all items with loadings less than 0.5 were removed from the model one at a time, and the indices assessed. This process continued until the model achieved a suitable reliability and validity.

To test the reliability of reflective measurement model, we assessed three indices: Cronbach's alpha, composite reliability or Dillon-Goldstein's p, and communality. For

Cronbach's alpha, values higher than 0.7 indicated acceptable reliability, and alpha values between 0.6 and 0.7 were acceptable for exploratory models. Also, composite reliability was utilized to evaluate internal reliability of constructs, wherein reliability was not calculated absolutely but in regard to their correlation with each other. Composite reliability values higher than 0.7 indicated suitable internal consistency of the measurement model and values lower than 0.6 showed lack of reliability.

The reflective measurement model was homogeneous if the absolute value of a loading factor corresponding with a construct in the model was at least 0.7, equal to a communality of 0.5 (0.7*0.7) [28]. To assess the validity of the reflective measurement model, we checked both convergent validity and discriminant validity. For convergent validity, average variance extracted (AVE) was used which indicates shared average variance between every construct with its indices. This shows the correlation of an index with itself with higher correlation reflecting a better fit. Discriminant validity measures the ability of the reflective measurement model for discrimination in the model [24]. AVE values higher than 0.5 showed acceptable convergent validity and discriminant validity was acceptable when the AVE for every construct was more than the shared variance between that construct and others (i.e., the square of the correlation coefficient between constructs) [29, 30].

Using *goodness* of *fit* (*GOF*) criteria, we assessed the general fit of the model. GOF is the square root of multiplying the "average coefficient of determination" by the "average communality index of construct" [31]. Wetzels et al. introduced the values of 0.01, 0.25, and 0.36 for weak, middle, and strong *GOF* of a general model [32]. The R squared values indicate the coefficient of determination, with values of 0.19, 0.33, and 0.67 indicating weak, middle, and good prediction ability [33].

Next, the relationship between total stress scores and the PLS-indicated components with background variables was modeled using generalized estimating equations (GEE). The model was built in a multivariate manner and included the explanatory variables of gender, education level, marital status, working shift, patient-to-nurse ratio, collaboration, supportive supervisor, working in holiday, ICU type, ICU system, age (years), clinical experience (years), BMI (kg/m^2), children (number), and ICU beds (number). The compound symmetry covariance structure took into account the structure introduced by the 31 cities. The categorical variables were entered in the model as indicators. Regression coefficients and their standard error were estimated.

14.3 Results

From 21,767 administered surveys, 17,414 cases returned valid surveys with a confidence interval (CI) = 79.5–80.5%. The surveys were completed in ICUs (n = 370), coronary care units (n = 240), and dialysis units (n = 180) at 180 educational and private hospitals. The details on demographic characteristics of the participants are provided elsewhere [1]. Briefly, the mean age of participants was 29 years (SD = 5.4; range = 21–43 years), 31% of the participants were male, and the ratios of patients to nurses were 3, 2, and 1 for 5.4%, 10.2%, and 84.4%, respectively. The mean job experience of the participants was 16.5 years (SD = 6.4; range = 4–27 years). The mean stress score was 69.2 out of 100 points (SD = 3.2; range = 62–84). Approximately 71% (95% CI = 70.3–71.7%) exceed the cutoff score of 67 for significant stress.

14.3.1 Content Validity

Based on the opinion of 36 experts in the field, an impact score > 1.5, CVI values >0.75, and CVR > 0.42 confirmed face validity and content validity of the items in this instrument (Table 14.2). For the qualitative analysis, required modifications were made according to the integrated experts' opinions.

Table 14.2 Face and content validity of the instrument

	Value
Face validity (impact score)	2.09–4.67
Content validity (CVR)	0.42–0.68
Content validity (CVI)	0.63–0.93

14.3.2 Construct Validity

A second-order PLS-SEM was fitted. In the first step, three subscales of interactive, managerial, and situational stress comprised the items, and in the second step, the stress scale comprised the subscales. The initial model (Fig. 14.1) was modified by iteratively removing each single item (with <0.5 lowest loadings). In the modified model (Fig. 14.2), the reliability and validity of the model and the adequacy were assessed. The model showed adequate fit ($R^2 = 0.77$, 0.83, and 0.63 for interactive, managerial, and situational subscales) indicating middle to good predictive ability of the subscales. This suggested a generally suitable fit of the CFA-PLS model (GOF = 0.642 > 0.36). In addition, path coefficients relating items to the subscales and those relating subscales to the stressful total scale were all significant ($P < 0.05$).

14.3.3 Reliability

For stability reliability, composite reliability, and internal consistency reliability, indices with values >0.7 confirmed the reliability of the instrument (Table 14.3). Cronbach's alphas for all subscales were in the range of 0.63–0.79, indicating suitable internal consistency reliability of the indices. For the total scale, the Cronbach's alpha was 0.85, indicating a good level of internal consistency of the stress scale (Table 14.3). The values of composite reliability for all constructs were also >0.7, which indicated suitable internal consistency of the constructs (Table 14.3).

14.3.4 Convergent Validity

The AVE value for all subscales was higher than 0.5, indicating suitable convergent validity (Table 14.3).

14.3.5 Discriminate Validity (the Fornell-Larcker Criterion)

Application of the Fornell and Larcker method showed that the model had acceptable divergent validity as the values of the principle diameter (i.e., the correlation among the subscales by itself) were higher than the correlations between a variable and other variables (Table 14.4), indicating the discriminate validity of the instrument [30].

14.3.6 Relationship Between Stress Components and Background Variables

The results of GEE on assessment of the relationship between total stress scores and PLS-indicated components with background variables are given in Table 14.5. The results were significantly higher for males, higher education levels, and separated or widowed individuals. Also the finding indicated significantly higher interactive, managerial, and total stress in rotation working shift and significantly lower scores of situational stress in rotation working shifts. High-level collaboration was associated with significantly lower stress scores, and supportive supervisor was associated with significantly lower interactive and total stress scores. Working in holidays was associated with significantly higher managerial stress. Age, clinical experience years, and number of children had an inverse relationship with stress.

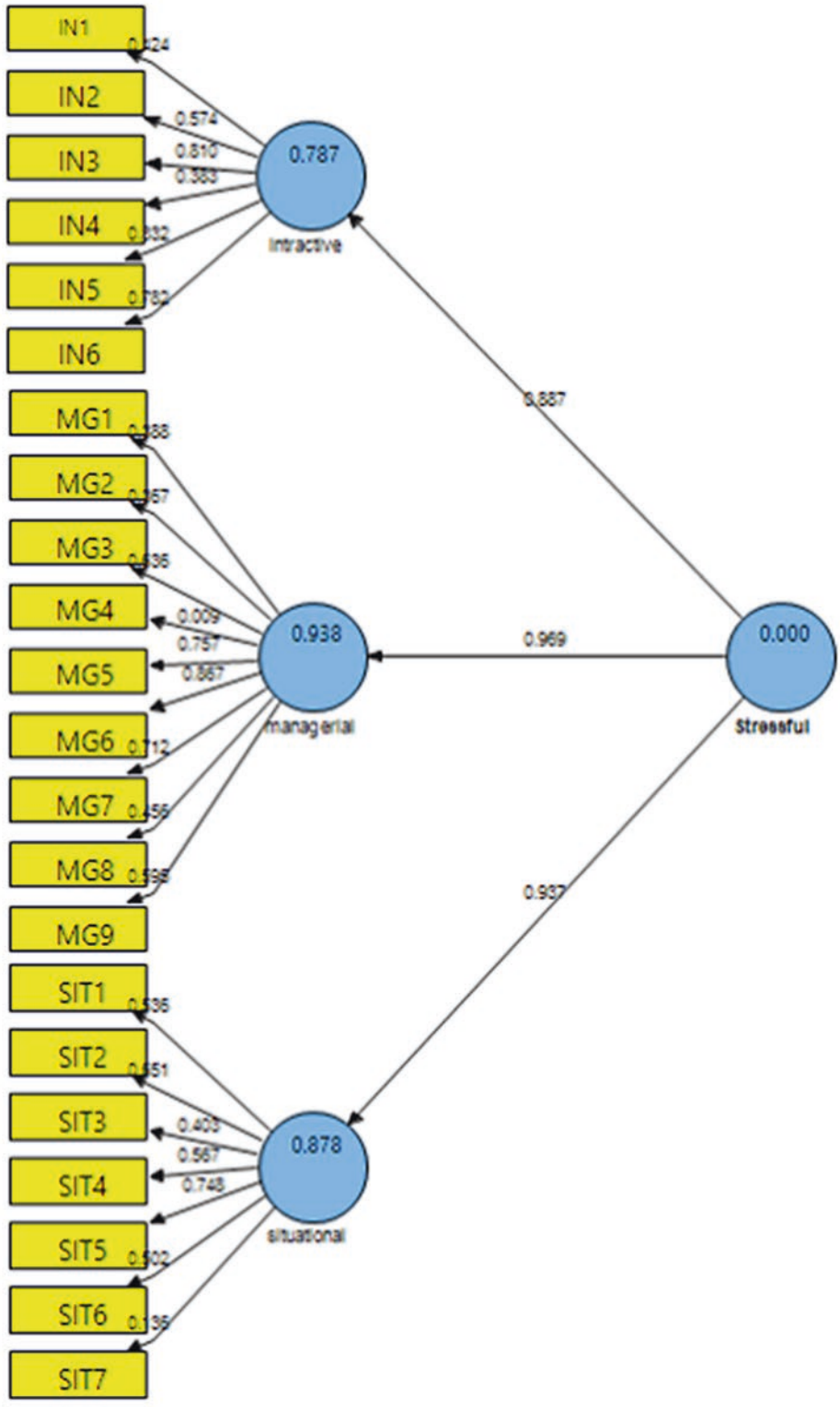

Fig. 14.1 Outer loadings for initial second-order PLS-SEM for stress scale

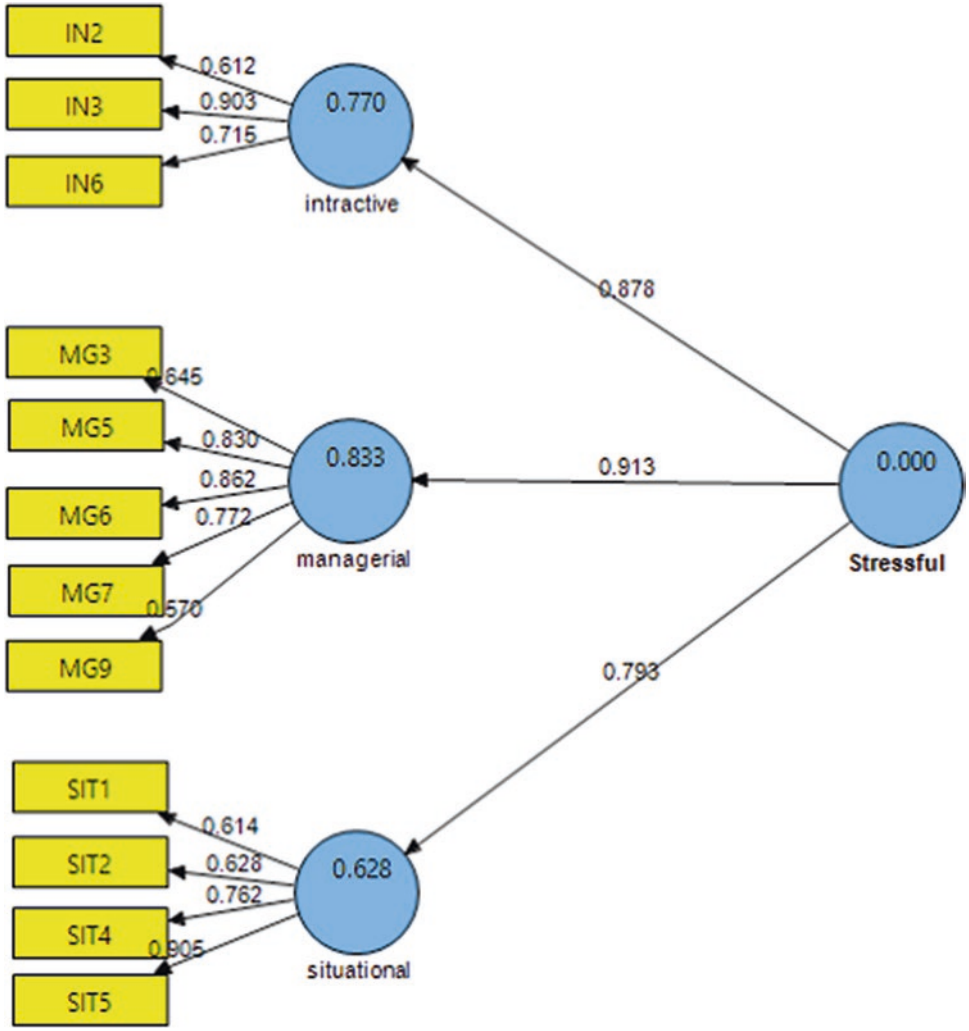

Fig. 14.2 Outer loadings for modified s*econd-order PLS-SEM* for stress scale. *All paths were significant ($P < 0.05$)

Table 14.3 Stability, composite reliability, internal consistency reliability, and average variance extracted of the instrument

Reliability	Stability (test–retest)	Composite reliability	Internal consistency (Cronbach's alpha)	Average variance extracted
Stressful	0.87	0.850	0.890	–
Interactive	0.81	0.793	0.632	0.567
Managerial	0.88	0.859	0.794	0.554
Situational	0.84	0.822	0.713	0.543

14.4 Discussion

This study has described the use of the working nurses stress scale in critical care units and confirmed its reliability and validity in Iran based on a PLS-SEM approach. This is the first time that such a tool has been developed with a sufficiently large sample size across different hos-

Table 14.4 Discriminate validity (Fornell-Larcker criterion) of the instrument based on latent variable correlations

	Interactive	Managerial	Situational
Interactive	**0.760**		
Managerial	0.727	**0.744**	
Situational	0.589	0.544	**0.736**

pital units in Iran, in accordance with Iranian culture. The face and content validities of the scale were confirmed by the opinions of a panel of experts. The construct validity was also satisfied by the PLS method. The model had a good level of adequacy and high coefficient of determination values. All item-subscale and subscale-scale relationships were statistically significant. The Fornell-Larcker criterion and AVE assessments indicated that the convergent validity and discriminant validity of the measure were satisfied. The test-retest reliability, internal consistency reliability, and composite reliability were also at satisfactory level for subscales and whole scales.

It is important to mention that we used the PLS approach of SEM, and not a covariance-based method, since PLS has minimal requirements on measurement scales, sample sizes, and residual distributions. In addition, the PLS approach focuses on maximizing the variance of the dependent variables explained by the independent variables and thereby avoids the problems of inadmissible solutions and factor indeterminacy associated with a covariance-based approach [34]. The algorithm involved in the PLS approach comprises a series of ordinary least squares equations and, therefore, identification is not a problem for recursive models. In addition, second-order PLS procedures were used in this study, which can be estimated by the standard PLS algorithm [24]. Finally, PLS is considered a better approach for clarifying complex relationships [34, 35]. PLS assumes consistency of the parameter estimates and this was satisfied in our study considering the large sample size. Standard errors need to be estimated in PLS through resampling procedures such as jackknifing or bootstrapping, and p-values of coefficients can be estimated by the jackknife method resulting from a blindfolded resampling technique [36].

The application of the tool developed here revealed that age, gender, education, marital status, working shift, system collaboration and support difference, working experience, and child number were significantly associated with stress levels of critical care nurses in Iranian hospitals, supporting the discriminant validity of the scales and subscales. Previous instruments designed for stress evaluation of nurses have been conducted in different countries, although these were limited with respect to the type of hospital ward and sample size. The most established and widely used tool designed for measuring the frequency and major sources of stress experienced by nurses on hospital units is the Nursing Stress Scale (NSS), which uses a 34-item, 4-point Likert scale [37].

The results of the current scale showed consistency with previous studies [38], and the high Cronbach's alpha (0.85) revealed a good level of internal consistency, confirming its reliability as an instrument for assessment of stress in Iranian nurses. The final version of this tool included 22 items in 3 domains: *interactive and communicative* (6 items), *managerial and administrative* (9 items), and *exclusive and situational* (7 items) subscales. In previous studies, job stressors for nurses were categorized into six broad domains: (1) intrinsic job characteristics; (2) organizational roles; (3) work relationships; (4) career growth issues; (5) organizational factors including climate, structure, and culture; and (6) the home-work interface. All six of these components are included in three domains of the instrument developed in the present study. This resulted in the present instrument having fewer items than previous tools. We suggest that the use of lower numbers of items increases the willingness of participants to the tool.

The items of the first subscales (interactive and communicative) include dealing with patients' pain and suffering, family presence, relatives reactions, patients' reactions, poor cooperation in the intensive care unit, and poor cooperation and communication in other departments. These items are in line with other studies which found that poor relationships with individuals from other

Table 14.5 Results of GEE model to assess relationship between the stress components with background variables

Variables	Interactive score			Managerial score			Situational score			Total score		
	B	SE	*P*-value	B	SE	*P*-value	B	SE	*P*-value	B	SE	*P*-value
Gender (male)	**0.95**	**0.09**	**<0.01**	**1.16**	**0.13**	**<0.01**	**0.77**	**0.14**	**<0.01**	**1.55**	**0.10**	**<0.01**
Education level												
Associate	**−0.91**	**0.33**	**0.01**	**−1.61**	**<0.01**	**<0.01**	NA	NA	NA	**−1.57**	**0.58**	**0.01**
Baccalaureate	−0.21	0.33	0.51	**0.16**	**<0.01**	**<0.01**	NA	NA	NA	−0.43	0.58	0.46
Master	**0.71**	**0.33**	**0.03**	**0.97**	**<0.01**	**<0.01**	NA	NA	NA	−0.15	0.58	0.80
PhD	**Referent**	–	–	–	–	–	–	–	–	–	–	–
Marital status												
Not married	0.32	0.94	0.73	**−0.49**	**<0.01**	**<0.01**	−0.05	1.73	0.98	0.05	0.66	0.94
Married	−0.11	0.93	0.91	**−1.33**	**<0.01**	**<0.01**	−0.53	1.72	0.76	−0.34	0.65	0.60
Separated or widowed	**Referent**	–	–	–	–	–	–	–	–	–	–	–
Working shift												
Morning	−0.06	0.05	0.22	**−0.15**	**0.07**	**0.04**	**0.28**	**0.08**	**<0.01**	**−0.20**	**0.07**	**<0.01**
Evening	**−0.21**	**0.05**	**<0.01**	**−0.51**	**0.07**	**<0.01**	**0.54**	**0.08**	**<0.01**	**−0.39**	**0.07**	**<0.01**
Night	**−0.17**	**0.05**	**<0.01**	**−0.17**	**0.08**	**0.03**	**0.67**	**0.08**	**<0.01**	**−0.73**	**0.06**	**<0.01**
Rotation	**Referent**	–	–	–	–	–	–	–	–	–	–	–
Patient-to-nurse ratio												
One	−0.18	0.93	0.84	−0.31	1.29	0.81	−0.42	1.71	0.80	−0.26	0.65	0.69
Two	−0.11	0.11	0.32	−0.17	0.16	0.29	−0.33	0.18	0.07	−0.06	0.13	0.66
Three	**Referent**	–	–	–	–	–	–	–	–	–	–	–
Collaboration												
low	**3.12**	**0.04**	**<0.01**	**2.56**	**0.04**	**<0.01**	**5.75**	**0.04**	**<0.01**	**2.17**	**0.06**	**<0.01**
moderate	**5.40**	**0.04**	**<0.01**	**4.64**	**0.06**	**<0.01**	**2.32**	**0.07**	**<0.01**	**1.23**	**0.06**	**<0.01**
high	**Referent**	–	–	–	–	–	–	–	–	–	–	–
Supportive supervisor (yes)	**−0.20**	**0.08**	**0.01**	0.22	0.12	0.07	<0.01	0.13	0.97	**−0.20**	**0.09**	**0.02**

Working In holiday days (yes)	−0.08	0.08	0.33	**0.07**	**<0.01**	**<0.01**	−0.01	0.14	0.97	−0.03	0.11	0.81
ICU types												
Surgical	−0.01	0.07	0.94	−0.07	0.11	0.54	0.04	0.12	0.75	**−0.31**	**0.09**	**<0.01**
Medical	−0.01	0.07	0.92	0.03	0.10	0.76	−0.01	0.11	0.95	−0.02	0.09	0.85
Toxicological	−0.02	0.08	0.81	0.00	0.12	0.98	0.04	0.14	0.78	0.13	0.10	0.23
Neurology	0.00	0.09	0.96	0.00	0.13	0.98	−0.02	0.14	0.88	−0.07	0.11	0.52
Trauma	0.14	0.08	0.10	−0.03	0.12	0.82	0.03	0.14	0.83	0.07	0.11	0.51
Adult mixed	0.00	0.09	0.98	0.13	0.14	0.36	0.18	0.15	0.24	0.12	0.12	0.31
Pediatric	0.01	0.09	0.89	0.03	0.13	0.85	−0.01	0.15	0.95	−0.04	0.11	0.69
Neonatal	−0.04	0.09	0.69	0.02	0.13	0.87	−0.01	0.15	0.94	−0.02	0.11	0.85
Pediatric and neonatal mixed	0.07	0.10	0.47	−0.06	0.15	0.67	−0.05	0.16	0.74	−0.07	0.13	0.60
Coronary	0.07	0.06	0.30	−0.04	0.10	0.65	−0.02	0.11	0.83	0.05	0.08	0.58
Dialysis	**Referent**	–	–	–	–	–	–	–	–	–	–	–
ICU system (semi-close)	0.06	0.05	0.23	0.07	0.07	0.31	0.08	0.08	0.34	0.10	0.06	0.11
Age (yrs)	**−0.09**	**0.01**	**<0.01**	−0.03	0.01	0.05	−0.02	0.01	0.11	**−0.08**	**0.01**	**<0.01**
Clinical experience (yrs)	−0.02	0.01	**0.01**	**−0.08**	**0.01**	**<0.01**	**−0.05**	**0.01**	**<0.01**	**−0.14**	**0.01**	**<0.01**
BMI (kg/m²)	0.00	0.00	0.55	−0.01	0.01	0.13	0.00	0.01	0.88	0.00	0.00	0.78
Children (number)	0.01	0.02	0.52	−0.03	0.03	0.36	−0.01	0.03	0.83	**−0.06**	**0.02**	**0.02**
ICU bed (number)	−0.01	0.01	0.40	0.00	0.01	0.93	0.00	0.01	0.98	0.00	0.01	0.75

GEE generalized estimating equation, *B* regression coefficient, *SE*, standard error
NA not applicable in the GEE model

professions may lead to lower levels of confidence and higher stress levels and better relationships with other professions [39]. In addition, exposures to pain, suffering, and traumatic life events that nurses experience on a daily basis can contribute to stress [40]. Similarly, another study showed that the least stressful subscale was inadequate preparation to deal with emotional needs of patients and families (feeling inadequately prepared to help with the emotional needs of patients and their families), and factors of the intense emotional support needed for patients and families are another burden of stress placed on nurses [41].

Studies have shown that an individual nurse may behave differently in their perception of stress. The results of the previous study [1] showed that age, marital status, working shift, and years of experience of nurses had significant associations with levels of stress. However, sex, education, and BMI showed no significant association with stress level. Similarly, Li et al. showed that gender and education were not linked with stress but marital status did show a significant effect [42]. In addition, Chang et al. concluded that education, marital status, and number of children did not have a significant association with the level of stress [43].

Similar to our findings about decision-making power in the subset of exclusive and situational subscale and physician dependency in the subset of managerial and administrative subscale, Kang et al. found that lack of autonomy and independency in making decisions was frequently stressful for staff nurses in clinical area [43]. The majority of staff nurses sometimes felt unable to make decisions and powerless to change unsatisfactorily situations. Workload and staff shortage were two other aspects of the managerial and administrative subscale, similar to the findings of a tool developed to assess nurse stress in Saudi Arabia [44]. Another study showed that staff nurses did not always utilize their training and experience despite the fact that some felt inadequately trained or equipped for their job [45]. Transition programs specifically designed to bridge the gap between the academic and service setting and prepare nurses to utilize critical thinking skills in management of acutely ill patients are therefore likely to be important to ensure nurses have sufficient confidence to deal with the degree of autonomy they are required to demonstrate.

The strengths of this study were the use of multistage random sampling methods and the consideration of different nurse groups in different wards and the large sample size across 31 cities in Iran. This resulted in a sample size significantly greater than the minimal requirement to conduct the PLS-CFA. There was a robust correlation between the level of stress and social and cultural status. The construct validity showed that stress scale items were grouped under three components which may provide greater incentive to participants in completion of the study compared with other studies on stress scale development that used components on the scale ranging from four to seven components [46–49]. This is supported by the finding that most of the above studies identified three major components linked with stress among nurses (lack of adequate staffing, dealing with difficult patients, and high workload). The large sample size of this investigation resulted in a higher response rate (about 80%) as compared to other studies (about 55.1 and 76.2%) [50, 51].

A potential limitation of the present tool relates to the fact that it was developed to assess stress of nurses in critical care units in Iran. Thus, it is not necessarily generalizable for assessment of nurses in healthcare institutions in other countries. Moreover, there are some aspects of the Iranian healthcare system that limit the generalizability of the findings. For example, most academic and tertiary care ICUs in the USA are closed systems and those in Iran are generally semi-closed or open units. We did not collect data on individual nurse's psychiatric symptoms or diagnoses. We also did not collect data on workplace violence or lateral hostilities.

14.5 Conclusion

The study demonstrated a valid and reliable scale to assess stress-related factors in the home and workplace for nurses. As the tool is short and simple to use, it is convenient for assessment of

nurses in critical care units. Further studies applying this developed tool are recommended to further elucidate the dimensions of stress in Iranian nurses, with the overall aim of improving working conditions for these critical workers in healthcare. Finally, this approach should be translated for use in other countries and cultures affected by the current COVID-19 outbreak. As a second wave of COVID-19 or outbreaks of further viruses can occur, such a response becomes even more critical to protect our healthcare professionals working on the frontlines.

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15 Targeting Age-Related Neurodegenerative Diseases by AAV-Mediated Gene Therapy

Umut Cagin

Abstract

Age-related neurodegenerative diseases have detrimental consequences on health of many patients and result in mortality. The current treatment options are limited and usually fail to correct the underlying pathology. AAV-based gene therapies have proved to be safe based on the data available on clinical trials for several monogenic diseases. Therefore, such therapies can pave the way to treat neurodegenerative diseases likes Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Here, the advantages of AAV-based gene therapies are discussed with emphasis on efforts of developing novel capsids with superior therapeutic efficacy. Furthermore, the results of clinical trials on AD, PD, and ALS are summarized.

Keywords

Neurodegenerative diseases · Neurodegeneration gene therapy · Central nervous system · CNS · AAV · Capsid

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15.1 Introduction

Neuronal networks are complex systems which require efficient communication between neuronal cells to maintain vital activities of the body. Neurodegeneration is a process which involves accumulation of dysfunctional molecules, proteins, and organelles, which progressively damage neuronal cells and result in neuronal cell death [1]. Neurodegenerative diseases are commonly observed in the aged population with detrimental consequences on quality of life and higher risks of death. The limited regenerative potential of neurons makes neurodegenerative diseases difficult to treat. Many widely observed neurodegenerative diseases are caused by genetic mutations, but epigenetic events are also observed in Parkinson's disease (reviewed by Pavlou et al. [2]) and have also been recently reported to occur in Alzheimer's disease [3, 4].

Commonly observed neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) have common dysregulated processes such as mitochondrial dysfunction and oxidative stress [5]. In line with this, approaches ameliorating mitochondrial dysfunction have been shown to have beneficial effects in various animal models of neurodegenerative diseases [6–9]. Importantly, one of the commonly observed pathological features of neurodegenerative diseases is

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the accumulation of misfolded protein aggregates, which have toxic effects and thereby cause neuronal cell death [1]. In AD, the toxic accumulation of amyloid-β (Aβ) and tau has been observed [10]. On the other hand, α-synuclein aggregates mainly occur in PD but can also be observed in AD [11]. Huntington's disease is a monogenic condition that occurs due to aggregation of the huntingtin protein via an expanded CAG repeat region [12]. These observations further suggest the possibility of targeting autophagy to reduce protein aggregation as a novel therapeutic avenue for neurodegenerative diseases [8, 13, 14].

Most of the drugs in common use for these disorders have limited efficacy due to low permeability of the blood-brain barrier (BBB). A study published in 2019 by Cummings and colleagues reported 132 agents currently being investigated in clinical trials for the treatment of Alzheimer's disease [15]. Unfortunately, many clinical trials for Alzheimer's disease have failed at different stages of clinical development [16]. The current treatment options can reduce some of the symptoms experienced by the patient; however, many drugs do not target the underlying pathology and are therefore not curative. Although these drugs can have some beneficial effects for the everyday life of the patient, novel therapeutic strategies are needed. On the contrary, gene therapy has the potential to target and correct the underlying pathological mechanisms and ultimately treat the root cause of a disease. One promising avenue is the use of adeno-associated virus (AAV) vectors as a delivery system for the genes of interest. The current challenges include, but are not limited to, the correct choice of delivery route, optimization of the gene expression cassette, and efficient expression in the desired cell type or brain region [17]. Delivery routes widely performed at the moment include intraparenchymal, intrathecal, intracerebroventricular, subpial, and intravenous injection [18]. It is important to note that other central nervous system (CNS) delivery routes are also under development. A recent study showed safe delivery of the gene cargo to the cisterna magna region of the brain for treatment of Tay-Sachs disease [19].

This review aims to summarize the advances in the AAV-based gene therapy in the context of neurodegenerative diseases as a novel and promising therapeutic avenue. The advantages and the current challenges of AAV-based gene therapy are also explained. Following this, the recent progress in choosing the most optimal AAV serotype for CNS transduction and designing therapeutically superior viral capsids for better neuronal targeting is discussed. Finally, examples of novel therapeutic options that have been entered into clinical trials using AAV-based gene therapy for AD, PD, and ALS are summarized and compared.

15.2 AAV-Based Gene Therapy

Gene therapy serves as a promising option for a one-time permanent treatment for genetic diseases. In short, gene therapy can be used to either silence the expression of a disease-causing gene, to modify a mutation (by gene editing), or simply to deliver an un-mutated copy of the mutated gene, simply known as gene replacement [20]. Viral vector-mediated gene therapy using different types of viruses including lentivirus, adenovirus, herpes simplex virus, vaccinia virus, and adeno-associated virus (AAV) can serve as vehicles for this purposes [21].

Thanks to the advances in vector biology and genetic engineering, AAV-based gene therapies are currently more than an investigation tool and are used as treatment options in the clinic. To date, many patients with detrimental diseases have been injected with AAVs with no major adverse events observed, highlighting the safety aspects of these treatments [22]. In particular "gene replacement" strategies have shown high success rates in cases of monogenic diseases. Several clinical trials targeting diseases such as hemophilia A [23], hemophilia B [24–26], retinal disorders [27–29], and spinal muscular atrophy (SMA) [30] have proved AAVs as safe and efficient therapeutic tools.

AAV is from the *Parvoviridae* family with a genome that contains four non-structural Rep proteins, three capsid proteins (VP1, VP2, and

VP3), and the assembly-activating protein (AAP) [31, 32]. AAVs have particular advantages making them ideal for in vivo gene transfer such as having a low risk of insertional mutagenesis. Generation of recombinant AAVs by elimination of all open reading frames (ORFs) makes them replication-defective thereby rendering them safe to use. This further allows cargo sizes of approximately 4.7 kb. Efficient production of AAVs with high yield and purity is an important factor for gene therapy as this can have a significant impact on the transduction efficiency [33]. The most commonly used approach is the triple-transfection method which is based on co-transfection with three plasmids: (1) the transgene of interest flanked by inverted terminal repeats (ITRs), (2) rep and cap genes for packaging, and (3) adenoviral helper genes [34]. For more detailed information on AAV production and purification, refer to Ayuso et al. [35].

The 4.7-kb cargo limit of AAVs is a limiting factor for delivery of large genes; therefore, alternative strategies such as production of oversized vectors and dual vectors are currently used to overcome this. Production of oversized AAVs can result in heterogeneous vector preparations and can affect transduction efficiency [36, 37]. On the other hand, dual-vector strategy is based on splitting the transgene into two (head and tail), and co-transfection therefore results in reassembly of the full-length expression cassette [38]. The therapeutic potential of this strategy has been proven via gene delivery to the retina [39, 40] and in a mouse model of Stargardt disease [41]. Furthermore, the dual AAV approach has shown positive results in muscle diseases such as dysferlinopathy [42–44] and Duchenne muscular dystrophy [45].

Progress in investigational and clinical AAV-based research and therapy increased following the discovery and characterization of 13 AAV serotypes and over 100 AAV variants from different species [46, 47]. The infectivity and specificity of these AAV serotypes depends heavily on cell type-specific receptors and co-receptors, although a universal multi-serotype receptor (AAVR) for AAV infection has been described [48]. Recently, GRP108, a member of the G protein-coupled receptor family, was characterized as a novel AAV entry factor conserved between mouse and humans [49]. Although this receptor was shown to affect the transduction of more than 20 divergent AAVs, the transduction of AAV5 was unaffected in a GRP108 knockout model [49].

The potential limitation of AAV-based gene therapy is not only limited to the size of the expression cassette, but transgene potency and vector genome persistence are other factors which can influence the outcome of the therapy [50]. Other than the vector genome and the expression cassette, another challenge can arise from the viral capsid. For example, a $CD8^+$ T-cell response to AAV capsids in humans was demonstrated by Mingozzi and colleagues which highlights that their modulation is important to achieve sustained AAV-mediated gene transfer [51]. Vector administration to seropositive patients and also to patients requiring re-administration usually limits the population size which can be treated by AAVs. Therefore, ongoing efforts are aimed at modulating AAV immunogenicity to allow vector re-administration using such approaches as tolerogenic rapamycin nanoparticles [52]. Furthermore, two recent studies described methodologies such as immunoadsorption and the use of AAV-specific plasmapheresis columns which can ultimately make vector re-administration possible [53, 54]. The importance of AAV immunogenicity for successful gene transfer in humans is reviewed elsewhere [55]. In the context of CNS gene delivery, achieving high specificity and efficacy is probably the current major challenge. Therefore capsid choice has vital importance for therapeutic efficacy. For this purpose, the following section summarizes the pre-clinical development of AAV capsids for gene delivery to the CNS.

15.3 Optimizing AAV Capsids for Efficient CNS Transduction

One of the advantages of AAV-based gene transfer is the availability of different AAV serotypes with specific tissue tropism [56]. In this aspect,

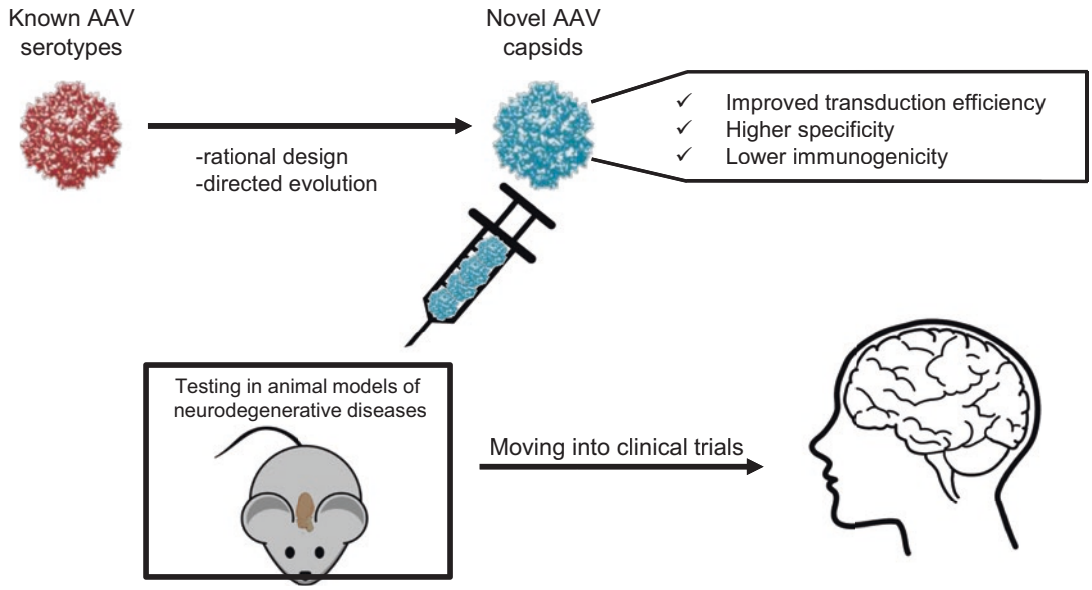

Fig. 15.1 Development of novel AAV capsids with superior CNS transduction. Current attempts have focused on rational design or directed evolution to develop capsids with better specificity, improved transduction efficiency, and lower immunogenicity. The therapeutic potential of candidate capsids can be tested on animal models of neurodegenerative diseases (e.g., Alzheimer's disease and Parkinson's disease). Novel capsids with superior therapeutic efficacy will potentially be used in clinical trials for improved treatment of patients suffering with such neurodegenerative disorders

AAV capsids with efficient CNS transduction can serve as valuable tools for the treatment of neurodegenerative diseases. Deeper investigations in AAV vector biology have resulted in advances in capsid discovery and engineering aiming for those with better tissue targeting and low genotoxicity and immunogenicity, created either by rational design or directed evolution [57]. Capsids with better therapeutic indices not only result in better delivery but can also reduce the optimal therapeutic dosage, thereby lowering potential immunogenicity as well as production costs (Fig. 15.1). Capsid-specific immune responses have been documented in both in pre-clinical and clinical trials. This includes the invaluable work of Nathwani and colleagues in this area for the treatment of hemophilia B [24, 25, 51, 58, 59].

Several AAV serotypes have been used to target and express the gene of interest in various cell types of the CNS. For example, AAV2, a widely used serotype in clinical trials, can target cerebral vascular endothelial cells [60]. In the last 15–20 years, many comparative studies have been performed in order to search for optimal AAV vectors for CNS-targeted gene therapy in mice [61–63], cats [64], dogs [65], and macaques [66] as models. Foust and colleagues showed that AAV9 can target both neurons and astrocytes in mice [67]. Importantly, AAV9 has been shown to have wider CNS expression possibilities including areas such as the substantia nigra, hippocampus, cerebellum, motor cortex, and cervical spinal cord following neonatal intracerebroventricular injection [68]. Although AAV9 has many advantages, due to questions on translatability to non-human primates [69] and immune responses [70], innovative novel AAV serotypes have been developed with higher therapeutic efficacy and lower immunogenicity.

Interestingly, modifications have not only focused on AAV9 but also using AAV2 due to its proven clinical safety in clinical trials. Heparan sulfate proteoglycan (HSPG) receptors are responsible for entry of AAV2 although other capable receptors have been characterized recently. A modified version of AAV2 incapable of binding to HSPGs, AAV2-HBKO, was shown to have an enhanced transduction efficacy depend-

ing on the route of administration [71]. Furthermore, Choudhury and colleagues created AAV-AS by inserting a poly-alanine peptide to the N-terminus of VP2 capsid protein which resulted in higher transduction in the spinal cord and cerebellum with particularly efficient targeting of striatal neurons in mice [72]. The same group also performed a single round of in vivo selection to characterize novel capsids with superior CNS transduction properties. This led to identification of AAV-B1 as an efficient transducer in multiple areas of the CNS and also in muscle, β-cell, pulmonary alveoli, and retinal vasculature in mice [73]. Another study generated and characterized AAV-S and AAV-F novel capsids with efficient CNS transduction [74]. Importantly, AAV-F had a 65-fold higher expression in astrocytes and 171-fold higher expression in neurons compared to the parental AAV9 vector [74].

One of the most important works in this field was performed using a cre-dependent evolution approach which led to generation of an AAV9 capsid mutant, AAV-PHP.B, with enhanced CNS transduction efficiency [75]. In mice, AAV-PHP.B can pass the BBB and transduce CNS cells at 40-fold higher levels than AAV9 due to the presence of a 7-amino acid insertion in the VP1 capsid protein [75]. However, a comparative study between AAV9 and AAV-PHP.B showed that intravenous injection did not result in enhanced efficacy in marmoset brain [76]. A later study by Hordeaux and colleagues showed that the CNS transduction efficiency of AAV-PHP.B in mice is limited to the C57BL/6Jbackground [77]. It is important to note that there are reports suggesting AAV-PHP.B has differential expression distributions in mice and non-human primates [78]. Interestingly, Liguore and colleagues observed a broad cortical and spinal transduction in 1–2-year-old rhesus macaques after intrathecal administration, although intravascular administration resulted in low transduction [78]. Regardless of these observations, it is worth noting that AAV-PHP.B-GBA1 treatment of A53T a-synuclein Parkinsonism mouse model led to reduced synucleinopathy and recovered behavior, highlighting the potential use of this vector for treatment of neurodegenerative diseases [79].

15.4 AAV-Based Gene Therapy for AD, PD, and ALS: Highlights from the Clinic

15.4.1 Alzheimer's Disease (AD)

AD is one of the most commonly observed age-related neurodegenerative diseases characterized by progressive degeneration of neurons and synapses in the cerebral cortex [80]. Although full mechanistic understanding is missing, neuroinflammation and mitochondrial defects are two factors which can contribute to disease pathology [9, 81]. There have been some successful attempts at reducing neuroinflammation in animal models of Alzheimer's disease, although the results have thus far not been translated to the clinic [82]. Additionally, AAV-mediated expression of CD74 showed beneficial effects by binding to the amyloid precursor protein, therefore inhibiting Aβ production [83].

One of the clinical trials (NCT00876863) aimed at stopping degeneration of the nucleus basalis region in the basal forebrain via delivery of nerve growth factor (NGF). Unfortunately, the stereotactically guided intracerebral injections of AAV2-NGF showed no effect highlighting the need of more accurate targeting [84]. A post-mortem analysis performed following this clinical trial demonstrated a need for improved vector delivery in order to achieve the full potential of the AAV2-NGF treatment [85]. Another therapeutic avenue which made progress in the clinic took advantage of the therapeutic potential of one of the apolipoprotein E (APOE) alleles, specifically APOE2. It has been reported that AAV-mediated delivery of APOE2 in a mouse model resulted in reduction in brain amyloid pathology [86]. Following this, the AAVrh.10hAPOE2-HA vector was tested using several delivery routes in non-human primates, and intracisternal delivery was shown to be the most optimal to deliver APOE2 to the CNS [87].

This approach is currently being tested in the clinic (NCT03634007).

15.4.2 Parkinson's Disease (PD)

Developing therapeutic drugs for the treatment of Parkinson's disease has been challenging, and many attempts resulted in failure, which has shifted the trend toward drug repurposing [88]. Importantly, gene therapy-based clinical trials for the treatment of Parkinson's disease showed promising results although there is room for improved therapeutic efficacy. The use of non-human primate models of Parkinson's disease has been instrumental in the pre-clinical development of gene therapy approaches for Parkinson's disease and helped candidate therapies to move into the clinical testing stage [89]. The current approaches for the treatment of Parkinson's disease can be classified into three groups: (i) enhancing dopamine synthesis, (ii) delivering trophic factors, and (iii) neuromodulation [90]. Below, some of the results obtained from clinical trials are summarized.

The most commonly used method for the enhancement of dopamine synthesis is the delivery of L-amino acid decarboxylase (AADC), an enzyme responsible for dopamine synthesis, using AAV2. Intra-putamen infusion of AAV2. hAADC was shown to be well tolerated with some beneficial effects in two studies [91, 92]. Following these attempts, a new clinical trial (NCT03065192) is currently ongoing using real-time magnetic resonance imaging as a read-out, with an estimated completion date of end of 2021. It is worth highlighting that a recent phase 1 study which used varying doses of AADC1 in an AAV2 vector saw clinical improvements in 3 cohorts of 15 patients [93]. Additionally, there are have been attempts to deliver multiple genes involved in dopamine synthesis, specifically AADC, tyrosine hydroxylase (TH), and GTP cyclohydrolase 1 (GCH1), using lentiviral vectors to enable increased production of endogenous dopamine. These approaches have been clinically evaluated primarily by Palfi and colleagues and followed by studies optimizing the expression cassette for better therapeutic efficacy [94–96].

For delivery of neurotrophic factors to enhance neuronal survival, glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN, a homologue of GDNF) have been tested. Several early clinical trials using protein infusion showed positive effects; however, this showed limited efficacy, possibly due to limited tissue spread [97, 98]. However, delivery of growth factors by gene therapy may result in a more continuous supply of protein and better tissue spread. A currently clinical trial (NCT01621581) aims to test this idea by putaminal injections of an AAV2-GDNF vector. Delivery of NRTN via AAV systems has also been tested in clinical trials with some beneficial effects, although full recovery of the disease pathology was not observed [99–101].

Another approach involved the use of AAV2-mediated delivery of glutamic acid decarboxylase (GAD), which showed some clinical benefits [102, 103]. This treatment was aimed at neuromodulation by transformation of glutamatergic neurons to GABAergic neurons to increase the proportion of inhibitory neurons in subthalamic nucleus. Although more extensive trials are needed, the results of the above studies have shown that AAV-GAD treatment can have beneficial effects for Parkinson's disease patients. The following articles can be consulted for a more detailed analysis and discussion reporting the pre-clinical and clinical progress of gene therapy approaches for Parkinson's disease [90, 104].

15.4.3 Amyotrophic Lateral Sclerosis (ALS)

ALS is the most common motor neuron disease which results in paralysis and premature death, with no current cure [105, 106]. However, there have been some pre-clinical studies which have shown progress, aimed at silencing and reducing the expression of superoxide dismutase 1 (SOD1) as a potential treatment. AAV9-mediated silencing of SOD1 by a short hairpin RNA (shRNA) approach was found to slow disease progression

and extend survival in a mouse model of inherited ALS [107], and these studies are ongoing [108]. Similarly, both AAV9 and AAV.rh10 have been used by different groups for testing the therapeutic efficacy of silencing SOD1 by using microRNA approaches [109–111]. Importantly, this latter approach has been shown to be safe and efficacious in cynomolgus macaques [112]. Biferi and colleagues reported an AAVrh10-based approach to deliver anti-sense sequences embedded in U7 small nuclear RNA to successfully silence SOD1 that increased survival in SOD1-G93A mice [113]. Silencing strategies for ALS are also in pre-clinical development for targeting C9orf72 (a hexanucleotide expansion in chromosome 9 which causes ALS) using RNAi-based gene therapy [114, 115]. There have also been attempts for increasing neuroprotection by AAV-mediated expression of neurotrophic factors such as brain-derived growth factor (BDNF), glia-derived growth factor (GDNF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) [116].

Various AAV capsids such as AAV6, AAV9, and AAV.rh10 have been used in pre-clinical studies as potential ALS treatment approaches [117]. The work of Cappella and colleagues can be consulted for a more detailed report for summarizing various aspects of this work [118].

15.5 Conclusions

Advances in genetic engineering have resulted in rapid progression in many areas of biomedical sciences, including gene therapy. To this end, AAVs have been used in pre-clinical studies of a variety of diseases with promising safety and efficacy profiles. This review has focused on the use of AAVs as promising therapeutic tools in age-related neurodegenerative diseases, for which current treatment options are limited and far from effective. To date, many pre-clinical studies using model organisms have shown beneficial effects of AAV-mediated treatments in this area, and ongoing efforts in capsid engineering may result in development of vectors with improved therapeutic indices. Furthermore, recently completed and ongoing clinical trials may help the scientific and medsical communities in the design of new strategies for targeting the underlying pathologies of neurodegenerative diseases.

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16 Is Adipose Tissue the Fountain of Youth? The Impact of Adipose Stem Cell Aging on Metabolic Homeostasis, Longevity, and Cell-Based Therapies

Hanél Sadie-Van Gijsen

Abstract

Aging is driven by four interlinked processes: (1) low-grade sterile inflammation; (2) macromolecular and organelle dysfunction, including DNA damage, telomere erosion, and mitochondrial dysfunction; (3) stem cell dysfunction; and (4) an accumulation of senescent cells in tissues. Adipose tissue is not immune to the effects of time, and all four of these processes contribute to a decline of adipose tissue function with advanced age. This decline is associated with an increase in metabolic disorders. Conversely, optimally functioning adipose tissue generates signals that promote longevity. As tissue-resident progenitor cells that actively participate in adipose tissue homeostasis and dysregulation, adipose stem cells (ASCs) have emerged as a key feature in the relationship between age and adipose tissue function. This review will give a mechanistic overview of the myriad ways in which age affects ASC function and, conversely, how ASC function contribute to healthspan and lifespan. A central mediator in this relationship is the degree of resilience of ASCs to maintain stemness into advanced age and the consequent preservation of adipose tissue function, in particular subcutaneous fat. The last sections of this review will discuss therapeutic options that target senescent ASCs to extend healthspan and lifespan, as well as ASC-based therapies that can be used to treat age-related pathologies, and collectively, these therapeutic applications may transform the way we age.

Keywords

Adipose stem cells · Aging · Longevity · Senescence · Cell-based therapy

16.1 Introduction

Prolonged survival is a product of our "protected aging" in the absence of predation or exposure. As there was historically no evolutionary pressure to survive beyond reproductive age, there was also no pressure to select for genetic mechanisms that would preserve tissue homeostasis in advanced age [1]. As a result, aging is a major risk factor for a host of chronic diseases that affects organs and tissues throughout the body,

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including diabetes, cardiovascular disease, osteoporosis, dementia, and physical frailty (reviewed in [2]). In young organisms, tissue-resident stem cells contribute to general tissue maintenance and repair after injury, but these stem cells deteriorate with age and lose their capacity to perform these functions, resulting in a gradual erosion of tissue function [1].

The four processes that drive biological aging are (1) low-grade sterile inflammation (in other words, inflammation not stimulated by pathogens); (2) macromolecular and organelle dysfunction, including DNA damage, telomere erosion, and mitochondrial dysfunction; (3) stem cell dysfunction; and (4) an accumulation of senescent cells (reviewed in [2]). These processes are intricately linked, and factors that target or promote one of these processes are likely to impact the others as well. All four of these processes occur within adipose tissue, and collectively they bring about age-related adipose tissue dysfunction through a multitude of molecular mechanisms and pathways, as will be discussed in this review.

Fat mass peaks around middle age and is subsequently lost during advanced aging in humans and in animal models (reviewed in [3–5]). However, this often constitutes a loss of total fat mass, while the total percentage body fat remains constant or even increases, as a result of loss of lean muscle mass and the redistribution of fat mass to non-adipose sites such as muscle and liver [4, 5]. With age, there is also a redistribution of fat mass from subcutaneous (SAT) to abdominal visceral depots (VAT) [5, 6]. SAT is usually the biggest adipose depot in the body, with up to fourfold greater volume than VAT [7], although this may be impacted by gender and is obviously affected by visceral obesity. Increased VAT is associated with an adverse metabolic risk profile and predisposes individuals to developing metabolic syndrome [8, 9], and therefore the age-related increase in VAT may form a crucial part of the mechanism underlying the well-documented age-associated increase in metabolic disorders (reviewed in [5, 10]). Correspondingly, increased VAT is associated with reduced lifespan, independent of total body adiposity [11–13].

Apart from adipocytes, adipose tissue contains a significant fraction of non-fat cells, collectively called the stromal-vascular fraction (SVF). This fraction includes endothelial cells, fibroblasts, and macrophages but also contains the tissue-resident preadipocyte progenitors called adipose stem cells (ASCs) [14, 15]. ASCs are a subset of mesenchymal stromal cells (MSCs) and express typical MSC cell surface markers, such as CD73, CD90, and CD105, but not hematopoietic or endothelial markers (reviewed in [16]). ASCs from SAT and VAT (scASCs and vASCs, respectively) are fundamentally different and can be used in cell culture to study adipose depot-specific biological responses and molecular mechanisms [16]. Adipogenesis, the process whereby ASCs differentiate into mature adipocytes, involves profound functional alterations in these cells, including morphological changes, intracellular lipid accumulation, the acquiring of insulin sensitivity, and the production of secreted factors, including adipokines (reviewed in [17]). Adipogenesis is under transcriptional control of master regulators such as C/EBPβ, C/EBPα, and PPARγ2 [18, 19].

A dominant function of adipose tissue is to incorporate cytotoxic free fatty acids into neutral triglycerides within intracellular lipid droplets. Nutrient availability varies widely, and adipose tissue, especially SAT, needs to respond to these variations by maintaining expandability. Fat mass expansion in response to nutrient excess can occur via either adipocyte hyperplasia (an increase in cell numbers) or adipocyte hypertrophy (an increase in cell size). However, adipocyte hypertrophy can have pathological consequences, including hypoxia and inflammation, while adipocyte hyperplasia, which is driven by the differentiation of ASCs into new adipocytes, is metabolically more favourable [16, 20].

Adipose tissue is at the nexus of various (patho)physiological processes, including aging, metabolic homeostasis, and inflammation. As will be discussed below, aging and adipose tissue actually exist within a reciprocal relationship: chronological aging affects adipose tissue function, and adipose tissue dysfunction in turn drives biological aging, loss of function, and reduced

lifespan, while optimal adipose tissue function is associated with longevity. This review will discuss the reciprocal relationship between aging and adipose tissue on a mechanistic level, paying particular attention to the role of ASCs in this relationship. ASCs not only serve as a reservoir of newly formed adipocytes but also actively contribute to the function and dysfunction of adipose tissue [16], and it is therefore not surprising that ASCs have emerged as central mediators of the role of adipose tissue during aging and metabolic disease. However, ASCs have also become extremely popular within the arena of regenerative medicine and stem cell-based therapies, and therefore the final sections of this review will discuss the implications of aging and metabolic dysfunction on the applicability of ASCs for therapeutic purposes.

16.2 The Effects of Chronological Aging on ASC Biology and Function

Both the birth of new adipocytes from ASCs and the lipid turnover within these adipocytes diminish with age, even in relatively young adults (23–38 years old), indicative of functional decline with age [21]. Adipocyte hypertrophy, a marker of impaired adipogenesis [16, 22], was also found to increase with age, independent of body mass index (BMI) [23]. Hypertrophied adipocytes promote a pro-inflammatory milieu within adipose tissue (reviewed in [16, 22]) and may therefore play a causal role in the age-associated increase in adipose tissue inflammation that will be discussed in detail below. However, due to the embedded and scattered nature of ASCs within adipose tissue, mechanistic studies focusing specifically on ASCs in vivo are virtually impossible with currently available technology. Consequently, the effects of physiological parameters such as aging on ASC biology and function are more effectively studied in an in vivo/ex vivo experimental set-up [24], where ASCs from different in vivo backgrounds are isolated and manipulated in primary cell culture ex vivo. Such studies have provided a wealth of mechanistic information on the impact of aging on ASCs. Many studies have found that aging decreases the ex vivo replicative capacity and adipogenic potential of rodent and human ASCs [3, 25–30], and the mechanisms involved will be discussed in more detail in the following sections. However, not all studies are in agreement, as discussed elsewhere [16]. A possible explanation for these discrepancies may lie in the unclear definitions and inconsistent application of age brackets referring to middle age and old age in humans and the corresponding age brackets in mouse and rat models. Specifically, many of the studies on "old" human ASCs discussed below examined cells from individuals younger than 60 years old, while many countries in the world have an average life expectancy of over 80 years of age [31]. As a result, our understanding of ASC (dys)function in truly advanced human age is limited, and most of the available mechanistic knowledge was derived from animal studies. The sections below will discuss findings in animal and human ASCs, detailing the effects of donor age on ex vivo ASC function on a molecular level.

16.2.1 Age-Induced ASC Senescence

16.2.1.1 Senescence Mechanisms: A Brief Overview

Senescence can be simplistically defined as irreversible cell cycle arrest, but actually involves complex molecular consequences for the cell itself and for the environment in which the cell resides. ASCs acquire features of senescence during aging, but whether senescence underpins all of the age-related dysfunctions of ASCs is not clear, as the relative contributions of the various senescence-associated pathways and mechanisms have not been well described in aging ASCs. However, our limited understanding of senescence in ASCs has to be viewed within the context of established senescence mechanisms in other cells. Many excellent publications have given exhaustive mechanistic descriptions of senescence pathways [32–40], mostly from work performed in senescent fibroblasts, and it is not within the scope of this review to recount those

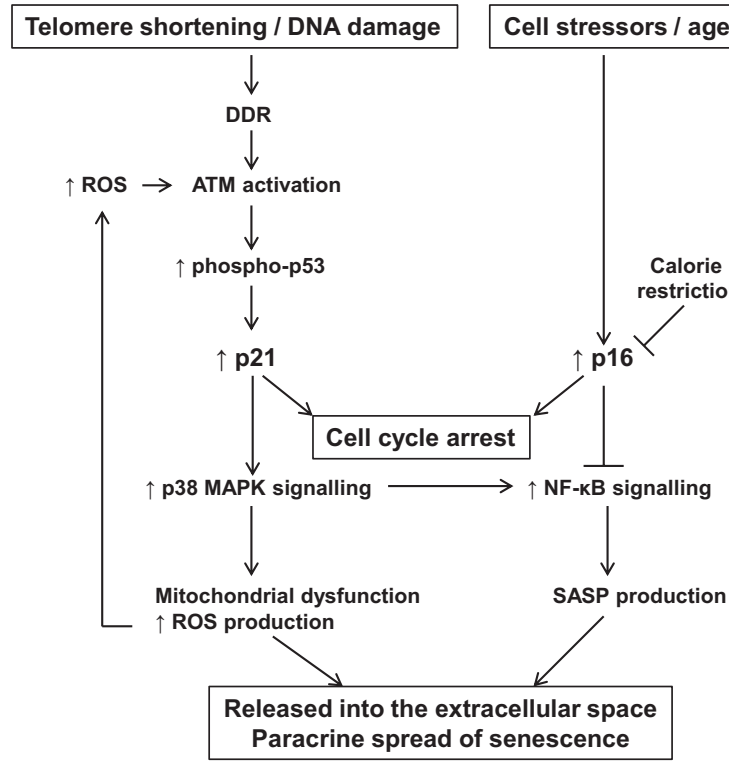

Fig. 16.1 A highly simplified overview of mechanisms and signalling pathways involved in cellular senescence. More detailed information on the individual signalling pathways and molecular connections can be found in Refs. [32–41, 43, 49–59]

descriptions. The tumor-suppressive role of senescence will also not be discussed here. However, a few highlights and salient points on senescence in aging non-cancerous cells will be presented in the following paragraphs and in Fig. 16.1.

The ends of chromosomes are capped by repetitive DNA sequences called telomeres, but telomere repeats are lost during successive cell divisions due to incomplete replication by DNA polymerases. Critical telomere shortening results in chromosomal instability and loss of cell viability. To compensate for telomere erosion and to extend cellular lifespan, new telomeres are added through the action of telomerase enzymes (reviewed in [41]). Beyond the embryonic stages, mammalian telomerase expression is restricted to proliferating cells such as progenitor cells and cancer cells [42]. The maintenance of telomere length may be essential to retain proliferative capacity, especially in adult stem cells [43], as shortened telomeres are regarded as a main trigger of replicative senescence [41]. In non-adipose tissues, the tissue-resident stem cells have the longest telomeres, but these telomeres also shorten with age, suggesting that telomere shortening may contribute to stem cell dysfunction with age [44–46]. Correspondingly, human ASCs possess telomerase activity [47] but also exhibit telomere shortening with age [48].

Senescence is a state of replicative arrest which can be triggered by critical telomere shortening or other forms of DNA damage, or by other cellular stressors independent of DNA damage [33]. Telomere attrition can be accelerated by extrinsic factors such as reactive oxygen species (ROS) [49, 50], suggesting that telomere damage may be a trigger for the onset of premature senescence in response to oxidative stress. Short/damaged telomeres or DNA double-stranded breaks (DSBs) trigger a DNA damage response (DDR) (reviewed in [32, 33, 37]), activating ATM (ataxia telangiectasia mutated) protein kinase which blocks cell cycle progression through the phosphorylation and stabilization of the tumor suppressor p53 and the subsequent transcriptional

upregulation of the cyclin-dependent kinase (CDK) inhibitor p21 (CIP1) [37, 51]. ATM can also be directly activated by ROS such as H_2O_2 in the absence of DNA damage [51], providing another mechanistic link between oxidative stress and senescence. Long-term activation of p21 promotes mitochondrial dysfunction and increased ROS production through complex p38 MAPK-dependent signalling, resulting in a sustained DDR-ROS feedback loop and the establishment of deep irreversible senescence [34]. Elevated intracellular ROS is therefore both a cause and a consequence of senescence (reviewed in [40]). The diffusion of ROS molecules such as H_2O_2 and nitric oxide (NO) across cell membranes can also induce oxidative DNA damage and senescence in neighboring cells [52, 53].

Deep irreversible senescence is established over a number of days and is characterized by extensive chromatin remodelling and upregulation of genes encoding for secreted factors such as chemokines, cytokines, growth factors, and proteases, especially matrix metalloproteases (MMPs). Combined, these factors constitute the pro-inflammatory senescence-associated secretory phenotype (SASP) that disrupts tissue homeostasis [34, 37, 54]. The SASP is a key feature that distinguishes senescent cells from all other non-proliferating cells. Although the list of potential SASP factors is long, inflammatory factors such as interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1/CCL-2), and plasminogen activator inhibitor-1 (PAI-1) feature prominently in the SASP of several cell-types, while it is unclear whether tumor necrosis factor-α (TNF-α) should be considered a SASP factor [54]. Master regulators of SASP production include NF-κB and p38 MAPK [35, 54, 55].

The cyclin-dependent kinase (CDK) inhibitor p16 (Ink4A) arrests cell cycle progression at G1 [56], but p16 upregulation has to be sustained over days before it can induce senescence [57]. Expression of p16 can be upregulated by a variety of cellular stressors to trigger stress-induced premature senescence (SIPS) (reviewed in [58]), but p16 is also upregulated with age, which likely plays a crucial mechanistic role in the age-related decline of replicative potential of adult stem cells [33, 57]. Conversely, p16 upregulation in some tissues can be delayed by calorie restriction [59], which enhances longevity (see Sect. 16.3). p16 does not appear to promote SASP production and may paradoxically inhibit SASP production through the blunting of NF-κB signalling [35].

It can therefore be concluded that the impact of senescent cells within tissues is twofold: (1) senescent progenitor cells are inherently dysfunctional and cannot participate in tissue repair and homeostasis, and (2) through the production and release of SASP factors and ROS, this dysfunction is spread through the tissue microenvironment, inducing senescence in neighboring progenitor cells and creating a tissue-level chronic inflammatory state that has additional deleterious effects on tissue function [37].

16.2.1.2 Markers of Senescence

A commonly used marker to identify individual senescent cells within tissues or among other cells in cell culture is staining for senescence-associated beta-galactosidase (SA-β-gal) activity, although increased β-gal staining is not always a conclusive indicator of senescence (reviewed in [33]). Most studies therefore measure a selection of senescence markers, such as upregulated p53, p21, and p16 (Fig. 16.1), or increased numbers of DNA damage foci that contain γ-phosphorylated forms of the histone H2AX (γH2AX) [60]. By utilizing these markers, it has been demonstrated that senescent cells accumulate within many tissues with age and also specifically at sites of age-related pathology (reviewed in [33, 37]).

16.2.1.3 Characterization of Age-Associated Senescence in ASCs

Some comparisons between young and old human ASCs have demonstrated an age-associated loss of ex vivo replicative potential and adipogenic potential with aging [28–30], although findings from several other studies are not in agreement, in particular with regard to the impact of age on adipogenic potential [61–66]. More recent studies [29, 30, 61–66] have also started to map the impact of age on the expression of senescence markers in human and rodent

Table 16.1 Summary of findings on the effects of age on ex vivo ASC function and expression of senescence markers

Species, depot	Age groups	Effects of age	Reference
Human, SAT	Young: 27 ± 1 y Old: 71 ± 2 y	↓ Proliferation ↓ Adipogenesis ↑ TNFα release from SAT	[28]
Human (women only), orbital fat pad	Young: 20–38 y Old: 50–67 y	↓ Adipocyte size ↓ Frequency of ASCs ↑ Population doubling time ↑ Senescent cells (β-gal staining, p53, p21) ↓ Adipogenesis, ↓PPARγ2	[29]
Human (women only), abdominal SAT	Young: 23.8 ± 0.4 y Old: 57.6 ± 0.9 y	↓ Proliferation, ↓ plastic adherence ↓ Viability, ↑ apoptosis ↑ Senescence (p53, p21, p16) ↑ Pro-inflammatory gene expression ↓ Adipogenesis, ↓ PPARγ	[30]
Human, chest SAT	Children: < 13 y Young adult: < 30 y Old: > 60y	↓ Frequency of ASCs ↑ Senescence (β-gal, p21) ↑ ROS production ↓ Migration potential Delayed, but not impaired adipogenic response	[61]
Human, SAT	Young: < 40 y Old: > 50 y	↓ Frequency of ASCs ↑ Population doubling time ↑ Senescence (β-gal, p16, p21) ↓ SOD activity = adipogenesis	[62]
Human, SAT	Young: 20–29 y Old (1): 50–60 y Old (2): 60–69 y Old (3): 70–79 y	↓ Replicative potential ↑ Population doubling time Non-linear changes in senescence markers (p53, p21) = β-gal staining ↑ Adipogenesis ↑ ROS production, ↓SOD activity	[63, 64]
Mouse (depot unclear)	Young: 6–7 mo Old: 28–31 mo	↓ Proliferation ↑ Senescence (β-gal, p21) ↑ SASP production (IL-6, MCP-1)	[65]
Rat, SAT	Young: 1 mo Old: 24 mo	↑ Senescence (β-gal, p16) ↑ ROS production ↓ Adipogenesis	[66]

Abbreviations and symbols: *β-gal* β-galactosidase staining, *mo* month, *ROS* reactive oxygen species, *SAT* subcutaneous adipose tissue, *SOD* superoxide dismutase, *TNFα* tumor necrosis factor alpha, *y* year, ↑ increase, ↓ decrease; = no change

ASCs. The results of these studies are summarized in Table 16.1.

Most of the studies cited in Table 16.1 demonstrated an age-associated increase in a variety of senescence markers (β-gal, p53, p21, and p16) in human and rodent ASCs, but these changes were not always associated with decreased adipogenic potential. However, age-associated increases in intracellular ROS [63, 64, 66] and decreased superoxide dismutase (SOD) activity [62–64] were reported in several studies, suggesting a failure of anti-oxidant defense mechanisms with increasing age. Notably, scASCs from aged rats were also found to be more sensitive to functional impairment by extracellular ROS than their younger counterparts [67], and the combined findings of these studies [62–64, 66, 67] suggest an age-associated loss in the capability of ASCs to prevent or compensate for oxidative stress.

Genomic instability in adult stem cells may increase during aging, but this remains to be fully characterized [68]. In their comparison of orbital fat ASCs from young (17–25 years) and

older women (50–59 years), Zhang et al. [68] reported that there was no difference in γH2AX levels, a marker of DNA DSBs, between the two groups and that DSB repair pathways were not impaired in older ASCs. However, the efficiency of the base excision repair (BER) pathway was reduced in old ASCs, which resulted in increased sensitivity to ROS damage [68]. In contrast to these findings, scASC cultures from old men (±71 years) exhibited increased levels of γH2AX, together with loss of the proliferation marker Ki67 and higher numbers of β-gal-positive cells than that of younger men (±31 years) [69]. Cultures of perirenal ASCs from very old (30 months) rats also had higher numbers of senescent cells, higher γH2AX levels, and decreased Ki67 expression [69]. Similarly, scASCs from elderly people with atherosclerosis exhibited increased levels of γH2AX, compared to ASCs from younger counterparts [70]. Combined, these studies show that age negatively impacts genomic integrity in ASCs, but that the nature of the DNA damage may be determined by the extent of aging and may possibly be influenced by the adipose tissue depot.

Many studies investigating the mechanisms of cellular senescence have utilized senescent fibroblasts. However, fibroblasts are terminally differentiated and post-mitotic, and therefore cellular aging processes in these cells may be different from those in adult stem cells such as ASCs that maintain some degree of turnover [21, 71, 72]. Therefore, findings on senescence mechanisms in fibroblasts might not be applicable to ASCs. Correspondingly, Shan et al. [73] showed that aged human scASCs did not share a gene expression profile with senescent fibroblasts and other senescent cells and that the transcriptome of aging ASCs is more stable than in other senescent cells. Expression of genes promoting cell cycle progression and protein translation initiation was found to be maintained in old ASCs, but not in other senescent cells, and this may form part of a mechanism whereby ASCs could maintain their "stemness" and support adipose tissue function into old age.

16.2.2 Downregulation of the Adipogenic Gene Program

Even though a loss of adipogenic potential does not always occur with age in ASCs (Table 16.1), it has been shown in rat epididymal ASCs that age has considerable negative effects on the adipogenic gene program [25–27], specifically the expression and activity of the C/EBP family of transcription factors that play an integral role in modulating adipogenesis [74]. These age-related changes culminate in impaired adipogenesis, as shown in Fig. 16.2. A major upstream mediator of changes in this pathway is CUG triplet repeat-binding protein-1 (CUGBP1), which was increased in rat ASCs with age and which has also been shown to increase the translation of p21 in senescent fibroblasts [75], and although this has not been demonstrated in ASCs, it provides a possible molecular connection between ASC senescence and the loss of adipogenic capacity during organismal aging.

16.2.3 Age, Senescence, and Inflammation in ASCs

Aging is associated with chronic low-grade systemic inflammation (reviewed in [54]). Within adipose tissue, the production of pro-inflammatory factors such as IL-6 [76] and TNFα [28] also increases with age. ASCs themselves may be a major source of adipose tissue inflammatory mediators during aging, contributing to the local inflammatory state, but they are also profoundly affected by paracrine inflammation. The conditioned media from aged rat ASCs was found to contain higher levels of TNFα than that of young ASCs and inhibited adipogenesis in young ASCs, demonstrating the paracrine effects of ASC-secreted factors [27] and providing evidence that pro-inflammatory ASCs could suppress adipogenesis in neighboring cells in vivo. In particular, TNFα inhibits adipogenesis through a variety of mechanisms [26, 77, 78], shown in Fig. 16.3, and pro-inflammatory macrophages within adipose tissue may also inhibit adipogenesis through their secretome [79].

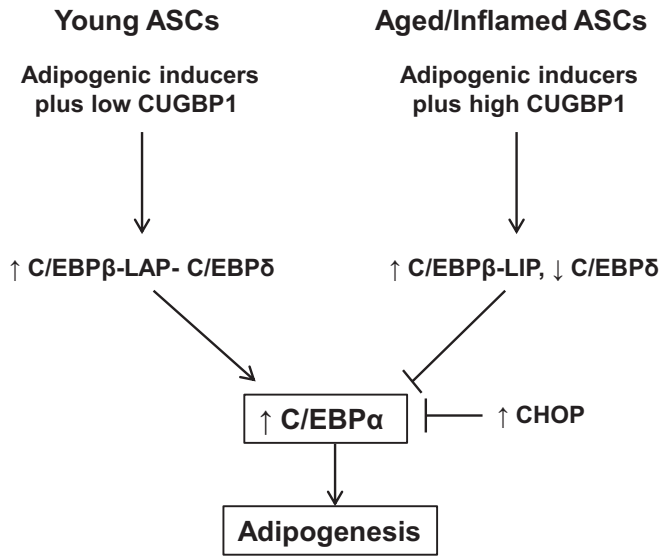

Fig. 16.2 Downregulation of adipogenesis by age or inflammation via changes in the signalling cascade involving C/EBP proteins. C/EBPα is a major pro-adipogenic factor, but while C/EBPα mRNA levels in undifferentiated ASCs do not differ with age, the adipogenesis-related induction of C/EBPα expression is blunted with increased age. During optimal adipogenesis, C/EBPα expression is induced by the full-length C/EBPβ-LAP (C/EBPβ-liver activating protein) in cooperation with C/EBPδ [74]. However, in old rat ASCs, C/EBPβ protein expression during adipogenic induction shifts from the full-length LAP isoform to the truncated dominant-negative LIP (liver inhibitory protein) isoform. CUG triplet repeat-binding protein-1 (CUGBP1) is upregulated in rat ASCs with age, binds to C/EBPβ mRNA, and preferentially increases the translation of C/EBPβ-LIP over C/EBPβ-LAP. Furthermore, expression of C/EBPδ, the heterodimeric partner of C/EBPβ-LAP, is downregulated in aged ASCs, while C/EBP homologous protein 10 (CHOP), an inhibitory heterodimeric partner of C/EBPα and C/EBPβ, is upregulated in rat ASCs with age. Collectively, these events impair the ability of C/EBPβ-C/EBPδ dimers to upregulate C/EBPα expression and stimulate adipogenesis [25, 26]. Decreased C/EBPα and increased C/EBPβ-LIP levels with age also occur in isolated rat adipocytes and adipose tissue [25]

Very few studies have characterized the SASP of ASCs. High p21 expression in ASCs from aged mice was associated with an upregulation of several pro-inflammatory pathways and increased secretion of several pro-inflammatory SASP components, including IL-6, MCP-1/CCL-2, GROα (CXCL-1), and IL-15 [65]. Similarly, compared to their younger counterparts, ASCs from elderly people with atherosclerosis secreted higher levels of the pro-inflammatory SASP components IL-6, IL-8, MCP-1/CCL-2, and MIF (macrophage migration inhibitory factor) [70]. However, aside from these two studies, information is lacking about age-related changes in the composition of the ASC secretome.

Independent of aging, inflammatory cytokines can also induce a SASP-like secretome in ASCs. TNFα treatment (but not IL-6 treatment) of undifferentiated human scASCs induced the production of a pro-inflammatory secretome containing factors such as TNFα itself, macrophage inflammatory protein (MIP-1α), and IL-1β, along with SASP components such as IL-6, IL-8, MCP-1/CCL-2, and MMPs [77]. MCP-1/CCL-2 [80] and IL-6 [81] in adipose tissue promote macrophage infiltration, thereby fuelling adipose tissue inflammation (Fig. 16.3), and correspondingly, the conditioned medium from LPS-activated monocyte-derived macrophages and from adipose tissue-derived macrophages was shown to upregulate the production of the pro-inflammatory SASP components IL-6, IL-8, and MCP-1/CCL-2 by ASCs and to downregulate the secretion of the anti-inflammatory adipokine adiponectin [79].

Circulating lipopolysaccharide (LPS) levels may also increase with age, possibly due to changes in the intestinal epithelial barrier or the

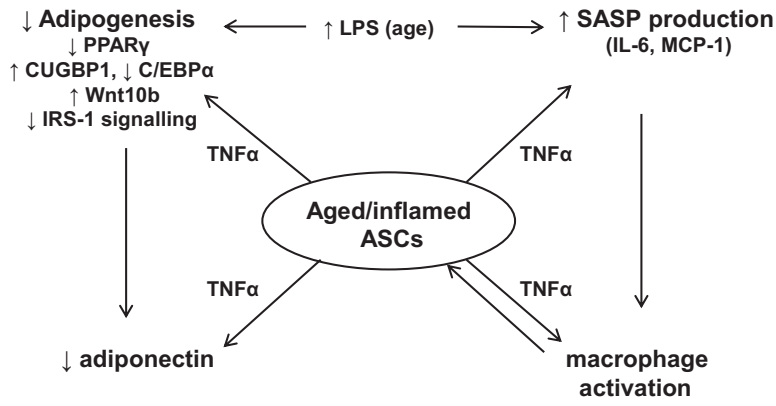

Fig. 16.3 The interlinked nature of ASC aging, senescence, and inflammation, with TNFα as a central mediator. Aged ASCs produce higher levels of TNFα, but TNFα can mimic chronological aging of ASCs on a transcriptional level through the premature upregulation of age-associated C/EBP inhibitors such as CUGBP1 and CHOP in young ASCs, resulting in a blunted adipogenic response [26] (refer to Fig. 16.2). TNFα also inhibits adipogenesis in ASCs by increasing Wnt10b expression, thereby activating anti-adipogenic Wnt signalling [77]. Furthermore, TNFα causes insulin resistance through the alternative phosphorylation and resultant de-activation of insulin receptor substrate-1 (IRS1) and through the downregulation of genes associated with insulin sensitivity and adipocyte function such as adiponectin and GLUT4 (reviewed in [78]). Other pro-inflammatory factors can be produced by ASCs themselves or by macrophages (tissue-resident or infiltrating) [79], or may originate from the circulation, such as lipopolysaccharide (LPS) [82]. Regardless of the source, inflammation establishes a self-sustaining feed-forward loop of senescence and inflammation within adipose tissue. Taken together, these findings present a mechanistic connection between age-related increased inflammation and loss of adipocyte differentiation and function in vivo

intestinal microbiota, resulting in low-grade endotoxemia [82]. In cultured mouse scASCs, LPS was shown to decrease adipogenesis and lipogenesis through the downregulation of PPARγ expression. LPS also induced senescence in these cells, characterized by increased p53 phosphorylation, β-gal staining, and ROS production, but telomere length was not affected. Furthermore, the expression of TNFα and several SASP components, including IL-1β, IL-6, MCP-1/CCL-2, and vascular endothelial growth factor-α (VEGFα), was increased by LPS [83]. Collectively, the studies mentioned in this section provide strong evidence for the negative impact of local and systemic inflammatory factors on ASC function in vivo and describe the complex interrelated feedback mechanisms between aging, inflammation, ASC senescence, and adipose tissue dysfunction (Fig. 16.3).

16.2.4 The Role of ASCs in the Age-Related Loss of Lipid Storage Capacity in Adipose Tissue

The loss of total body fat mass with aging [3–5] suggests that the lipid storage capacity of adipose tissue deteriorates with age. Apart from the impaired storage capacity that would inevitably result from defective adipogenesis in vivo, the lipid handling of individual adipocytes may also deteriorate with age. Guo et al. [84] demonstrated that aged ASCs were more susceptible to fatty acid-induced apoptosis than young ASCs. The fatty acid oleate upregulated the expression of PPARγ2 and C/EBPα in young ASCs, but not in old ASCs, indicating that young ASCs can launch an adipogenic response to exogenous lipid overload, resulting in increased lipid storage and reduced lipotoxicity, while this response may be impaired in old ASCs. β-Oxidation in ASCs was also reduced with age, due to mitochondrial dysfunction. These age-related alterations in lipid

handling may establish a cycle of lipotoxicity, with exogenous free fatty acids inducing preadipocyte apoptosis, reduced adipogenesis, and failure to store fatty acids, resulting in increasingly elevated local levels of free fatty acids and aggravated fat tissue dysfunction. Release of these fatty acids into the circulation may also cause ectopic lipid deposition and lipotoxicity in other tissues, such as muscle and pancreas [84].

In addition to the loss of total adipose tissue mass, a specific loss of SAT volume is often observed with age [5, 6] and may involve the accelerated replicative exhaustion of scASCs, relative to vASCs. Cultures of human scASCs were found to contain a higher proportion of rapidly dividing cells than omental ASCs (oASCs) [85], but human scASCs also have shorter telomeres than oASCs [86]. Combined, these two factors may result in scASCs losing their replicative capacity and entering senescence before ASCs in other depots, resulting in loss of adipogenic potential specifically in SAT.

Adipose tissue inflammation may also contribute to the loss of SAT with age. Caso et al. [28] found that the loss of replicative and adipogenic capacity in scASCs from older humans was associated with increased TNFα release from the originating SAT. In addition, the adipogenic potential of isolated scASCs was positively correlated with the SAT/VAT ratio of the donors [28], and taken together, these findings indicate that the age-associated loss of SAT may result from the suppression of adipogenesis in scASCs by increased local concentrations of inflammatory mediators, via the mechanisms discussed above (Figs. 16.2 and 16.3).

The consequence of the age-related loss of SAT can also impact on the ability of the individual to compensate for metabolic insults such as a high-fat diet (HFD). In mice, HFD was initially associated with increased energy expenditure, but aged HFD mice exhibited lower energy expenditure and glucose intolerance. Older mice on HFD developed non-alcoholic fatty liver disease (NAFLD) to a far greater extent than younger HFD-fed mice, indicative of poor adipose tissue storage capacity and lipid spill-over with age. While this study found that there was no age-specific increase in senescence markers in the SAT of lean animals, HFD induced senescence in the SAT of aged mice [87]. These findings support the idea that the SAT is an important site of metabolic compensation and that the loss of this function with age may underpin many age-related metabolic disturbances.

When considering the findings of the studies described in this section, it can therefore be concluded that, consistent with the general features of senescent cells described above, senescent ASCs are dysfunctional in two main ways: (1) they have impaired capacity for adipogenesis and lipid storage, and (2) they secrete SASP factors that negatively impact the adipogenesis and lipid storage of neighboring ASCs and mature adipocytes. Collectively, these two mechanisms drive adipose tissue dysfunction through the interrelated pathways of lipotoxicity, inflammation, and insulin resistance (reviewed in [20]).

16.3 Adipose Tissue as a Source of Longevity Signals

The mechanisms described in Sect. 16.2 underscore the notion that aging has a profound impact on the biology and function of adipose tissue and ASCs. However, it has emerged that the reverse is also true, that adipose tissue can indeed play a major role in how we age, in terms of both healthspan and lifespan, suggesting that adipose tissue may generate systemic signals that can regulate organismal aging. This may take the form of physical signals, i.e., circulating factors that emanate from adipose tissue to impact the function of both adipose and non-adipose tissues, thereby influencing the risk for age-related metabolic disorders and other diseases affecting mortality. Two factors appear to hold the key to the effects of adipose tissue on longevity: (1) the relative distribution of fat mass between SAT and VAT and (2) the modulation of adipose tissue function by calorie restriction (CR). The interlinked nature of these two factors and the role of ASCs in regulating lifespan and healths-

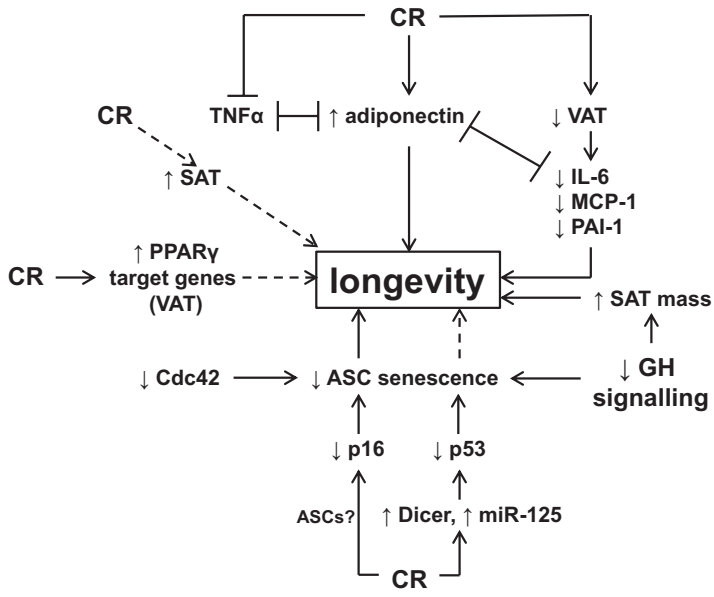

Fig. 16.4 The multitude of mechanisms whereby calorie restriction (CR) and other signals may enhance longevity through the regulation of adipose tissue and ASC function. Mechanisms which have been directly demonstrated are shown with solid arrows, while putative or inferred mechanisms are shown with dashed arrows. CR reduces VAT mass [106, 107], and decreased VAT is associated with reduced circulating levels of IL-6 and MCP-1/CCL2 [91]. Circulating IL-6 levels are positively associated with mortality [93, 94], and therefore a reduction in IL-6 levels may promote longevity. Dietary restriction (DR) in humans decreases circulating PAI-1 levels [108], which may prevent atherothrombosis [92] and promote longevity. DR also increases circulating adiponectin levels [112]. Adiponectin is independently associated with longevity [97], but adiponectin also suppresses TNFα [101] and IL-6 [99] production, while both IL-6 [100] and TNFα [102] suppress adiponectin production. CR also downregulates other components of the TNFα signalling cascade [109, 110]. It is not clear whether the effects of adiponectin on lifespan are mediated solely through the suppression of IL-6 and TNFα, or whether other mechanisms are involved as well. CR was found to increase expression of PPARγ target genes in VAT [111], but a direct connection to extended lifespan was not demonstrated. CR was also found to prevent the age-related downregulation of Dicer and miR-125 and the subsequent upregulation of p53 in ASCs, thereby reducing ASC senescence [116, 117], but a direct connection to lifespan was not demonstrated in these studies. CR delays the age-related upregulation of p16 in some tissues [59], but this has not been demonstrated for ASCs. However, clearance of p16-expressing cells is associated with extended lifespan [123, 124]. Cdc42 activity increases with age and is associated with reduced lifespan and healthspan and increased ASC senescence [30, 66, 119], but it is not clear whether CR can reverse this. CR may also preserve SAT mass independent of effects on VAT [121], but this has not been directly demonstrated. Reduced GH signalling is associated with increased lifespan, preserved SAT mass, and decreased senescent cell burden in adipose tissue [113–115]

pan will be discussed below and are summarized in Fig. 16.4.

16.3.1 Adipose Depot-Specific Contributions to Lifespan Determination

While general obesity is in itself a risk factor for many diseases [88], increased VAT has specifically been linked to increased morbidity and mortality associated with insulin resistance, T2DM, and cardiovascular disease [89], likely due to the heightened pro-inflammatory nature of VAT, compared to SAT (reviewed in [13, 90]). In humans, the ratio of VAT to SAT increases with age (reviewed in [5]), but increased VAT reduces lifespan and increases mortality, independent of total body adiposity [11, 13]. VAT mass is positively associated with the circulating levels of several pro-inflammatory cytokines, including IL-6 and MCP-1/CCL-2 [91], and is also a major

source of PAI-1 [92]. Plasma IL-6 levels have emerged as a predictor of mortality [93, 94], while PAI-1 contributes to the pathogenesis of atherothrombosis [92]. Therefore, in addition to predisposing individuals to insulin resistance and diabetes, VAT also produces circulating factors that directly impacts mortality, although these may form part of converging pathophysiological mechanisms. By extension, removal of VAT or reduction of VAT-derived factors should then reduce mortality. Correspondingly, in rats, surgical removal of VAT enhanced lifespan [12] and also strongly downregulated the expression of TNFα in SAT [95, 96], which would reduce inflammation and SASP production and improve lipid storage capacity in SAT, as discussed above.

16.3.2 Circulating Factors and Longevity

One adipose-derived factor (adipokine) that may be pivotal in enhancing longevity is adiponectin. High circulating levels of adiponectin were found in individuals of extremely advanced age (>95 years old), independent of BMI [97], and was also associated with an increased SAT/VAT ratio [98]. A causal relationship between adiponectin and longevity may reside in the ability of adiponectin to downregulate IL-6 production. In cultured porcine ASCs, adiponectin prevented the upregulation of IL-6 expression by inflammatory mediators such as LPS [99]. Conversely, in cultured human scASCs, IL-6 treatment reduced adiponectin gene expression [100]. Given the positive association of IL-6 levels with mortality [93, 94] and the increase in IL-6 expression in adipose tissue with age [76], it may be hypothesized that the balance between IL-6 and adiponectin production could be a determining factor in lifespan, although this has not been conclusively demonstrated in humans. In addition, Maeda et al. [101] showed that adiponectin suppresses TNFα expression in adipose tissue and reduces TNFα levels in circulation, which would reduce inflammation and inhibit SASP production within adipose tissue (Fig. 16.3). Conversely, TNFα blocks the release of adiponectin from adipose tissue [102]. These "balancing acts" may form part of a mechanism whereby adiponectin could preserve ASC and adipose tissue function into advanced age, or alternatively, whereby a reduction in lifespan could result from the negative impact of chronic inflammation on adiponectin production (Fig. 16.4).

CR is the only documented non-genetic, non-surgical, and non-pharmacological ("life-style") intervention to date that can delay the onset of many age-related disorders and extend lifespan across vertebrate and non-vertebrate species, although the evidence in humans is understandably scant [88, 103]. In the laboratory, CR is achieved by restricting the calorie intake of the animals by 20–40% of that of ad libitum-fed counterparts. This reduction in food intake results in a substantial reprogramming of energy balance, body mass, and body composition, with a typical reduction in fat mass (reviewed in [13]). Nutrient signalling such as the mTOR pathway is also profoundly affected by CR [104, 105]. While it is controversial whether the reduction in fat mass actually contributes to the effects of CR on longevity [13, 103], CR affects the function of adipose tissue and ASCs on a molecular level, as will be discussed below (Fig. 16.4), and these adipose-specific effects have a broader impact on a systemic level. Findings from animal models suggest that CR may preferentially target VAT, which may account for at least some of the lifespan-extending effects of CR, due to the impact of VAT on lifespan as discussed above. In rats, CR over 18 months reduced total fat mass to 33% but VAT mass to less than 20% of that of their ad libitum-fed counterparts [106], and CR in monkeys reduced VAT mass within 12 months [107]. In addition, the surgical removal of VAT in young rats achieved the same extension of lifespan as CR [12]. Dietary restriction (DR, the human equivalent of CR) induced moderate weight loss and reduced circulating PAI-1 levels in elderly obese individuals [108], suggestive of direct effects on VAT function by DR.

The combination of VAT reduction and CR may promote insulin sensitivity and decrease the risk of developing age-related insulin resistance and T2DM (reviewed in [90]), but CR also has

dramatic effects on gene expression in adipose tissue that may directly contribute to preserving adipose tissue function. Long-term CR in mice upregulated the expression of numerous genes involved in metabolism, substrate utilization, and energy production in epididymal fat, while a large number of inflammatory genes were downregulated, in particular genes in the TNFα signalling cascade [109, 110]. In rats, CR was found to have a far greater transcriptional effect in VAT, compared to the heart, with the majority of the transcriptional effects of CR in rat VAT serving to reverse age-associated changes. PPARγ target genes involved in adipogenesis were downregulated in VAT with age, but not with CR, suggesting that CR does not reduce fat mass by downregulating adipogenesis, but rather that it preserves adipogenic potential to reduce the risk of adipocyte hypertrophy and inflammation. CR also downregulated immune response genes and preserved the expression of cell cycle regulatory genes in VAT, which may delay the onset of senescence [111]. Moreover, DR increased the circulating adiponectin levels of both diabetic and non-diabetic subjects [112], suggesting a possible convergence of mechanisms involved in the lifespan-extending effects of CR and adiponectin (Fig. 16.4).

Findings in genetic models of longevity have also demonstrated a role for ASCs in the relationship between adipose tissue function and lifespan. Decreased growth hormone (GH) activity is associated with increased lifespan in rodent models, while overexpression of GH has the opposite effect (reviewed in [113]). This is consistent with observations in humans that a low level of circulating insulin-like growth factor-1 (IGF-1), which acts downstream of GH, is associated with longevity, especially in women [114]. Long-lived GH-deficient or GH-resistant mice are not subject to the age-associated loss of SAT mass observed in their wild-type littermates [113, 115], and the adipogenic potential of scASCs is accordingly enhanced in GH receptor (GHR) KO mice [113]. GHR deficiency also results in a decreased senescent cell burden in various adipose tissue depots. These findings indicate a role for GH in modulating ASC function as we age. However, as alterations in GH levels and signalling affect circulating levels of IGF-1, insulin, and glucose [113], the mechanism involved is not clear at this stage.

16.3.3 Molecular Mediators of Longevity: Dicer and Cdc42

Mouse and human adipose tissue and ASCs were found to exhibit an age-related downregulation of miRNA processing machinery, in particular Dicer, a cytoplasmic enzyme involved in the late processing events of miRNAs. This was associated with decreased expression levels of several miRNA species, but a concomitant accumulation of miRNA precursors, consistent with impaired miRNA processing capacity. Cultured Dicer-KO mouse scASCs exhibited increased population doubling time and upregulation of senescence markers such as increased total p53 and phospho-p53, increased p21 expression, and higher numbers of β-gal-positive cells [116]. Loss of Dicer expression downregulated miR-125 expression, which allowed for the upregulation of its target gene, p53 [117], thereby driving senescence in ASCs. Mice with white adipose tissue-specific KO of Dicer had higher levels of phospho-p53 in adipose and other tissues, indicating that adipose Dicer KO had effects beyond adipose tissue that mimicked premature aging, although it is not clear how these effects were communicated at the systemic level. The downregulation of Dicer expression and subsequent deficiencies in miRNA processing was prevented with CR, providing another candidate mechanism whereby the effects of CR on adipose tissue and ASCs could promote longevity [116] (Fig. 16.4). It is noteworthy that CR has also been found to enhance the capacity of hepatocytes to repair oxidative DNA damage [118], and although this has not been shown in ASCs, a similar mechanism may contribute to the CR-mediated delay in senescence in ASCs.

Comparisons between ASCs from young and old rats and humans also identified a role for Cdc42 (cell division control protein 42), a member of the Rho GTPase family, in lifespan as well

as ASC senescence and dysfunction. Increased age was associated with increased levels of Cdc42-GTP, the active form of Cdc42, in both rat and human ASCs [30, 66]. In rat ASCs, this correlated with decreased replicative and adipogenic potential, increased expression of senescence markers, and increased ROS production, all of which was at least partially reversed by pharmacological inhibition of Cdc42-GTP by CASIN [66]. Similar to these findings in rat ASCs, pharmacological inhibition of Cdc42-GTP with ML141 reversed the age-related decrease in proliferation, viability, and plastic adherence, as well the enhanced pro-inflammatory gene expression profile and loss of adipogenic potential in aged human ASCs [30]. In mice, Cdc42 activity was also found to increase with aging in various tissues, and constitutive activation of Cdc42 resulted in reduced lifespan, loss of SAT, and several age-associated impairments [119] (Fig. 16.4). These findings are consistent with the idea that longevity is closely tied to the preservation of SAT and ASC function, and taken together, these studies imply a mechanism whereby Cdc42 activity may affect lifespan through the modulation of ASC function.

16.3.4 Calorie Restriction: Guardian of SAT Function?

The idea that a reduction in fat mass through methods such as CR is beneficial to lifespan has long been controversial [13, 103, 120]. The results of Stout et al. [113] and Wang et al. [119] suggest that preservation of fat mass, especially SAT, is associated with longevity, and given the various roles of SAT in maintaining metabolic homeostasis into old age, as described above, such a mechanism would make intuitive sense. In support of this, Liao et al. [121] observed that among various strains of inbred mice, the reduction of fat mass with CR, compared to ad libitum feeding, was actually negatively correlated with lifespan, although the effects of CR on individual fat depots were not determined. However, given that SAT is the biggest adipose depot in the body, it may be reasonably assumed that when fat mass loss is reflected in body mass loss, the SAT mass is being reduced, possibly in parallel with reductions of other adipose depots. Taken together, the findings described in this section demonstrate that adipose tissue function plays a fundamental role in longevity, by regulating glucose and lipid metabolism and by generating signals that modulate lifespan on a systemic level. In particular, the preservation of optimal adipose tissue and ASC function, in particular in SAT, forms an indispensable part of this mechanism. Given the information in the paragraphs above, perhaps the controversy may then be addressed as follows: the key to longevity does not reside in the actual fat mass, but in the function of individual adipose depots. Ideally, SAT mass should be preserved into old age, for optimal lipid storage and prevention of lipotoxicity, provided that the SAT does not become pro-inflammatory. Interventions that preferentially reduce VAT mass may be desirable, but even in the absence of a loss of VAT mass, a shift in the production of circulating factors toward a less inflammatory phenotype, in particular increased adiponectin production, may achieve the same goal. Crucially, the preservation of adipogenic potential and delayed onset of senescence in ASCs provides adipose tissue-level resilience into old age and protects against adipose inflammation that disrupts systemic metabolic regulation and predisposes to age-related diseases.

16.4 Alleviating the Effects of Senescent ASCs In Vivo

16.4.1 Targeting Senescent ASC: Senolytics

Aging is a major risk factor for a host of chronic diseases, which often cluster within individuals, resulting in multi-morbidity [122]. The "geroscience hypothesis" posits that if the interrelated aging processes of sterile inflammation, progenitor cell dysfunction, and increased burden of senescent cells can be targeted therapeutically, this can delay or prevent age-related diseases or disabilities as a group, rather than attempting to treat individual conditions. Consequently, if

senescent cells within adipose tissue mediate tissue-level and systemic metabolic dysregulation to reduce healthspan and lifespan, then the clearance of such cells should restore metabolic homeostasis, relieve age-related disorders and frailty, and promote longevity (reviewed in [2]), and there is indeed promising evidence to support this idea. Clearance of p16-expressing cells via a drug-induced "suicide" transgene in wild-type and progeroid mice resulted in delayed initiation and progression of age-related pathologies and frailty, preserved fat mass including SAT, restored adipogenesis, reduced inflammation, and extended lifespan [123, 124]. Of specific importance here was the association between preserved SAT mass and increased lifespan, supporting the notion that longevity signals originate in the SAT, as described in Sect. 16.3.

Transgene strategies to reduce senescent cell burden are not currently a feasible treatment option in humans, but efforts in the past 5 years have identified pharmaceutical compounds that may serve the same purpose. Senescent cells remain viable and metabolically active, in particular with regard to the production of SASP factors, but they are also more stress-resistant than non-senescent cells, with greater reliance on anti-apoptotic and pro-survival pathways, and are therefore more difficult to clear from tissues. Senolytic drugs can trigger the selective clearance of senescent cells from in vitro cultures and from tissues in vivo by circumventing the resistance of senescent cells to apoptosis through targeting senescence cell anti-apoptotic pathways (SCAPs) (reviewed in [10, 125]). Dasatinib (D) and quercetin (Q) (both broad-spectrum kinase inhibitors) have been identified as senolytics with specific activity to induce apoptosis in culture-senescent ASCs, but not proliferating non-senescent ASCs [125]. In addition, a single dose of D + Q was sufficient to reduce the number of senescent cells in the adipose tissue of aged mice within 5 days. However, senescent cells from different tissue origins vary in their susceptibility to individual senolytic drugs [125, 126], and therefore it may be advisable to devise tissue-targeted senolytic regimes for improved efficacy. Conversely, as senescent cells employ common SCAP pathways, many senolytics may have broad-spectrum effects and could alleviate a range of dysfunctions, albeit at varying degrees of efficacy.

Clinical trials of senolytics are only now starting to emerge, and therefore the long-term side effects and consequences of these agents are not yet known. As senolytics function to remove senescent cells from tissues, rather than continuously occupying a single molecular target, they can be administered intermittently through short-course treatments [10]. Such treatment regimens may reduce off-target side effects such as delayed wound healing, where senescent cells play a crucial role [127], while the therapeutic effects would be long-lasting, depending on the rate of senescent cell re-accumulation. In addition, as senescent cells do not divide, they also cannot acquire mutations to induce drug resistance [10]. Moreover, as many of the deleterious effects of senescent cells are mediated via the SASP, even partial clearance of senescent cells should have beneficial effects. However, the true endpoints of senolytic treatment in humans, such as increased lifespan and healthspan, occur over decades and are therefore impossible to measure in clinical trials [10]. Short-term surrogate markers therefore have to be utilized instead, but their validity and correlation with longer-term outcomes will only be determined in the future. In a phase I pilot study [128], a 3-day course of D + Q was administered to patients with diabetes and chronic kidney disease, and the effects were assessed 11 days after cessation of treatment. Adipose tissue histology demonstrated a decrease in the number of senescent cells within adipose tissue, and ASC cultures exhibited enhanced growth rate over time, consistent with the prior removal of non-replicating senescent cells from the SVF. In addition, circulating levels of several SASP components, such as interleukins and MMPs, were reduced, supporting the systemic impact of senolytic treatment [128]. This study provided evidence that senolytic treatment does have measurable short-term effects in humans that would be consistent with a long-term improvement of healthspan and lifespan and therefore paves the way for future senolytic trial design.

16.4.2 Targeting the SASP

An alternative strategy for mitigating the negative impact of senescent cells in adipose tissue may be to suppress the production of inflammatory SASP components with conventional drug treatment. In support of such an approach, glucocorticoids such as corticosterone and cortisol have been found to reduce the production and secretion of several pro-inflammatory SASP components by senescent fibroblasts in culture [129], although similar effects have not been demonstrated in ASCs or adipocytes. The anti-diabetes drug metformin, which blocks NF-κB signalling in senescent fibroblasts in vitro [130], was unexpectedly found to extend the lifespan of diabetic patients beyond that of non-diabetic counterparts [131]. Correspondingly, in healthy aging mice, chronic metformin administration had insulin-sensitizing and anti-oxidant effects in the liver and improved healthspan and lifespan, although the contribution of adipose-specific effects of metformin was not determined [132]. Similar to metformin, the mTOR inhibitor rapamycin was also found to inhibit NF-κB signalling and SASP production in senescent fibroblasts [133] and extended the lifespan of aged mice [134]. However, as is the case for metformin, the impact of rapamycin on senescence in ASCs and adipose cells has not been examined.

Ruxolitinib, an inhibitor of the Janus kinases (JAK)1/2 that form part of the JAK/STAT pathway, shows promise as an adipose-specific SASP inhibitor. In culture-senescent human ASCs, different JAK inhibitors, including ruxolitinib, were all found to downregulate the expression of various pro-inflammatory SASP components [69]. In aged mice, ruxolitinib administration decreased systemic and adipose tissue inflammation and reduced frailty, although the effect on lifespan was not reported [69]. In a related study, the same authors also found that ruxolitinib treatment of aged mice had several beneficial metabolic effects, including preservation of SAT mass, enhanced adipogenesis, and lipid storage in fat [135]. These effects were attributed to a reduction in the expression of the ASC SASP factor activin A [135]. Notably, increased serum activin A has been associated with increased cardiometabolic risk factors in humans, providing more evidence of the systemic impact of SASP factors [136]. Importantly, several of the effects of ruxolitinib only occurred in old animals, suggesting a specific targeting of senescent cells, of which the burden in adipose and other tissues increases with old age [69, 135]. In addition, IL-6, of which the expression in adipose tissue and ASCs increases with age [65, 76], activates the JAK/STAT pathway [137], which may also explain why inhibition of this pathway with ruxolitinib has specific effects in aged animals. Taken together, these findings indicate that ruxolitinib may enhance healthspan, if not lifespan, in aged organisms, by targeting adipose tissue and ASC inflammation. However, although ruxolitinib is FDA-approved for treating conditions such as myelofibrosis, it has considerable side effects in humans, including anemia and thrombocytopenia [138], and therefore further assessments need to be performed before this drug can be recommended for alleviating age-related metabolic dysfunction in the clinical setting.

16.5 Implications of Age for the Use of ASCs in Cell-Based Therapy

Stem cell therapy, where stem cells are used to treat or prevent a disease, can be either autologous, where the patient's own stem cells are used, or allogeneic, where cells from a genetically similar donor are used. Both of these strategies have advantages and disadvantages: autologous therapy will circumvent immune rejection, but stem cells from individuals may be compromised due to age or disease. Conversely, allogeneic therapy allows for "off-the-shelf" products and the choice of stem cells with increased therapeutic fitness, but may fail due to immune rejection. MSCs, including ASCs, possess several features that make them ideal for either autologous or allogeneic stem cell therapy. ASCs are easily accessible and highly proliferative in culture, and large numbers of cells can

therefore be generated with ease. In addition, they can migrate and home into sites of tissue injury, and they are immunoprivileged in that they do no elicit an immune response and furthermore generate an immunosuppressive environment through the paracrine actions of secreted cytokines (reviewed in [139]). For these reasons, ASCs are becoming increasingly popular for use in therapeutic interventions in a variety of degenerative or inflammatory diseases (reviewed in [140]). However, given the fundamental changes in ASC biology that occur with organismal aging outlined above, including senescence, loss of replicative potential, and adoption of a pro-inflammatory phenotype, it is vital to assess the impact of individual donor characteristics on the performance of ASCs in a therapeutic setting. Depending on the application, ASCs can be directly transplanted through injection or infusion, but the therapeutic effects of ASCs are mostly derived from their secretome, rather than their repopulation of injured sites or their multipotency for differentiation (reviewed in [141]). Therefore, aside from the potential loss of fitness of aged ASCs and the resultant failure of the ASC-based treatment, it is also possible that aged dysfunctional ASCs may actively introduce harmful effects in the recipient organism through their pro-inflammatory secretome. However, the effects of in vivo aging on human ASC function ex vivo vary quite considerably between individuals [142], suggesting that ASCs from individual donors should ideally be evaluated on own merit to determine suitability in therapeutic applications.

16.5.1 Therapeutic Implications of Donor Age

16.5.1.1 Loss of Function (Passive Dysfunction)

Limited information is available on the effect of donor age on the capacity of ASCs to promote angiogenesis, which is an important feature of injury repair. Duscher et al. demonstrated a loss of vasculogenic potential for aged mouse ASCs using in vitro and in vivo assays [143]. Similarly, in a rat model of myocardial infarction (MI), transplanted ASCs from old rats exhibited impaired capacity for engraftment, angiogenesis, and structural repair, which was attributed to the increased sensitivity of aged ASCs to ROS damage, in particular the dramatically elevated ROS levels in the MI micro-environment [67]. Of note, old ASCs performed better than old bone marrow stem cells (BMSCs) in animals undergoing surgical ventricular reconstruction, due to lower expression of several senescence markers and better angiogenic potential of old ASCs, compared to old BMSCs [144]. Surprisingly, given the interest in ASCs for regenerative medicine, the impact of age on the pro-angiogenic effects of ASCs in the clinical setting has not been studied, and therefore the clinical relevance of these animal studies still remains to be determined.

Wang et al. [65] demonstrated that ASC cultures from aged mice had a significantly higher proportion of cells expressing p21 than their young counterparts, and these cells also exhibited higher expression of pro-inflammatory SASP factors such as IL-6 and IL-8. Correspondingly, direct intraperitoneal transplantation of ASCs from young animals was able to reverse physical frailty in aged mice, whereas transplanted old ASCs could not, again indicating that senescent ASCs may result in failure of cell-based therapies. In contrast, Dufrane et al. [145] found that donor age did not affect the success of ASCs for use in manufacturing a complex osteogenic graft for bone non-union injury, suggesting that the applicability of aged ASCs may depend on the specific therapeutic application.

16.5.1.2 Acquisition of Pathological Features (Active Dysfunction)

Due to their immunomodulatory properties, ASCs are considered for use in the treatment of chronic inflammatory disorders. However, ASCs from aged individuals with atherosclerosis were found to have a predominantly pro-inflammatory secretome, with increased levels of IL-6, IL-8, and MCP-1/CCL-2 [70], rendering these cells useless for therapeutic purposes and creating the likelihood that these cells may actively

contribute to pathology. Accordingly, infusions of young ASCs into aged mice with bleomycin-induced pulmonary fibrosis (a model for progressive idiopathic pulmonary fibrosis that occurs mainly in older humans) reduced fibrosis, inflammation, apoptosis, and oxidative stress in lung tissue, while aged ASCs could not reverse disease progression and actually exhibited increased expression of MMP2, which is directly associated with disease severity [146]. These findings suggest not just a lack of therapeutic potential of old ASCs but also provide in vivo evidence that old ASCs may actively contribute to pathology under certain conditions.

Pre-clinical investigations have also provided evidence that the immunomodulatory actions of injected ASCs may be utilized to treat a diverse range of conditions, including mucosal immunosenescence [147, 148], ovarian aging [149], and diabetic osteoarthritis [150]. However, these treatments all rely on an optimal ASC secretome, and it is likely that such treatment strategies would fail if the administered ASCs are senescent and pro-inflammatory and may even result in aggravated pathology, although this has not been directly demonstrated. The menopausal status of female ASC donors may also influence the immunomodulatory capabilities of isolated ASCs [151]. Collectively, these findings highlight the need for comprehensive evaluation of both the ASC donors and the isolated ASCs, especially when autologous application of ASCs is being considered in aged or metabolically compromised individuals.

16.5.1.3 Transplanted ASCs as a Treatment for Metabolic Syndrome?

Various consequences of the age-related loss of SAT on healthspan and lifespan were discussed in detail in the preceding paragraphs, but SAT function can also affect the ability of the individual to compensate for metabolic insults such as a high-fat diet (HFD). Taketani et al. [87] demonstrated that HFD in aged mice induced glucose intolerance, NAFLD, and senescence in SAT. ASCs from aged HFD mice also displayed reduced expression of stemness markers and diminished capacity for differentiating into white and brown adipocytes. Injection of young ASCs into the SAT of aged HFD mice improved metabolic parameters and resolved NAFLD, although this was not achieved when using ASCs from aged HFD mice. ASC transplant also reduced SAT inflammation, reflected in decreased expression of inflammatory markers such as MCP-1/CCL2, TNFα, and PAI-1. Therefore, ASC transplant restored the capacity of SAT to compensate for HFD-induced metabolic disturbances, which holds tremendous potential for the treatment of obesity, adipose tissue dysfunction, and metabolic disorders that are otherwise not responsive to dietary or pharmacological interventions.

16.5.2 Ex Vivo Rejuvenation of ASCs

The accumulation of senescent ASCs within the adipose tissue may result in compromised fitness of isolated ASCs for use in autologous ASC-based therapies. In addition, harvested ASCs may have to be expanded in culture to achieve sufficient cell numbers for therapeutic applications, but ASC cultures from aged individuals often exhibit prolonged population doubling times in culture [28–30, 62–64]. Unfortunately, the aged population also represents the biggest market for ASC-based therapies, due to the large numbers of chronic conditions in this group, coupled with increased prevalence of vasculopathies and impaired tissue repair, which may present a problem when autologous ASC therapy is being considered [141, 143]. However, potential strategies and products have been identified that may be used in ex vivo cell culture to rejuvenate senescent ASCs isolated from aged individuals before these cells are applied in the therapeutic setting. These products include anti-oxidant and/or anti-inflammatory compounds such as zinc sulfate [152], L-carnitine [153, 154], and resveratrol [155]. Chaker et al. also reduced ASC senescence and restored ASC function through treatment with the Cdc42 inhibitor ML141 [30]. These studies support the principle that the ex vivo reju-

venation of ASCs can be achieved, but were largely performed in ASCs from young or middle-aged subjects (<60 years old), and should ideally be repeated in ASCs from geriatric subjects (>65 years old) to provide more evidence for the clinical potential of these compounds with regard to cell-based therapy in elderly patients. Rejuvenation strategies that have shown promise in rodent ASCs include curcumin [156], glutathione, and melatonin [157]. It is also noteworthy that *Alpinae oxyphyllae fructus* (AOF) extract, used in traditional Chinese herbal medicine, promoted an anti-inflammatory and pro-survival secretome in cultured rat epiASCs [158]. Taken together, these findings open up the possibility that ASCs can be rejuvenated ex vivo, in terms of both replicative potential and secreted factors, to improve the applicability of these cells in the therapeutic setting.

Intriguingly, Son et al. [159] demonstrated that intravenous administration of autologous ASCs in old rats improved several measures of biological aging, including improved serum levels of anti-oxidant enzymes. Although the performance of these cells was not compared to that of young ASCs, these aged ASCs were not treated with any rejuvenation agents before transplantation, suggesting that the 2 weeks (four passages) that these cells spent in ex vivo culture was sufficient to rejuvenate them to the extent that they could subsequently stimulate functional improvement in their original aged environment. In contrast, Wang et al. [65] found that intraperitoneal injection of old ASCs into aged mice resulted in increased frailty, compared to aged recipients of young ASCs. In this study, ASCs were only maintained in culture for one passage before transplantation, which may not have been sufficient to achieve ex vivo rejuvenation, possibly contributing to these contradicting outcomes. Alternatively, the site of administration (intravenous vs. intraperitoneal) may also have affected the outcome of the ASC transplants. These findings may have implications for ASC therapy in aged individuals, but need to be further explored in humans.

16.6 Concluding Remarks

From the findings reviewed here, it can be concluded that the age-related changes in the functional capacity of WAT are underpinned by the age-related changes in ASC biology. Aging ASCs lose their capacity to proliferate and differentiate into adipocytes, become senescent and inflamed, and "infect" surrounding cells with senescence and inflammation. These changes on a cellular level result in metabolic disturbances at an organismal level and reduce lifespan. In contrast, where these changes can be avoided through genetics or reversed with therapy, good health can be insured into advanced age.

However, much remains to be examined and elucidated with regard to the effects of age on ASC function. For instance, given the distinct roles of SAT and VAT in metabolic (dys)function as we age, it is surprising that no studies have directly compared the effects of aging on ASCs from different adipose tissue depots. Oxidative stress damage may become more pronounced in VAT than in SAT with age [6], and given the central role for oxidative stress in driving senescence in other cell types [34, 38–40], this may possibly bring about depot-specific features of senescence in ASCs. Depot-specific effects of aging on telomere length in ASCs have been shown [86], further supporting the idea that aging may proceed differently in scASCs and vASCs. The SASP of aging ASCs has also not really been characterized, other than a few studies measuring individual candidate SASP components, but comprehensive proteomics analyses of the SASP may be more informative. Inter-species comparisons of ASC SASP are also lacking. This is an important issue to address, as fundamental differences in senescence mechanisms between mouse and human cells have been identified [37], and therefore experimental findings in animal models should be viewed with this caveat in mind.

The molecular mechanisms and signalling pathways governing senescence in ASCs have also not been studied in detail. Nutrient excess and the resultant chronic activation of mTOR sig-

nalling promote senescence in other cell types and reduce lifespan (reviewed in [40, 105, 160]), but the role of mTOR signalling in age-related ASC senescence has not been studied, despite the importance of adipose tissue and ASCs as nutrient sensors. The actions of major adipose tissue influencers such as IGF-1, insulin, and glucose all converge on mTOR signalling (reviewed in [113]), but these mechanisms remained to be elucidated in ASCs. In other cell types, the regulation of senescence by mTOR has been shown to occur within the context of a complicated relationship with p53 [36, 105, 161, 162], but knowledge on the exact actions of p53 in ASCs is scant. Although several studies mentioned in this review describe an increase in p53 and p21 expression in aged ASCs, the p53-p21 pathway can be pro- or anti-senescent, depending on the nature and the level of the cellular stress [37, 40], and therefore more work is needed to understand the biological relevance of these molecular events.

Although there is clear evidence for the negative impact of chronological aging on ASCs, it is also important to note the resilience of aging ASCs, compared to other senescent cells, especially with regard to the preservation of cell cycle gene expression and protein translation functionalities [73]. Numerous studies [29, 30, 48, 61, 62, 66–68, 70, 146] have also shown that there are no differences in the expression of mesenchymal cell surface markers, such as CD90, CD73, and CD105, between young and old ASCs from rodent and human origin, indicating that the underlying stemness of ASCs do not decrease with age, even when senescent markers are upregulated. This feature of ASCs may serve to "soften the blows" of aging by avoiding a total loss of ASC function and may also form part of the mechanism that allows ASCs from aged individuals to be rejuvenated ex vivo, as was described in Sect. 16.5.2.

In the future, therapeutic approaches such as senolytics or ASC-based treatments may very well transform the way we age. A long list of age-related disorders for which ASC-based therapies could be developed was mentioned here, while the cosmetic applications of ASC-derived products in skin rejuvenation were not even discussed here. While it may feel intuitively "comfortable" to assume that ASCs from young individuals would perform better in therapeutic applications, there is some evidence to suggest that ASCs from older patients could also achieve successful outcomes, or alternatively, could be supported by ex vivo rejuvenation before application, although this has to be evaluated for each individual. While the field of senolytic drug development is in its infancy [2, 10], such compounds hold real promise to reduce frailty and metabolic dysregulation as we age.

Then, to conclude, is adipose tissue the fountain of youth? In the 1990s, recombinant human growth hormone supplementation was touted as the fountain of youth, essentially based on results from 1 study on 12 men [163], but this was of course later discredited [164]. In contrast, the present review demonstrates clearly, through the combined results of scores of studies, that maintaining healthy functional adipose tissue over time is associated with improved healthspan and lifespan. Preservation of functional non-inflamed adipose tissue, especially SAT, protects against age-related metabolic dysregulation, and healthy adipose tissue is a source of circulating longevity signals with demonstrable impact at distal sites. Optimally functioning ASCs form the foundation of healthy, resilient adipose tissue into advanced age. While much remains to be discovered, there is ample evidence to suggest that the key to a long and healthy life may reside within our ASCs.

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17 Proteomics for Target Identification in Psychiatric and Neurodegenerative Disorders

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Abstract

Psychiatric and neurodegenerative disorders such as schizophrenia (SCZ), Parkinson's disease (PD), and Alzheimer's disease (AD) continue to grow around the world with a high impact on health, social, and economic outcomes for the patient and society. Despite efforts, the etiology and pathophysiology of these disorders remain unclear. Omics technologies have contributed to the understanding of the molecular mechanisms that underlie these complex disorders and have suggested novel potential targets for treatment and diagnostics. Here, we have highlighted the unique and common pathways shared between SCZ, PD, and AD and highlight the main proteomic findings over the last 5 years using in vitro models, postmortem brain samples, and cerebrospinal fluid (CSF) or blood of patients. These studies have identified possible therapeutic targets and disease biomarkers. Further studies including target validation, the use of large sample sizes, and the integration of omics findings with bioinformatics tools are required to provide a better comprehension of pharmacological targets.

Keywords

Proteomics · Target identification · Schizophrenia · Alzheimer's disease · Parkinson's disease

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17.1 Introduction

Psychiatric and neurodegenerative disorders are a current health issue whose burden and prevalence is expected to increase with the ever-growing elderly population in the world [1, 2]. Canonical psychiatric and neurodegenerative diseases, such as schizophrenia (SCZ), Alzheimer's (AD), and

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Parkinson's (PD) disease, present distinct clinical features, yet they seem to share the underlying feature of altered energy metabolism in the brain [3, 4], along with disruptions in emotional control. In neurodegenerative disorders specifically, progressive neuronal loss and disabilities occur in motor function. Sadly, despite it being over a century since these diseases were first described, their etiology remains largely unknown and disease-attenuating drugs are still much needed. Hope in tackling this unmet need has grown in the past decades with the progress of omics technologies, which allow for a high-throughput—and, at times, a global assessment—of a set of biological molecules. In this way, they provide a deeper and more detailed understanding of human health and disease states at the molecular level.

Genomics, transcriptomics, proteomics, and metabolomics refer to the analysis and screening of biological samples at the DNA, RNA, protein, and metabolite levels, respectively. Further to these canonical omics technologies, additional layers of information can be gained from biological samples through pharmacogenomics, epigenomics, phosphoproteomics, glycoproteomics, chemoproteomics, lipidomics, and several other omics approaches. In the context of psychiatric and neurodegenerative disorders, these technologies have had a pivotal effect in shifting us away from the old concept of "one gene, one disorder" toward the currently accepted view of a multifactorial origins nature. In addition, they have enabled a better understanding of central nervous system (CNS) disorders and identified numerous molecular targets [5, 6]. In line with the paradigm shift toward the multifactorial nature of CNS disorders, omics technologies have demonstrated the necessity of targeting networks rather than single biological targets [7–9]. In general, CNS diseases are affected by multiple pathways and some pathways are shared among several diseases [10]. This underlies the need of an integrated approach for the identification of targets in the development of novel therapies.

In this review, we focus on the contribution of proteomics to the understanding of psychiatric and neurodegenerative disorders. We provide a brief overview of the main proteomics technologies and discuss how these have helped to progress our overall understanding of SCZ, PD, and AD. We will then discuss the role of proteomics in the identification of protein targets, delineating how the identification of single targets and networks impacts the therapeutic landscape for these CNS disorders.

17.2 Overview of Proteomic Technologies

After completion of the human genome project nearly two decades ago, technical advances in DNA and RNA sequencing as well as in mass spectrometry technologies have ushered in a new period of scientific progress defined as the post-genomic era. In this new era, the main omics technologies such as genomics, transcriptomics, and proteomics and their subfields (e.g., epigenomics, pharmacogenomics, phosphoproteomics) have provided scientists with an unprecedented level of resolution in the assessment of biological samples at the genomic, transcriptional, and post-transcriptional levels. Today, omics technologies are at the core of our progress in understanding molecular mechanisms underlying complex diseases such as psychiatric and neurodegenerative disorders. These methodologies enable us to generate genome-wide datasets, to link genes to the transcriptome and to phenotypes, and to consider pathophysiological mechanisms. In addition, they have been pivotal in the identification of novel targets for drug development. Furthermore, omics data can be exploited to identify the mechanism of action of drugs and to predict their side effects.

In this section, we will focus on providing a brief overview of the main proteomics technologies, followed by its use in understanding SCZ, AD, and PD.

17.2.1 Proteomic Methods

Over the past two decades, mass spectrometry has been extensively employed in proteomics

approaches and has become increasingly relevant in translational studies [11, 12]. Proteomics based on mass spectrometry can be divided into four different approaches: bottom-up or shotgun, middle-down, top-down, and targeted proteomics. Shotgun mass spectrometry is so far the most commonly used method for the analysis of biological samples and the understanding of complex physiopathological mechanisms, as well as in the identification of potential biomarkers and therapeutic targets [13, 14]. In shotgun proteomics, proteins extracted from a biological sample are cleaved with trypsin at arginine and lysine amino acid residues, generating large numbers of peptides. These peptides are then normally separated by liquid chromatography coupled to a mass spectrometer, thereby creating large numbers of spectra that can be used for quantitation and identification of the parent proteins. Advances in software with the ability to automatically interpret data from middle-down and top-down proteomics and the creation of new data banks offer a favorable scenario for the expansion of proteomics studies using the shotgun approach. This method can also produce information about the protein structure and post-translational modifications, which are of great interest for researchers [15, 16].

In the field of mass spectrometry, an evolution in sensitivity, speed of ion acquisition, better ion separation, and the possibility of combining different fragmentation methods have improved the reliability of the data generated. Moreover, spectra complexity produced by eluted peptides partially separated by liquid chromatography makes protein analysis from biological samples a challenging task, which could benefit from improvements not only in the mass spectrometer analyzers, instrument settings, and software analysis but also in sample preparation methods. Currently, many new methods for sample preparation are being developed aiming to increase the sensitivity and number of proteins that can be identified and to remove impurities from the peptide mixture, which can increase the background noise on the mass spectrometry analysis and suppress ion intensity of low abundant peptides [17].

Shotgun proteomics can be an important tool in the study of multifactorial diseases such as psychiatric disorders and neurodegenerative diseases, which have pathophysiological mechanisms that have not been completely elucidated. For many of these diseases, there is no well-defined biomarker or therapeutic target, and thus this large-scale approach can identify thousands of proteins at once in a qualitative and quantitative fashion. Mass spectrometry proteomics has proven to be more suitable for the elucidation of molecular mechanisms and the identification of targets than other proteomics methods, such as immunoprecipitation, antibody-dependent, or multiplex techniques, since no previous knowledge is needed about the sample or the biological system being analyzed. Therefore, mass spectrometry proteomics removes the need of a previously known molecular target or a protein panel to achieve answers [18–20].

Several studies have applied shotgun proteomics to investigate the dysregulation in multiple metabolic pathways and molecular disturbances in psychiatric and neurodegenerative disorders from postmortem brain tissue and body fluids. These investigations have revealed molecular mechanisms relevant in disease and health states by unraveling dysregulated pathways such as altered energy metabolism in the brain [3, 4]. There is still much progress to be done by taking advantage of new methods and technologies in mass spectrometry and introducing these to obtain new insights into complex disorders.

17.2.2 The Use of Proteomics in Target Identification

Target identification consists of identifying the direct biological target (e.g., protein or nucleic acid) of an existing or potential new drug. It is a critical step in the elucidation of biological processes and mechanisms that affect health and disease states as well as in drug development and validation. In the context of drug discovery, targets have been traditionally identified on the basis of experimental evidence that a given gene,

RNA molecule, or protein is involved in a particular disease process, thus having the potential of producing a desired therapeutic effect if targeted by a drug [21]. In the past decades, omics technologies have been widely used in target identification and drug development [22]. Since proteins are generally the ultimate effectors of biological processes, they have been the main focus in target identification. It is believed that only a fraction of the putative targets coded by the genome have been identified to date [23] and proteomics has been pivotal in this process through bottom-up and top-down approaches [13].

Target identification by proteomics begins with the construction of a suitable disease model in an animal or cell line, or the collection of biological samples (e.g., patient biopsies or post-mortem tissue). Next, samples are subjected to proteolytic digestion and mass spectrometry analyses before the acquired data are finally processed using computational tools. More recently, multi-omics approaches have been employed for target identification and drug discovery in complex diseases through the integration of proteomics data with pathways and network annotations commonly used for systems biology analysis [24–26]. Data available in public databases are also a source of information for bioinformatics analyses in the quest for target identification. Data mining, reverse docking, and network biology have been used for the identification of drug targets. Functional analysis is then performed and biomolecules are proposed as potential targets. The final step consists of pharmacological target validation using in vitro and in vivo models.

17.3 Proteomics in Psychiatric and Neurodegenerative Disorders

Psychiatric and neurodegenerative disorders represent a heterogeneous group of mental ailments with unclarified etiology and pathophysiological mechanisms. Due to the lack of disease-specific biomarkers, diagnostics are still based mainly on clinical symptoms. The post-genomic era has brought hopes of a better understanding of pathophysiological mechanisms and the identification of biomarkers and therapeutic targets. However, the proteins and associated pathways that have been identified so far are not specific for one psychiatric or neurodegenerative disorder. Thus, the identification of molecular targets in complex diseases has increasingly focused on protein sets or pathway networks and less on single molecules.

Omic technologies have been effective in offering novel insight into the pathophysiology of disease states and in target identification [27]. Until recently, proteomics studies were focused on merely measuring the abundance of differentially regulated proteins in whole cells and tissue samples, but increasing numbers of studies have turned their attention to measuring protein abundance in subcellular compartments as well as to the identification of post-translational modifications and protein-protein interactions. Here, we will explore recent advances in proteomic-based target identification in SCZ, PD, and AD.

17.3.1 Schizophrenia

SCZ is a neuropsychiatric disorder that affects about 1% of the population worldwide. Its pathophysiology is still poorly understood and the dawn of the post-genomic era has raised hopes for a better comprehension of disease mechanisms and novel treatments. In the past decade, proteomics studies, in particular, have increasingly appeared in the literature with novel insights about the pathology obtained directly from post-mortem brain samples of patients [28].

To date, the proteomes of different brain regions have been analyzed confirming the complexity of the disorder. Proteomics studies have identified alterations in energy metabolism of neurons and glial cells [29], oligodendrocyte function [30], neurogenesis and synaptogenesis [30, 31], and calcium homeostasis and the immune system [32]. For instance, findings of the involvement of glial cells in the pathophysiology of the disease [33] were later confirmed by the

analysis of the proteome and phosphoproteome of the corpus callosum [34, 35]. Among the differentially regulated proteins identified are members of the 14–3-3 family. These proteins play a regulatory role in neurodevelopmental processes and in protein-protein interactions in the brain and are, therefore, a potential target for a better understanding of disease processes and new therapeutics. The phosphoproteome analysis of the corpus callosum was the first one reported in an analysis of postmortem brains, and it revealed the involvement of signaling pathways such as those involving ephrin B and ciliary neurotrophic factor. Moreover, a recent study from Martins-de-Souza's group analyzed the cerebellum, the caudate nucleus, and the posterior cingulate cortex of postmortem brains and revealed myelin-associated proteins to be differentially regulated in these three regions [36]. Together, these studies provide compelling biochemical evidence of the participation of glial cells in the pathology of schizophrenia and implicate pathways that can be further dissected to indentify potential targets. Other brain regions such as the dorsolateral prefrontal cortex (DLPFC) [37], the ventral caudate [38], and the hippocampus [39] have also been analyzed at the proteome level, and this revealed several proteins and pathways involved in the pathology of SCZ, including synaptic transmission processes and effects on GABAergic interneurons.

More recently, postmortem proteomics analyses of subcellular compartments have started to emerge and have provided a more precise and detailed snapshot of the metabolic and biochemical processes implicated in SCZ. Based on the widely accepted knowledge of altered dopaminergic and glutamatergic neurotransmission in the disorder, Velásquez et al. [40] analyzed the synaptosome of postmortem brain samples and identified over 50 differentially regulated proteins using two different quantitative shotgun-based methods. Beyond the identification of differentially regulated proteins, the authors performed a network association analysis by investigating the protein-protein interaction of the differentially regulated proteins and identified calmodulin (CaM) as a point of convergence in the network. Another study focusing on a subcellular compartment analyzed the postsynaptic density in the anterior cingulate cortex in postmortem brains of SCZ patients vs. healthy controls and identified 143 differentially regulated proteins [41]. These included several proteins involved in clathrin-mediated endocytosis as well as N-methyl-D-aspartate receptor (NMDA-R)-interacting proteins. Moreover, pathway analysis of the differentially regulated proteins implicated processes involved in calcium signaling. Saia-Cereda et al. [42] compared the nuclear proteome of white vs. gray matter regions of the brain and found that heat-shock proteins and proteins belonging to the histone family were differentially regulated in both regions. In contrast, changes in proteins associated with calcium/calmodulin signaling were identified in white matter regions, confirming earlier findings, whereas proteins differentially regulated in the gray matter were closely associated with the spliceosome. This study provided a more integrated view of the processes affected by SCZ in the brain as a whole. Gray and white matter regions are enriched for neurons and glial cells, respectively, and most studies so far have focused largely on the neuronal elements of the brain.

It is undeniable that postmortem brain samples have provided significant information regarding molecules and pathways affected in SCZ. However, confounding factors exist when probing this kind of biological sample, such as postmortem molecule degradation, patient lifestyles, and use of and adherence to antipsychotic medication [43]. Furthermore, brain biopsies from patients are difficult to obtain and several studies suggest that alterations in the CNS might be reflected in the periphery [44]. In fact, analysis of the serum proteome of SCZ patients identified ankyrin repeat domain-containing protein 12 (ANKRD12) as a potential disease biomarker. ANKRD12 is a putative histone deacetylase (HDAC) recruiter, which is in line with the observation of histone-associated proteins reported to be differentially expressed in gray and white matter regions of SCZ patients [42, 45]. In another study researchers analyzed the proteomes of lymphoblastoid cells obtained from patients by fluo-

rescence two-dimensional differential gel electrophoresis (2D-DIGE) and identified 22 differentially regulated proteins associated with the disease. They validated the findings using Western blot analysis for eight of these proteins using an independent second sample set, and multivariate logistic regression analysis was performed to propose a four-marker protein panel for dysfunctional molecular pathways in SCZ [46]. The proposed panel is composed of proteins associated with antiviral response (MX1), nucleotide metabolism (GART), neurodevelopment (TBCB), and protein folding (HSPA4L) processes, all of which have been previously linked with SCZ. Moreover, the panel underscores the multifactorial nature of the disorder and highlights the shift toward the identification and validation of protein sets rather than single molecules as predictive or therapeutic targets.

Serum was also used by Cooper et al. [47] to identify 77 proteins differentially regulated between first-onset drug-naïve patients and controls. They then employed multiple-reaction monitoring (MRM) proteomics to test whether any of these could be detected on blood swatches from newborns and observed increased levels of alpha-2-antiplasmin, complement C4-A, and antithrombin-III in babies who later developed SCZ. This proof-of-principle study demonstrated the feasibility of using targeted proteomics for early target identification with possible implications for the development of preventive measures. MRM proteomics has also been integrated with cognitive and anatomical data to validate biochemical targets. Knöchel et al. [48] demonstrated that altered apolipoprotein C levels are associated with cognitive impairments and changes in hippocampal volume. In addition, a recent study using patient-derived induced pluripotent stem cells from monozygotic twins discordant for SCZ identified sex-specific gene and protein expression signatures which revealed different pathophysiological mechanisms between males and females [49]. These findings shed some light into sex-dependent differences in the manifestation of the disease, such as the time of psychosis manifestation after puberty, and may indicate the necessity for sex-specific treatments.

17.3.2 Parkinson's Disease

PD is characterized by progressive degeneration of the dopaminergic neurons in the substantia nigra leading to severe motor complications, affecting around 1–2% of the population over 55 years old. It is the most common movement disorder and the second most common neurodegenerative disease of the human brain after AD with a higher incidence expected as people are living longer lives [50]. Nearly 200 years after it was first described, the ultimate cause of the disease still remains unknown. PD is a complex multifactorial disorder with variable contributions of environmental factors and genetic susceptibilities. Genetic predisposition accounts for 10% of the cases but the other 90% are described as idiopathic (also known as sporadic), with age being one of the main risk factors [51]. There is strong evidence that suggests that aberrant α-synuclein deposition in the brain is the driving force in PD pathogenesis and α-synuclein, a hydrophilic protein abundant in presynaptic terminals, is a major component of the Lewy bodies and Lewy neurites, which lead to neuronal loss [52]. In addition, aberrant mitochondrial function and increased oxidative stress have also been implicated in the pathogenesis of PD [53].

Poor access to fresh brain tissue and the caveats of using postmortem samples have promoted the development of in vitro models, which have become increasingly robust in the past decade. Human iPSC-derived neurons are now well-established and have aided researchers in the elucidation of the pathophysiology of PD and in target identification. In fact, in vitro analyses of patient-derived dopaminergic neurons from a group of young-onset PD patients revealed α-synuclein accumulation, a hallmark of PD [54]. These neurons were subjected to transcriptomics and proteomics analyses, which revealed a previously unknown genetic contribution in the development of PD in this group. In addition, proteins associated with the lysosomal machinery were significantly downregulated relative to healthy controls. Treatment with a specific phorbol ester compound was able to normalize the disease signature through an increase in the abundance of

lysosomal-membrane proteins and to promote a proteasome-mediated decrease of α-synuclein accumulation. This study is a clear example of how omics technologies can shed some light on the mechanistic aspects of PD and reveal potential therapeutic candidates. Transcriptome- and proteome-wide analyses have also allowed the identification and validation of mRNAs, which are amenable to small molecule targeting. Using a designed small molecule able to selectively bind to the 5′ untranslated region of *SCNA*, the gene coding for α-synuclein, Zhang et al. [55] were able to block its translation, thus offering a promising approach to tackle α-synucleinopathy and opening an avenue for targeting other proteins that, like α-synuclein, are not druggable at the peptide level. Proteomics studies have further aided the development of α-synucleinopathy targeting as a therapeutic approach by revealing an enrichment of disaggregase members in Lewy bodies of PD [56]. Based on the proteomic identification of potential targets, Hsp110, a disaggregase member shown to accelerate the rate-limiting step in the disassembly of fibril aggregates, was overexpressed in an α-synuclein model of PD and was found to reverse the proteomic signature of α-synuclein mutants and prevent α-synuclein templating and spreading in the brain [57]. In addition to target identification in the α-synucleinopathy component of PD, proteomics has provided mechanistic insight into its pathophysiology. Using redox proteomics, Ludtmann et al. [58] analyzed the post-translational modifications of ATP-synthase of mitochondrial fractions exposed to oligomeric α-synuclein and demonstrated that it was able to trigger an oligomer-dependent redox imbalance in one of the protein subunits. This led to mitochondrial swelling and ultimately to cell death, thus providing a potential mechanistic link between α-synuclein aggregation and the aberrant mitochondrial function in PD.

More recently, target genes and pathways were identified in an endogenous neural stem cell population in the human brain. Transcriptome and proteome profiling of $CD271^+$ cells revealed differential regulation of genes and proteins involved in metabolism, cytoskeletal organization, and transcriptional activity. Moreover, the transcriptomic and proteomic signature of this neural stem cell population suggests they may transit into a primed-quiescent state, thus opening up new possibilities for the development of novel therapeutic strategies for the replacement of lost dopaminergic neurons from this endogenous neural stem cell pool [59].

Neuronal accumulation of α-synuclein aggregates is a well-established hallmark of PD, although access to brain tissue for diagnostic purposes is impracticable. In the search for accessible sources of biological samples for biomarkers and target identification in PD, the cerebrospinal fluid (CSF), blood, and even tear fluid have been explored. Posavi et al. [60] analyzed the blood proteome of PD vs. healthy people using discovery and replication cohorts and found bone sialoprotein (BSP), osteomodulin (OMD), aminoacylase-1 (ACY1), and growth hormone receptor (GHR) to be consistently differentially regulated in PD patients. Moreover, lower GHR levels at baseline were associated with faster rates of cognitive decline. This study is one of the first ones to identify diagnostic and prognostic biomarkers for PD from an easily accessible biological sample. Tear fluid has also been exploited as a potential source of biomarker discovery, and studies have found effects on the immune response and lipid metabolism pathways in the disease [61].

Exosomes are products of cells which have gained increased attention for their role in neurodegenerative diseases [62]. In fact, the proteome analysis of blood-derived exosomes revealed a distinct protein profile that was progressively upregulated from mild to severe in PD patients [63]. Among the identified proteins was gelsolin, which has been previously shown to occur in Lewy bodies [64].

In addition, targeted MRM proteomics of CSF samples has been employed to assess proteins that are part of pathways involved in PD such as lysosomal, ubiquitin-proteasomal, and autophagy pathways and identified significantly reduced levels of chromogranin B [65].

17.3.3 Alzheimer's Disease

AD is the most common neurodegenerative disorder in the world, accounting for 50–70% of dementia cases [66]. It is characterized by progressive loss of neurons leading to cognitive impairment and late dementia. AD pathogenesis consists of the development of intracellular neurofibrillary tangles (NFTs) and the deposition of senile plaques [67]. NFTs arise from the collapse of the neuronal cytoskeleton due to Tau hyperphosphorylation [68], while senile plaques are formed by the accumulation of protein fragments (amyloid-β, Aβ) from the abnormal proteolytic processing of the amyloid precursor protein (APP) [69]. Both processes induce neuroinflammation [70], axonal degeneration [71, 72], and disruption of synaptic integrity [73], thus leading to impairment of physiological neural connectivity. Both genetic and environmental factors have been described as AD risk factors, and these can be classified as two different disease forms: late-onset sporadic AD and early-onset familial AD [74]. A small proportion of cases has a clear genetic origin and demonstrates point mutations in the genes that code for APP, presenilin 1 (PS1), presenilin 2 (PS2), and the apolipoprotein E (APOE) ε4 allele [75–77]. The vast majority of patients suffer from the sporadic origin-type AD, which has age as the main risk factor and is associated with a series of biochemical, molecular, and cellular abnormalities in the brain, including increased activation of genes and pathways of cell death signaling, chronic oxidative stress, and impaired insulin response [78, 79].

Similar to SCZ and PD, the underlying causes of AD are not completely understood. However, in contrast to the other diseases, a core panel of biomarkers has been already established for disease diagnosis, consisting of Aβ42, total tau (T-tau), and phospho tau (P-tau) [80]. Despite the establishment of a few key markers for AD diagnostics, the disorder is heterogeneous and presents multiple genotypes and phenotypes; thus the identification of additional targets and biomarker panels is needed for accurate patient stratification [81].

In the last decade, the existence of different Aβ peptides has been reported in the brain and CSF of AD patients [82, 83], while ApoE levels appear to be downregulated in plasma [84]. A recent multiplex proteomics analysis of plasma and CSF from elderly participants identified proteins involved in the regulation of the inflammatory response, apoptosis, endocytosis, leukocyte proliferation, and other processes believed to be downstream of Aβ and tau deposition. Among these were chemokines, interleukins, and other immune markers, thus revealing relatively unexplored candidate biomarkers and reinforcing the potential of targeting pathways beyond Aβ and tau in AD [85]. Involvement of glial cells in AD has been previously suggested by a proteomic study of postmortem brain samples [86]. Moreover, RNA-binding proteins appeared to be differentially regulated between symptomatic and asymptomatic AD patients, opening a new avenue for targeting cognitive decline in AD. A comprehensive label-free quantitative proteomics analysis of over 2000 brains and 400 CSF samples identified astrocyte and microglial metabolism, mitochondrial function, synaptic function, and RNA-associated proteins as being altered in AD. Moreover, altered pathways associated with energy metabolism, more specifically certain lipids, insulin, amino acids, and glucose, emerged as strongly associated with AD and cognitive decline [87, 88].

17.3.4 Shared and Distinct Molecular Pathways Between SCZ, PD, and AD

Knowing the molecular similarities and distinctions among SCZ, PD, and AD may help to define the importance of certain biochemical pathways and molecular targets, which aid in the development of new treatment strategies. Thus, we searched for proteins found to be associated with the three diseases detailed in this review at UniProt (http://uniprot.org) and analyzed these using the pathway analysis software Metascape [26]. The results are displayed in Fig. 17.1.

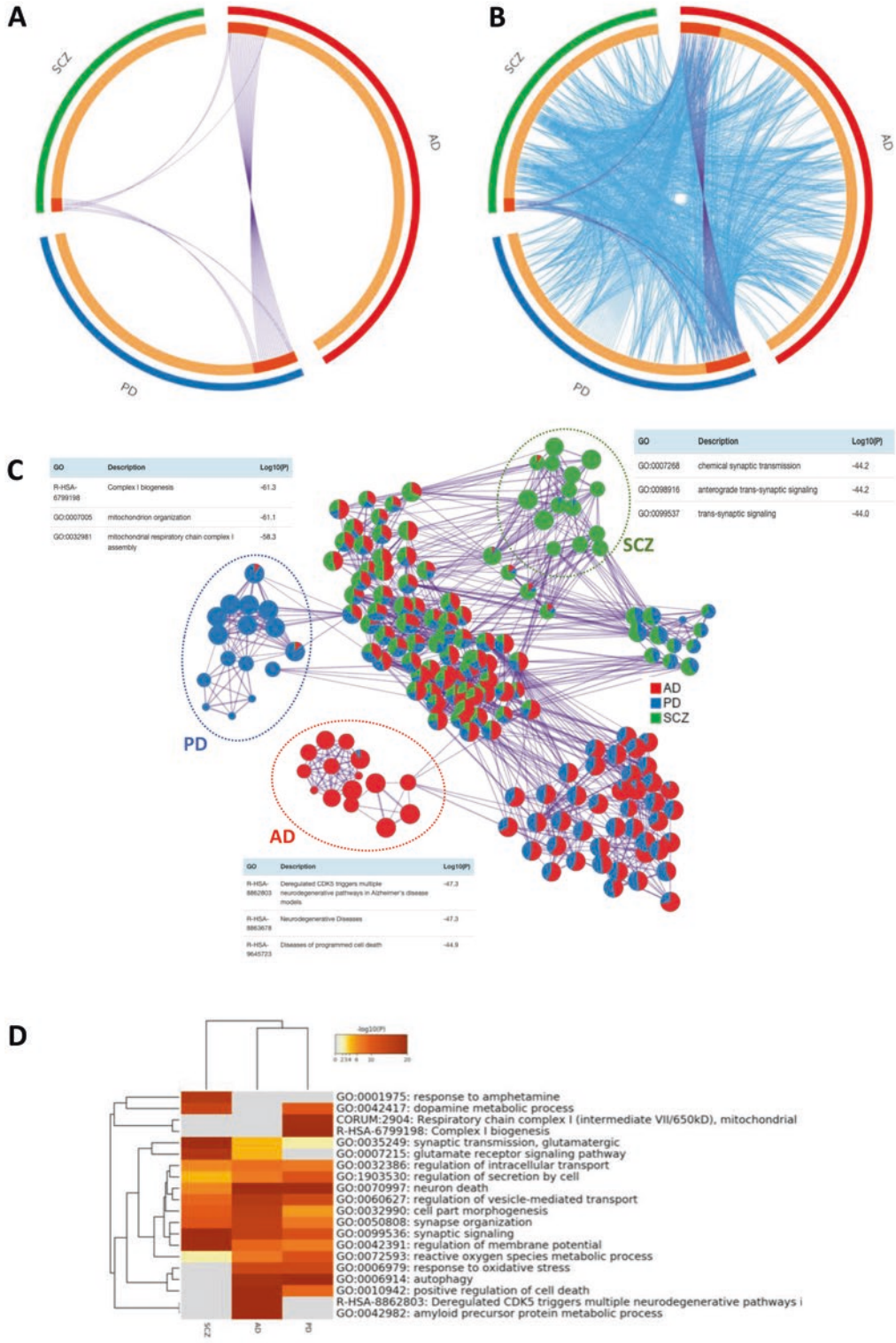

Fig. 17.1 Cord diagram depicting (**a**) overlap of proteins and (**b**) biological processes in SCZ, PD, and AD. (**c**) Visualization of interactome networks formed by differentially regulated proteins in SCZ (green), PD (blue), and AD (red). Tables depict the top three pathways according to the *p*-value. (**d**) Heatmap of the top enriched clusters using annotated curated databases for each disease. Gray represents a lack of significance and the color intensity represents -log10 (*p*)

As might be expected, AD and PD should be more similar in terms of molecular pathways compared to SCZ, considering the neurodegenerative nature of the former illnesses. This is confirmed in Fig. 17.1a, which shows a greater overlap of proteins between AD and PD. It is interesting to note that an enrichment of biological processes is shared among the three diseases, indicating that SCZ is somehow close to PD and AD in ontology terms (Fig. 17.1b). While SCZ is not considered a classical neurodegenerative disease [89, 90], there might be some kind of degeneration, even if only at the white matter level [33, 91, 92]. Alternatively, all three conditions are associated with disruptions in synaptic connectivity, and some of the similarities might reflect this [93].

The shared molecular characteristics among the three disorders are also evident in Fig. 17.1c, where most of the affected pathways are common to the three diseases. Some of these commonly shared pathways can also be observed in Fig. 17.1d. On the other hand, certain molecular pathways are specifically affected in each disease. For example, mitochondrial pathways are more affected in PD, degenerative processes and cell death are more affected in AD, and neurotransmission is more specifically affected in SCZ (Fig. 17.1c, d).

Protein databanks and gene/proteins meta-analysis platforms are crucial in the elucidation of common and unique pathways as well as protein networks from targets identified in proteomics studies. Furthermore, they can be explored for better disease classification and patient stratification by unraveling differences within the pathological spectrum of these heterogeneous diseases.

17.4 Conclusions

Omics technologies and their integration have led to significant progress in our comprehension of the pathophysiological mechanisms that underlie psychiatric and neurodegenerative diseases and greatly facilitate therapeutic target and biomarker identification. While genomics and transcriptomics developed rapidly over the last two decades and made high-throughput screening of gene risk variants and transcript levels commonplace, proteomics assay platforms have not evolved at the same pace [93]. However, given that proteins are the main ultimate effectors of biological processes and their modulation is pivotal in maintaining a healthy state, proteomics is an essential technology for the identification of novel targets and biomarkers. Proteomics data such as subcellular localization, post-translational modifications, and protein-protein interactions have only recently begun to be integrated with other omics data. Significant advances in proteomics have been achieved through improvements in mass spectrometry resolution, accuracy, and speed [94], and future developments in computational tools will allow better integration of different omic datasets and provide more robust identification of targets and biomarkers.

Despite the significant progress in target and biomarker identification in recent years, the limited number of subjects or samples analyzed in individual studies represents a caveat for the statistical power of the findings. Furthermore, despite the pathways and common features shared among different neurological disorders, they are heterogeneous in nature and therapeutic target identification could benefit from improved disease classification and patient stratification, prior to analysis [5].

Robust in vitro disease models and easy-to-access biological samples have proven to be valuable tools in target identification. iPSC-derived neurons and brain organoids have been valuable in providing the omic signatures of neurological disorders. However, the neurodevelopmental component of disorders like schizophrenia could be further explored with these models. Nevertheless, the brain is still the primary site affected in psychiatric and neurodegenerative disorders. Thus, issues in postmortem brain analyses such as transcript and protein stability, post-translational modifications, and protein-protein interaction stability, as well as the effect of confounding factors such as medication uptake by patients, should be monitored.

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