

A microscopic view of biological tissue, showing various cellular structures and fibers in shades of pink, red, and white. The image is used as a background for the book cover.

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Mitochondria and Brain Disorders

Edited by Stavros Baloyannis



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Lovelesh Kumar Nigam, Aruna V Vanikar, Rashmi D, Kamal V Kanodia, Kamlesh S Suthar, Hideo Tsukada, Xianquan Zhan, Na Li, Cheril Tapia-Rojas, Angie K. Torres, Margrethe A. Olesen, Claudia Jara, Ekaterina Alexandrovna Nikolaeva, Ilgar Mamedov, Irina Zolkina, Stavros J Baloyannis

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Meet the editor



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Coenzyme Q₁₀ and L-Carnitine Disturbances in Children
with Mitochondrial Diseases

by Ekaterina A. Nikolaeva, Ilgar S. Mamedov and Irina V. Zolkina

Preface

The mitochondrion is a unique and ubiquitous organelle that contains its own genome, encoding essential proteins that are major components of the respiratory chain and energy production system. It is reasonable therefore that mitochondria play a dominant role in the life and function of eukaryotic cells including neurons and glia, as their survival and activity depend almost exclusively upon mitochondrial energy production and supply.

Thus, mitochondrial functional impairment is related to a substantial number of neurological disorders, most of them having high incidence in the range of neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, Friedreich's ataxia, and Huntington's disease.

New techniques improving mitochondrial DNA analysis and detecting mutation penetrance patterns have also increased the awareness of the rate of mitochondrial involvement in the pathogenetic mechanisms of a substantial number of neurological diseases. In addition, since mitochondria synthesize ATP through oxidative phosphorylation (OXPHOS) as the main energy providers for neurons and glia, their dysfunction induces an energy crisis with tragic consequences for cells of high energy demands.

Besides energy production, mitochondria also play a vital role in neuronal and glial calcium homeostasis due to their high capacity for accumulating Ca^{2+} . On the other hand, mitochondria comprise reactive oxygen species (ROS), which control redox status, intracellular Ca^{2+} levels, and may induce apoptosis by activating the mitochondrial permeability transition pore (mtPTP), a crucial point of excitotoxic neuronal injury.

In a parallel manner, ROS may play an important role in inflammatory conditions of the brain such as Multiple Sclerosis (MS) either by decreased activity of mitochondrial enzymes, or by peroxidation of lipids, proteolipid protein or myelin basic protein, therefore destabilizing the anoxic myelin sheath.

In reality, the role of the mitochondria in neuronal survival and function is multi-dimensional. Thus metabolic intermediates derived from Krebs cycle in the mitochondria may behave as co-factors for epigenetic modifications, playing an essential role in histone acetylation and in the addition and removal of epigenetic marks, controlling the expression of a substantial number of genes in the neuronal genome.

Mitochondria also contribute to the synthesis of steroid hormones such as glucocorticoids, mineralocorticoids, progestogens, estrogens, and androgens; and facilitate the interaction of catecholamines with their receptors, therefore promoting neuronal plasticity by stress mediators. Thyroid hormones also stimulate mitochondrial activity by enhancing oxidative phosphorylation. In turn, steroid hormones regulate mitochondrial respiratory capacity and oxidative stress, since mitochondria are particularly sensitive and dependable on hormonal stimulation. Given that sex hormones regulate mitochondrial biogenesis and function, it is expected that

mitochondria express sexual dimorphism and show different reactions to stress and disease in males and females.

Mitochondria modulate systemic energy homeostasis in both sexes by the production and release of mitokines, such as humanin, and by operating as the central organelle of adaptation and stress-response modulation.

From the morphological point of view, mitochondrial alterations are accurately visualized in light microscopy in a properly fixed material by means of a number of special staining reactions. Transmission electron microscopy has also contributed substantially in the morphological study of mitochondria in neurological diseases revealing the disruption of the cristae and the disarrangement of their inner architecture, taking in consideration also that mitochondria exhibit high morphological variability according to activity and metabolic demands of the cell. Using transmission electron microscopy, it was also observed that in Alzheimer's disease, mitochondrial cristae are disrupted from the initial stages of the disease even in areas of the brain with minimal Alzheimer's pathology.

A considerable number of syndromes have been described with marked neurological phenomena in the spectrum of mitochondrial disorders. It must be highlighted that the severity of the clinical manifestation of mitochondrial dysfunction varies considerably, given the threshold in the degree of mitochondrial deficiency for the clinical expression of the disease. Thus, the symptoms and clinical phenomena are continuously aggravated, in the majority of the cases of mitochondrial diseases, as the age of the patient advances and the energy deficiency increases in various organs. However, the most frequent clinical manifestations that have been reported in mitochondrial diseases include cognitive impairment, epilepsy, myopathies, renal and liver dysfunction, cardiopathy and disorders of inner secretion resulting in hormonal imbalance.

Molecular genetic testing and muscle biopsy for histochemical investigation in light microscopy and ultrastructural study of the specimens in electron microscopy are essential diagnostic procedures for the documentation and final verification of the diagnosis of mitochondrial disorders. In addition, biochemical testing in blood, urine, and spinal fluid associated with neuroimaging would be useful diagnostic procedures in following up the progression of mitochondrial diseases.

A final escape from the labyrinth of mitochondrial related neurological disorders is extremely difficult and less pragmatic under the present circumstances. Thus the treatment for the majority of mitochondrial diseases remains mostly symptomatic. Therapeutic factors enhancing mitochondrial biogenesis and restoring nitric oxide production might be beneficial at the initial stages of the diseases. The administration of cardiolipin protector and agents aiming at ameliorating mitochondrial function, such as antioxidants, riboflavin, idebenone, CoQ10 (ubiquinone) and thiamin might be reasonably prescribed in attempting to improve the physical and mental condition of the patients who suffer from mitochondrial disorders.

Prospectively, an efficient treatment could be based on a stable modulation of mtDNA heteroplasmy, whereas gene therapy, liver transplantation, gene transfer and tRNA-targeted therapeutic attempts as well as stem cell therapy for nuclear DNA mutations are promising therapeutic endeavors.

In conclusion, an efficient and easy treatment of mitochondrial dysfunction would open new bright horizons in the therapy of inflammatory and neurodegenerative disorders, being beneficial in the amelioration of the quality of life of a substantial number of patients.

In this book, the authors attempted to describe the complex relationship of mitochondrial disorders with neurodegeneration for further clarification and analysis of the multidimensional background of the pathogenetic mechanisms of neurological diseases.

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Section 1

Introduction

Introductory Chapter: Mitochondrial Alterations and Neurological Disorders

Stavros J. Baloyannis

1. Introduction

Mitochondria (from Greek mito, μίτος, thread; and chondrion, χόνδριον, thick granule) are principal cell organelles, which participate in a wide spectrum of essential cellular functions, being the main energy providers for living eukaryotic cells, especially for neurons and glia, which are characterized by high metabolic activity and energy consumption.

Thus, it is expectable that mitochondrial dysfunction, having pleotropic effect on the cell, may play a crucial role in a substantial number of serious neurological disorders including Alzheimer's disease (AD) [1, 2], Parkinson's disease (PD) [3] Huntington's disease [4, 5], amyotrophic lateral sclerosis (ALS) [6], multiple sclerosis (MS) [7, 8], as well as some of the major psychiatric diseases [9], given that both, neurons and glia, are particularly sensitive and vulnerable to energy decline [10].

Mitochondria hypothesis of those devastating diseases advocates reasonably in favor of the important role that mitochondrial dysfunction may play in the early stages of neurodegeneration by inducing energy deficiency and oxidative stress [11].

However, the majority of the mitochondrial diseases, being maternally inherited, which are designated as mitochondrial encephalomyopathies [12], are closely connected either with the impairment of nucleus-to-mitochondria signaling or with mutations in mtDNA or nuclear genome that affect seriously the mitochondrial respiratory function even from the initial steps of the life [13], inducing defective oxidative phosphorylation (OXPHOS).

2. The genetic background of mitochondrial dysfunction

It is well known that mitochondria, as very specific organelles, include several copies (2–10 copies) of their own DNA (mtDNA), which consists of a 16.5 kb circular DNA molecule, being particularly prone to mutation [14]. mtDNA encodes for 37 genes, 13 of them encoding 13 polypeptides, which all are major components of OXPHOS complexes I, III, IV, and V, along with 22 tRNAs and 2 rRNAs, which play an essential role for the expression of the 13 subunits [15].

Mutations in mtDNA may be related to 25% of childhood-onset diseases [16] and to 75% of adult-onset ones [17], depending on the existing homoplasmy or heteroplasmy. In addition, the accumulation of mtDNA mutations can also induce or facilitate the aging process [18], since a common phenomenon in mammalian aging is the substantial decrease of electron transfer in mitochondria [19, 20].

3. Biological consequences of mitochondrial dysfunction

The mitochondria in addition to energy production compose also reactive oxygen species (ROS), which control redox status and intracellular Ca²⁺ levels and may induce apoptosis, by activating the mitochondrial permeability transition pore (mtPTP) [21]. In addition, mitochondria play a very important role in neuronal and glial calcium homeostasis due to their high capacity to accumulating Ca²⁺ [22].

Resting neurons contain usually minimal Ca²⁺ that can be increased by the activation of NMDA glutamate receptors, which induce a massive entry of Ca²⁺ into neurons, resulting in its high accumulation in the mitochondria [23]. Continuous activation of NMDA receptors would therefore induce Ca²⁺ overload of the mitochondria with the tragic consequence of the cell apoptosis, which frequently occurs as an epilogue of the excitotoxicity [24].

The apoptosis consists of a wide spectrum of biological phenomena [25] including the release of caspase activators [26], the alterations of the electron transport system, the change of mitochondrial transmembrane potential, the disruption of the cellular oxidation-reduction equilibrium, and the activation of the pro-apoptotic Bcl-2 family proteins [27, 28].

In the majority of the mitochondria-related neurological disorders, the functional or morphological alteration of the mitochondrial may be induced by increased ROS production, abnormal protein aggregates (Ab, tau) [29, 30], mutations in genes encoded by the mitochondrial and nuclear genome, and exposure of the cell to toxic factors [31].

4. The morphology of mitochondria in health and disease

Cell mitochondria could be visualized in light microscopy in properly fixed material by means of a number of special staining reactions [32–34]. It is observed that their size generally ranges from 0.5 to 1 micron in diameter, being changeable due to frequent divisions and fusions, which are controlled by mitofusin activity [35]. The shape of the mitochondria is also continuously changed due to their impressive active motility, controlled by calcium signal [36, 37], given that they are in constant flux, especially in brain's areas of high energy consumption in order to contribute in energy supply and to participate in the intracellular signaling actively [38].

Electron microscopy has been contributing greatly in the study of mitochondria in health and disease [39, 40]. Each mitochondrion in healthy condition is surrounded by a limiting double membrane and includes numerous longitudinal or tubular invaginations called mitochondrial cristae that are folds of the inner layer of the double membrane [41], which is four times greater than the outer one.

The cristae are mostly arranged perpendicularly to the long axis of the organelle, exhibiting a high morphological variability according to metabolic demands of the cell [42], being frequently lamellar, tubular, or triangle-shaped. In the majority of the mitochondria, the cristae are arranged parallel to one another inside a structureless matrix, which is clearly seen among the cristae.

Cardiolipin seems to play a crucial role in the morphology of cristae, since the disruption of cardiolipin biosynthesis induces obvious alteration of the cristae morphology [43]. In addition, Opa1, which is a GTPase, demonstrating dynamin-like properties, plays a substantial role in the modulation of the cristae structure and in their remodeling during mitochondrial fusion and fission [44] and apoptotic process [45]. The cristae have a high protein content [46], being also the principal site of the oxidative phosphorylation [47].

Electron microscope tomography, revealing the three-dimensional appearance of the cristae, shows that they are connected with the inner mitochondrial membrane by a narrow, tubular opening, characterized as “crista junction” (CJ), which is associated with protein import [48] and mitochondrial inner compartmentalization [49].

In neurodegeneration such as in Alzheimer’s disease, mitochondrial cristae are disrupted even from the initial stages of the disease, and concentric patterns of cristae membranes are frequently seen [50].

5. Mitochondrial trafficking and concentration

Mitochondria, like many other cell organelles, are oriented and positioned properly in neurons and glia in order to be able to fulfill the energy demands of the cells perpetually. Thus, neurons, axons, dendrites, and synapses, which are characterized by high ceaseless activity, have intensive mitochondrial motility and impressive concentrations [51], via various trafficking patterns [52].

Axonal transport of mitochondria [53] requires microtubules (MTs) [54, 55] or actin filaments in axons [56], which facilitate the movement of the mitochondria in areas of high metabolic demands and increased energy consumption [57]. It is noticed that disruption of axonal transport of mitochondria occurs as an early phenomenon in cases of neuroinflammation [58], including multiple sclerosis [59–61].

6. Clinical expression of mitochondrial dysfunction

A considerable number of syndromes have been described with marked neurological phenomena in the spectrum of mitochondrial disorders [62]. The severity of the clinical manifestation of mitochondrial dysfunction varies considerably, given that there exists a threshold in the degree of mitochondrial deficiency for the clinical expression of the disease [63, 64]. Thus, the symptoms and clinical phenomena are continuously aggravated, in the majority of the cases of mitochondrial diseases, as the age of the patients advances [65]. It is reasonable to accept that organs with high energy demand would be more seriously affected by the mitochondrial dysfunction than others with low level of energy necessity. Thus the brain, the skeletal muscles, and the heart have a typical involvement in adolescence and adulthood, though multi-system manifestation is not also an uncommon phenomenon, especially in childhood.

Many clinical syndromes have been described that are associated with mitochondrial dysfunction including encephalomyopathy, stroke-like episodes, myoclonic epilepsy, neuro-gastrointestinal phenomena, cranial or peripheral neuropathy, ataxia, retinitis pigmentosa, chronic progressive external ophthalmoplegia which are associated frequently with lactic acidosis, mental retardation, or progressive mental decline [66].

In addition, oxidative stress, due to mitochondrial dysfunction, plays a principal role, as causative factor, in the neurodegeneration [67] and in Alzheimer’s disease particularly [68, 69], and it is considered as been among the potential risk factors for the neurometabolic and neoplastic diseases, as well as obesity [70].

Molecular genetic testing on one hand and muscle biopsy on the other hand for the histochemical investigation in light microscopy and the ultrastructural study in electron microscopy of the muscle tissue are essential diagnostic procedures for approaching the diagnosis of mitochondrial disorders [71]. In addition, biochemical testing in blood, urine, and spinal fluid associated with neuroimaging [72]

would be useful diagnostic procedures in following in time the progression of mitochondrial diseases [71].

7. The final escape

A final escape from the labyrinth of mitochondrial-related neurological disorders is extremely difficult and less pragmatic under the present circumstances. Prospectively, an efficient treatment could be based on a stable modulation of mtDNA heteroplasmy [73], whereas gene therapy, gene transfer, and tRNA-targeted therapeutic attempts [74] as well as stem cell therapy for nuclear DNA mutations [75, 76] are very promising therapeutic endeavors with substantial medical and scientific value [77, 78].


In addition, an efficient and easy to apply treatment of mitochondrial dysfunction would open new bright horizons in the therapy of the inflammatory and neurodegenerative disorders [79], being beneficial in the amelioration of the quality of life of a substantial number of seriously suffering human beings.

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Section 2

Mitochondria and Hormones

Pathology Associated with Hormones of Adrenal Cortex

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Abstract

Adrenal gland is an endocrine organ comprising of an outer cortex and inner medulla. These secrete various hormones that have a vital role in maintaining the normal homeostasis of the body. Lesions of adrenal cortex are quite common to encounter and most of these are related to the hormones secreted by three layers of adrenal cortex: the zona glomerulosa, the zona fasciculata, and the zona reticularis. Also it is very infrequent to encounter metastatic lesions in the adrenal glands too. So it is very important as a part of a clinician as well as a pathologist to know the pattern in which these hormones are secreted along with their physiological roles. Thus this chapter includes the disease that are related to excess as well as deficiencies of the hormones secreted by adrenal cortex. The chapter also includes various genetic syndromes that are associated with the disorders associated with hormones of adrenal cortex. The last part of the chapter includes a brief description of various benign as well as malignant lesions, the pathological as well as the etiological aspects and the hormonal abnormalities associated. This chapter thus mainly focuses on the pathology associated with the adrenal cortex and hormones secreted by the various layers of adrenal cortex.

Keywords: adrenal cortex, hormones, Cushing syndrome

1. Introduction

Adrenal gland consists of an outer cortex and inner medulla; the cortex is further subdivided into three distinct zones: the zona glomerulosa, the zona fasciculata, and the zona reticularis. Mineralocorticoids (aldosterone) secreted from the zona glomerulosa are essential for fluid and electrolyte balance and the renin-angiotensin-aldosterone system. The fasciculata secretes glucocorticoids (mainly cortisol). The zona reticularis produces steroid sex hormones called androgens. These hormones play an important role in maintaining the normal homeostasis of the body [1–3]. However, it is quite common to encounter disorders related to the hormones of these three layers. These disorders could be possibly due to adrenal cortical masses secondary to cortical hyperplasia. It is very infrequent to encounter metastatic lesions in the adrenal glands.

This chapter mainly focuses on the pathology related to adrenal cortex which includes various forms of adrenocortical hyperplasia and benign and malignant neoplasms of the adrenal gland which lead to various hormonal imbalances encountered in clinical practice. Hormonal deficiency is due to inherited glandular or enzymatic

disorder, destruction of pituitary gland by autoimmune disorders, infection, infarction, or others [4, 5]. The major disorders of the adrenal cortex are characterized by excessive or deficient secretion of each type of adrenocortical hormone.

2. Functional manifestations

The lesions of the adrenal cortex could be functional as well as nonfunctional, which means that patients with these lesions may exhibit clinical symptoms that are due to hypersecretion of hormones released. Usually cortical hyperplasia and adenomas are nonfunctioning. The functional syndromes associated with pathology of adrenal cortex are hypercortisolism (Cushing's syndrome), adrenal insufficiency (Addison's disease), hyperaldosteronism, and androgen excess (adrenogenital syndrome) [4–9].

3. Adrenal hyperplasia

Adrenal hyperplasia is characterized as a smooth, diffuse, bilateral enlargement of the adrenal glands, wherein the glands retain their adreniform shape. Hyperplasia can be either macronodular or micronodular. They are commonly unilateral; however bilateral cases are also observed [7, 8]. Broadly adrenal cortical hyperplasia can be grouped into three main categories: ACTH-dependent (adrenocorticotrophic hormone), ACTH-independent, and congenital adrenal hyperplasia (CAH). Cushing's syndrome is one of the common functional manifestations of adrenal gland hyperplasia and therefore is discussed first [8, 10, 11].

3.1 Cushing's syndrome

3.1.1 Definition

It is a syndrome which encompasses various clinical features due to chronic excess of glucocorticoids. The incidence is nearly 1–2 per 100,000 population per year. Harvey Cushing was the first to observe pituitary adenomas associated with hypercortisolism in 1932 [10–13]. Cushing's syndrome, caused by prolonged exposure of tissues to high levels of cortisol, presents as constellation of symptoms including central obesity, muscle fatigue/atrophy, hirsutism, infertility, osteoporosis, moon facies, dorsocervical and supraclavicular fat pads, and wide purple striae [8, 10, 12]. The syndrome may be ACTH-dependent or ACTH-independent. A fair number of cases attributed to iatrogenic causes are also identified. Most of the cases of Cushing's syndrome are due to ACTH hypersecretion from the anterior pituitary and are associated with pituitary cortical adenoma. Majority of the cases, about 80–90%, show diffuse hyperplasia of the adrenal cortex [9, 10]. Nearly 15% of cases do present with ectopic ACTH secretion associated with small cell lung carcinoma or bronchial carcinoid. Thymic carcinoids, pancreatic islet cell tumor, pheochromocytomas, and medullary carcinoma of thyroid form minor group of tumors associated with ectopic ACTH secretion [12]. In a study by Ejaz et al., lung tumors constituted 44.4% of all cases of neoplasm-related ectopic ACTH secretion causing Cushing's syndrome [14]. Clinically patients with Cushing's syndrome present with diastolic hypertension, hypokalemia, and edema. Hypogonadism and amenorrhea can also be seen in these patients which are attributed to suppression of gonadotropin secretion secondary to excess glucocorticoid secretion [10–14].

3.1.2 Cushing disease

Cushing disease, resulting from a pituitary corticotrophic adenoma, and rarely carcinoma, makes up to 80–85% of endogenous Cushing's syndrome cases [8, 10, 15].

3.1.3 Investigating a case of Cushing's syndrome

A two-stage test is usually recommended in a patient to rule out Cushing's syndrome [8, 10, 15–17]:

1. The first group of tests are to screen for the evidence of hypercortisolism. These comprise urine cortisol excretion and low-dose dexamethasone suppression test.
2. The second group of tests comprise of the diagnostic tests which help to determine the cause of excessive production of cortisol. These are:
 - a. Plasma ACTH measurement: Low plasma ACTH level suggests an adrenal cause of the disease; however normal/high [ACTH] level suggests ectopic ACTH secretion or hypersecretion of ACTH from pituitary (Cushing's disease).
 - b. High-dose dexamethasone suppression test: In this test the patient is administered with 2 mg of dexamethasone, 6 hourly for 48 h, following which plasma cortisol levels are measured. In the case of ectopic ACTH secretion or adrenal limited hypercortisolism, there is a failure of suppression of cortisol secretion. Also it is important to remember that cortisol is not suppressed with either low- or high-dose dexamethasone suppression in adrenal hyperplasia associated with ectopic ACTH production [18].

3.1.3.1 The 24-h urinary-free cortisol test

This investigation is used primarily for the diagnosis of hypercortisolism due to Cushing's syndrome, and reference ranges for this test with respect to age are 1.4–20 µg/24 h (3–8 years), 2.6–37 µg/24 h (9–12 years), 4–56 µg/24 h (13–17 years), and 3.5–45 µg/24 h in individuals ≥18 years of age. A 24-h urine sample with boric acid (10 g) as preservative is advisable for performing this analysis [10, 17–19].

3.1.4 ACTH-independent Cushing's syndrome

Nearly 15–20% of Cushing's syndrome are associated with ACTH-independent hypercortisolism and are secondary to a functioning adenoma or carcinoma. Diagnosis of ACTH-independent Cushing's syndrome includes clinical features of hypercortisolism, absence of serum cortisol diurnal rhythm, elevated late-night cortisol levels, and incomplete suppression of cortisol production with low-dose dexamethasone suppression test [10, 17–20].

3.1.5 Pathological findings

Adrenal glands from patients with Cushing's syndrome/hyperplasia appear variably enlarged in size and weigh approximately 6–12 g. The cortical width is widened as compared to the reticulosa. The zona fasciculata usually shows nodular hyperplasia. Nearly 10–20% of the patients reveal bilateral nodular hyperplasia, and up to 30% of patients may have normal adrenal morphology [2, 20, 21].

3.2 Primary pigmented nodular adrenocortical disease (PPNAD)

Primary pigmented nodular adrenocortical disease is a rare cause of childhood Cushing's disease having female preponderance, whereas Cushing's disease is common in prepubertal males [20–22]. It is the main endocrine manifestation of Carney complex (a multiple neoplasia syndrome caused by mutation in *PRKAR1A* gene) [23]. This is an autosomal dominant syndrome and is characterized by cutaneous lentigines, myxoma, schwannomas, and endocrinopathy [11, 23]. It was first described by Aidan Carney and co-workers in 1985. Almost 25–30% of patients with Carney complex have ACTH-independent Cushing's syndrome. Cutaneous pigmentation is the commonest manifestation of the disease [24]. Lentigines are seen in most patients, and this characteristic manifestation can be used to make the definitive diagnosis. The name is derived from the macroscopic appearance of the adrenals that show characteristic small pigmented micronodules in the adrenal cortex. The disease typically involves bilateral adrenal glands. Grossly the adrenal glands may have variable size. The most characteristic finding is the presence of multiple brown-black pigmented cortical nodules that measure 1 mm to 3 cm in diameter. The adjacent cortical tissue invariably shows atrophy. These pigmented nodules may extend into corticomedullary junction or peri-adrenal fat [9, 11, 24].

On microscopy these tumors appear as sharply circumscribed, unencapsulated tumors composed of large eosinophilic lipid-poor cells similar to the zona reticularis arranged predominantly in trabecular growth pattern. However the nucleus appears enlarged, with a variable degree of pleomorphism and prominent nucleoli. There is prominent lipofuscin deposit. Lipid-rich fasciculata-like cells are also seen invariably. The tumor may have focal areas of necrosis, mitotic activity, myelolipomatous change, and lymphocytic infiltrates [9, 11, 24].

3.3 ACTH-independent macronodular adrenal hyperplasia (AIMAH)

(Synonyms: ACTH-independent massive bilateral adrenal disease, massive macronodular hyperplasia, giant macronodular adrenal hyperplasia, macronodular adrenal hyperplasia, macronodular hyperplasia).

AIMAH is a disorder characterized by bilateral adrenocortical nodules, associated with ACTH-independent hypercortisolism, without any clinical features of pigmented nodular adrenocortical disease and histological features consistent with atrophic internodular cortex [25]. It is a rare cause of ACTH-independent Cushing's syndrome with slightly male preponderance. The patients present usually at later age (average: 48 years) [24–26]. In few patients with AIMAH, ectopic expression and/or increased sensitivity to gastric inhibitory peptide, vasopressin receptors, and beta-adrenergic receptors is also seen [25].

Grossly these lesions are characterized by nodules in the adrenal cortex, ranging from 1 to 4.2 cm. The adrenal gland weighs approximately 16.7–218 g. The adrenal gland may have a large mass of cortical tissue and multiple bilateral nodules measuring up to 5 cm. Combined adrenal gland weight of more than 300 g has also been noted (normal range: 8–12 g). Histology demonstrates large, yellow macronodules comprising of small cells with eosinophilic cytoplasm. Bilateral adrenalectomy and well-controlled glucocorticoid replacement is the most accepted treatment modality [2, 3, 9, 25, 26].

3.4 Congenital adrenal hyperplasia

CAH is an autosomal recessive disorder characterized by impaired steroidogenesis finally leading to mineralocorticoid and cortisol deficiency secondary to reduced activity of enzymes required for cortisol biosynthesis in the adrenal cortex.

These patients usually present during the perinatal period with ambiguous genitalia in females and salt wasting in males. The milder forms of disease may present later with virilization at puberty or even as irregular menses. Most of the cases (nearly 95%) are attributed to deficiency of the 21-hydroxylase enzyme [27, 28].

Abnormal growth and development, adverse effects on bone and the cardiovascular system, and infertility are few long-term effects seen in these patients. These patients are usually managed by reducing glucocorticoid exposure and improving excess hormone control [29, 30].

Congenital adrenal hyperplasia can be of four forms [8–10, 27–30]:

1. Congenital adrenal hyperplasia: classical 21-hydroxylase deficiency
2. Simple virilizing congenital adrenal hyperplasia
3. Non-classic or late onset form of congenital adrenal hyperplasia
4. Congenital adrenal hyperplasia with steroidogenic acute regulatory (StAR) mutation

3.4.1 Congenital adrenal hyperplasia: Classical 21-hydroxylase deficiency

This form is the most common form of CAH, occurring due to 21-hydroxylase (21-OH) deficiency, accounting for almost 90% of the cases. It occurs with the frequency of 1:12000 to 1:15000 births, and nearly 75% of patients with classic 21-OH

Multiple endocrine neoplasia (MEN) type 1	Adrenocortical lesions are seen in nearly 36–41% of individuals with MEN type I syndrome, the commonest being bilateral nonfunctioning adrenal cortical hyperplasia or adenoma; adrenocortical carcinoma is exceedingly rare. The pathogenesis of these lesions is proposed to be due to influence of locally secreted insulin and insulin-like growth factors and not due to menin gene mutations
Carney complex	This syndrome encompasses multiple endocrine hyperplasia, with tumors of two or more endocrine glands, including primary pigmented adrenocortical disease (PPNAD), GH- and prolactin-producing pituitary adenomas, testicular neoplasms, thyroid adenoma or carcinoma, and ovarian cysts. This autosomal dominant syndrome is mapped to two genetic loci, one present on chromosome 2p16 and another locus at chromosome 17q22–24 encoding the PRKARIA gene [82]
Beckwith-Wiedemann syndrome	This syndrome is characterized by gigantism, ear lobe pits and/or creases, macroglossia, and defects in the abdominal wall and is associated with chromosomal aberration of 11p15.5. These individuals are at higher risk of developing benign or malignant tumors of multiple organs, commonest being Wilms' tumor, rhabdomyosarcoma, hepatoblastoma, and adrenal carcinoma
Li-Fraumeni syndrome	Rare, autosomal, dominant familial syndrome with high incidence of multiple malignancies at an early age, including breast cancer, leukemias, soft tissue sarcomas, gliomas, laryngeal carcinoma, lung cancer, and adrenocortical carcinoma. The pathogenesis of this syndrome is attributed to germ-line point mutations in the p53 tumor suppressor gene (chromosome 17p13) in pediatric age group with adrenocortical carcinoma and deletion of short arm of chromosome 17 (17p)
Familial adenomatous polyposis	This disease is an autosomal dominant disorder, characterized by the presence of multiple adenomatous polyps of the colon and rectum. The gene [adenomatous polyposis coli gene] is located at 5q21. These patients are at high risk to develop adrenocortical adenomas and carcinomas, the incidence being 7.4% higher than 0.6–3.4% reported for normal population

Table 1.
Hereditary adrenocortical tumor syndromes.

deficiency also have defect in synthesizing aldosterone. These patients die in the neonatal period due to shock from salt wasting. CAH is associated with multiple tumors like testicular tumors arising from ectopic adrenal cortical rests, testicular and ovarian Leydig cell tumor, and ovarian tumor of the adrenogenital syndrome as ovarian and paraovarian brown masses. Grossly the adrenal gland is marked enlarged having a cerebriform appearance. On cut surface the gland appears tan-brown in color. Under the microscope the adrenal gland reveals diffuse cortical hyperplasia. The cells are compactly arranged like how they are in the zona reticularis [2, 27–30].

Table 1 illustrates various syndromes associated with adrenocortical lesions [31].

4. Primary hyperaldosteronism (Conn's syndrome)

This disease was defined first by Conn in 1955, with a prevalence of 5–13%. This syndrome is characterized by an inappropriate increase in production of aldosterone which is relatively independent from the renin-angiotensin mechanism and is non-suppressible by sodium loading. This is one of the leading causes of secondary hypertension in hypertensive adults [32]. Patients with primary aldosteronism may exhibit adrenal cortical hyperplasia or adenoma in 30% of sporadic cases, and nearly 1% of sporadic cases may have adrenocortical carcinoma [33]. Clinically these patients present most commonly as normokalemic hypertension, and severe cases do show hypokalemia (**Table 2**).

S. no.	Tests	Procedure	Interpretation
1.	Postural testing	<ul style="list-style-type: none"> Performed after overnight recumbency IV catheter inserted at 7 AM (measure baseline aldosterone, cortisol, and PRA values at 8 AM) Ambulate the patient for 2 h Measure baseline aldosterone, cortisol, and PRA 	<ul style="list-style-type: none"> 30–50% of APAs respond to upright posture, and 20% of bilateral adrenal hyperplasia are unresponsive Diagnostic accuracy of 85%
2.	18-Hydroxycorticosterone level		<ul style="list-style-type: none"> Elevated (>100 ng/dL) in patients with APAs and is significantly lower in patients with IHA Diagnostic accuracy of 82% Hybrid steroid levels (18-OHF and 18-oxoF) are high (3–30 times normal) in FH-I, normal to mildly elevated in FH-II (3–4 times normal, as in sporadic PA), and mildly to extremely high in FH-III (3–100 times normal)
3.	Dexamethasone suppression test: used for patients with glucocorticoid-remediable aldosteronism (GRA) as well as for those patients who do not have GRA	<ul style="list-style-type: none"> 4-day dexamethasone suppression test (using a dosage of 0.5 mg every 6 h) Measure aldosterone, renin, and cortisol levels—before suppression testing, after 2 days and then after 4 days of testing Plasma cortisol suppression (i.e., <5 mcg/dL) is used as an index of the dexamethasone effect 	<ul style="list-style-type: none"> Patients without GRA: aldosterone levels typically fall by approximately 50% and return to the reference range by the end of testing Patients with GRA: persistent suppression of aldosterone levels to less than 4 ng/dL Sensitivity of 92% and a specificity of 100% for the diagnosis of GRA

S. no.	Tests	Procedure	Interpretation
4.	18-Oxocortisol and 18-hydroxycortisol (>100 nmol/day)		<ul style="list-style-type: none"> Markedly elevated level of GRA
5.	Adrenal venous sampling	<ul style="list-style-type: none"> Test to differentiate unilateral disease from bilateral disease in patients with PA ACTH may be infused into a peripheral vein (at a dosage of 50 mcg/h, starting 30 min before sampling) Can be performed with cosyntropin stimulation 	<ul style="list-style-type: none"> Calculate lateralization index (LI): Plasma aldosterone (PAC) and plasma cortisol concentration (PCC) in both adrenal veins is determined The ratio of the higher (dominant) over the lower (non-dominant) PAC/PCC ratio is used for the assessment of lateralization of aldosterone hypersecretion Cutoff values of 4 during cosyntropin stimulation and of 2 for unstimulated AVS—criteria to document lateralization of aldosterone excess

Source: [9, 10, 33, 34].

Table 2.
Investigations.

The aldosterone-to-renin ratio (ARR), a gold standard method to differentiate primary from secondary causes of hyperaldosteronism, is defined as the ratio of plasma aldosterone (expressed in ng/dL) to plasma renin activity (PRA, expressed in ng/mL/h). The cutoff value of ARR is 30 ng/dL per/mL per hour (or 750 pmol/L per ng/mL per hour). The principle behind this test is that as aldosterone secretion rises, PRA in ex vivo testing falls due to sodium retention. This negative feedback response should occur when the aldosterone levels are supraphysiologic for that individual patient, and PRA may fall well before plasma aldosterone is clearly increased. Primary aldosteronism is suspected if the ARR is >30 ng/dL per mL per hour. This method is also helpful in differentiating aldosterone-producing adenoma from bilateral adrenal hyperplasia [34].

4.1 Familial primary aldosteronism

Familial primary aldosteronism is mainly of three types, all of which are inherited in an autosomal dominant manner [8, 10, 32, 33]:

1. Familial hyperaldosteronism type I (glucocorticoid-remediable aldosteronism): accounts for less than 1% of cases. This disorder is caused by a recombination between the CYP11B2 and CYP11B1 genes.
2. Familial hyperaldosteronism type II: nearly 3–5% cases of primary aldosteronism belong to this category and are attributed to 7p22. This disorder still lacks a specific gene.

4.1.1 Gross features

Grossly, the adrenal gland in cases of idiopathic hyperaldosteronism is rather unremarkable or may exhibit slight enlargement. The enlargement could be due to the presence of micronodules or macronodules. Usually, adenomas are unilateral and solitary. However few cases of bilateral disease have also been reported. These adenomas

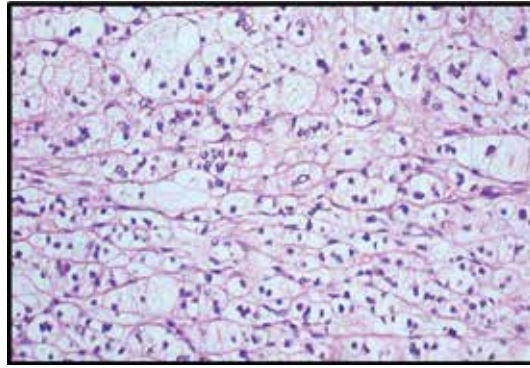


Figure 1. Section from a 22-year-old patient, presented with a 2 cm mass in the right adrenal gland. Histology reveals adenoma with clusters of cells with enlarged lipid-rich cytoplasm (hematoxylin and eosin stain, $\times 200$).

are mostly intra-adrenal and do not show a capsule. Few cases may reveal the presence of a true capsule or a pseudocapsule [2, 3, 35]. The cut surface of this tumor appears homogenous and golden yellow and is classically described as “canary yellow” [2]. Focal areas of hemorrhage or cystic changes can be present in few cases [35].

4.1.2 Microscopic features

Microscopically these adenomas appear encapsulated by compressed fibrous rim or fibrous “pseudocapsule.” The tumor cells are most commonly arranged in the form of nests or in alveolar pattern. Occasionally these cells may be arranged in short cords and trabeculae. Few cases may show mixed histological patterns. The tumor is composed of four different varieties of cells which may be present in varying proportions. More commonly seen are clear cells, having optically clear cytoplasm and centrally placed nuclei similar to those of the zona fasciculata cells; then there may be cells resembling to the zona glomerulosa or zona reticularis which appear small with compact eosinophilic cytoplasm. Then we have cells that are designated as “hybrid” cells. These hybrid cells have cytological features resembling both the zona fasciculata and glomerulosa (**Figure 1**). The uninvolved portion of adrenal cortex reveals atrophy. This atrophy is secondary to the negative feedback suppression effect of the hypothalamic–pituitary axis. Spironolactone bodies which appear as small, intracytoplasmic eosinophilic inclusions, round to oval, measuring 2–12 mm, are often encountered in adrenal cortical adenoma in patients on spironolactone treatment. These inclusions are delineated from the surrounding cytoplasm by a small, clear halo [2, 7, 9, 35].

5. Adrenal insufficiency

5.1. Introduction and definition

Adrenal insufficiency was first described by Thomas Addison in 1855 and was popularly known as Addison’s disease. This disorder can occur either due to failure of the adrenal gland or impairment of the hypothalamic–pituitary axis [36]. Clinically this syndrome is characterized by weakness, fatigue, anorexia, abdominal pain, weight loss, orthostatic hypotension, and salt craving. Characteristic hyperpigmentation is seen in patients with primary adrenal failure [37]. This disease has been reported in three forms [7, 9, 37]:

1. Primary disease also known as Addison's disease, a result of destruction of 90% or more of the adrenocortical gland or conditions that involve decreased production of adrenal steroids, resulting in subnormal synthesis of aldosterone, cortisol, and androgens.
2. Secondary and tertiary insufficiency occurs due to deficiency of secretion of corticotropin (ACTH) and corticotropin-releasing hormone (CRH), respectively.

Most of the cases (80–90%) of primary adrenal insufficiency are caused by autoimmune adrenalitis. Most of the cases fall under the autoimmune polyendocrinopathy syndrome (60%) [1, 2, 19, 32–34]. Cell-mediated immune mechanisms are implicated in pathogenesis. Various antibodies have been identified, antibodies against steroid 21-hydroxylase (85% cases) and autoantigens like steroid 17 α -hydroxylase and cholesterol side-chain cleavage enzyme. Other associations include cytotoxic T-lymphocyte antigen 4, protein tyrosine-phosphatase non-receptor type 22, and the MHC class II transactivator. Secondary adrenal insufficiency results from any process that involves the pituitary gland and interferes with corticotropin secretion. Tertiary adrenal insufficiency results from processes that involve the hypothalamus and interfere with secretion of corticotropin-releasing hormone, arginine vasopressin, or both. Suppression of the hypothalamic–pituitary–adrenal (HPA) axis by long-term administration of high doses of glucocorticoids is the most common cause [9, 36, 38].

5.2 Laboratory investigations

The patients of AI usually present with hyponatremia and hyperkalemia due to decreased aldosterone. Hypoglycemia also occurs due to cortisol. Decreased levels of this hormone also lead to an increase in lymphocytes and eosinophils, as a result of decreased immune-modulatory action of hydrocortisone. Measurement of baseline cortisol levels between 8:00 and 9:00 AM is the test used to diagnose AI. A serum cortisol level of value less than 5 $\mu\text{g}/\text{mL}$ favors diagnosis of AI. Stimulation test with cosyntropin which stimulates the cortex helps in differentiating primary and secondary AI. In this test 250 μg of cosyntropin is administered intramuscularly or intravenously, and serum cortisol is measured 30 min after infusion. Serum cortisol value of $\geq 18 \mu\text{g}/\text{dL}$ indicates a normal response. A cortisol peak $< 18 \mu\text{g}/\text{dL}$ confirms the diagnosis of AI. Serum cortisol level $\geq 100 \text{ pg}/\text{mL}$ confirms the diagnosis of Addison's syndrome. Serum cortisol value of $< 10 \text{ pg}/\text{mL}$ confirms diagnosis of secondary AI [35–39].

6. Adrenocortical carcinoma

6.1 Definition and introduction

ACC is a highly aggressive and a very rare malignancy. The incidence of this malignancy is approximately 0.72 per million cases per year according to the study by Surveillance, Epidemiology, and End Results (SEER) database [39]. The median age of diagnosis is usually fifth to sixth decade; however the German ACC Registry reports a median age at diagnosis of 46 years with a predilection for the female gender (female to male ratio: 1.5–2.5:1) [35, 38, 39].

Adrenocortical carcinomas (ACC) are rare tumors with an estimated annual incidence of 0.7–2 cases by year and a global prevalence of 4–12 cases per million with a 5-year survival rate inferior to 35% in most of the studies published.

6.2 Pathogenesis of adrenocortical carcinoma

Various mutations have been implicated in association with ACC. Most common are germ-line *TP53* mutations, associated with childhood ACCs. The adult population shows a prevalence of 3–7% of similar mutation. Childhood ACC can be found in association with Li-Fraumeni syndrome, Beckwith-Wiedemann syndrome, Lynch syndrome, and multiple endocrine neoplasia type 1. Of late an association with familial adenomatous polyposis (FAP), neurofibromatosis type 1, Werner syndrome, and Carney complex has also been postulated [39, 40].

6.2.1 Molecular mechanisms

In ACCs, chromosomal gains were frequently observed in regions 4q, 4p16, 5p15, 5q12–13, 5q32–qter, 9q34, 12q13, 12q24, and 19p, and chromosomal losses were observed at 1p, 2q, 11q 17p, 22p, and 22q. Microsatellite studies identified frequent allelic losses in regions 17p13, 11q15, and 2p16 (85%, 92%, and 90% of samples, respectively) [41–43].

Signaling pathways involved in adrenal malignant carcinogenesis [44–47]:

- p53 signaling pathway
- Wnt/beta-catenin signaling pathway
- Insulin growth factor II (IGF-II) locus

6.2.1.1 The *TP53* alterations

Protein p53, “guardian of genome,” is located at the 17p13 locus, and alterations in this gene have been noticed in various cancers including adrenocortical carcinoma, more so at the somatic level. p53 gene mediates cellular response to stress, and adult sporadic ACCs usually reveal loss of heterozygosity at this locus (nearly 85%) [48]. Stress leads to inhibition of degradation of p53 by E3 ubiquitin ligase MDM2, leading to inhibition of cell cycle arrest in response to DNA damage as well as apoptosis. These tumors tend to be larger and present at more advanced stage of tumor progression with shorter disease-free survival. Various genetic alterations have been reported in patients with adrenal cortex carcinoma like loss of *PTTG1* has been reported in nearly 84%, mutation in retinoblastoma protein (pRb) in nearly 27% cases and mutation in *RB1* gene in 7% of the cases. Inactivating mutations or homozygous deletions of *CDKN2A* have also been reported in 11–16% cases. High-level amplifications of *CDK4* and *MDM2* were reported in 2–7% ACCs [49–51]. It is surprising to see that majority of the *TP53* mutations occur at the DNA-binding domain. Some tumors also have shown abnormalities in genes that encode for negative regulators of *TP53*, like *PTTG1* which encodes for securin, noted in 84% of ACC. It is considered as a marker of poor survival [44, 46, 49].

6.2.1.2 *Wnt/beta-catenin signaling pathway*

Wnt family consists of highly conserved growth factors having similar amino acid sequences and is responsible for various developmental and homeostatic processes [4, 44, 46–48, 51]. A prevalence of 39 and 84% has been reported by various authors on immunohistochemistry for β -catenin. The Wnt receptor is composed of members of the frizzled family and low-density lipoprotein receptor-related

protein. β -Catenin accumulates in the cytoplasm and gets translocated into the nucleus and then binds with Wnt receptor leading to inhibition of the axin-adenomatous polyposis coli—glycogen synthase kinase 3 β (GSK-3) complex. This blocks the phosphorylation of β -catenin, leading to increased accumulation of β -catenin in the cytoplasm which further translocates into the nucleus. Interaction between β -catenin with the T cell-specific transcription factor/lymphoid enhancer-binding factor-1 family of transcription factors occurs in the nucleus, thus regulating transcription of Wnt target genes. If Wnt stimulation of GSK-3 phosphorylating β -catenin does not occur, degradation by proteasomes occurs following ubiquitylation of this receptor. Wnt pathway has been implicated in patients with familial adenomatous polyposis and in the development of colorectal carcinomas as well as ACCs. Wnt/beta-catenin pathway can be activated in both benign and malignant tumors by *CTNNB1* mutations and by *ZNRF3* inactivation in adrenal cancer. *ZNRF3* is a recent gene that encodes a cell-surface transmembrane E3 ubiquitin ligase which acts as a negative feedback regulator of Wnt signaling. Recently, *ZNRF3* was found to be the most frequently altered gene in study cohorts of ACC investigated by integrated genomics, with a prevalence of 21 and 19% in studies by Assié et al. and Zheng et al., respectively [50, 51].

6.2.1.3 *Insulin growth factor II (IGF-II)*

Nearly 85–90% of the adult adrenocortical carcinomas are attributed to *IGF-II* overexpression. This molecular abnormality is associated with DNA demethylation at *IGF-II* locus in most of cases. Various transcriptome studies have confirmed that *IGF-II* is the most upregulated gene in ACC [52, 53].

6.2.2 *Biochemistry*

ACC are the tumors characterized by adrenocortical hormone production in nearly 45–70% of patients. Hypercortisolism is the most common presentation of patients presenting with hormone excess leading to a plethora of symptoms like diabetes mellitus, hypertension, hypokalemia, muscle weakness/atrophy, and osteoporosis [40–43]. Excess of androgens which comprise nearly 40–60% of hormone-secreting ACCs can cause rapid-onset male pattern baldness, hirsutism, virilization, and menstrual irregularities in women. Estrogen production occurs in 1–3% of male ACC patients, causing gynecomastia and testicular atrophy (through suppression of the gonadal axis). In the evaluation of adrenal tumors, regardless of size, androgen or estrogen production should always raise the suspicion of a malignant tumor [44].

6.2.3 *Gross findings*

ACCs are generally large tumors, measuring on average 10–13 cm. Only a minority of tumors are less than 6 cm (9–14%), with only 3% presenting as lesions less than 4 cm [2, 3, 6, 9, 35].

6.2.4 *Microscopy findings*

Microscopically these tumors have variable architectural patterns. The tumor cells are arranged in a trabecular, alveolar, or diffuse pattern. Occasionally mixed patterns are also noted. Some areas may also exhibit free-floating tumor cells forming balls [2, 3, 6, 9, 35] (**Figure 2A, B**).

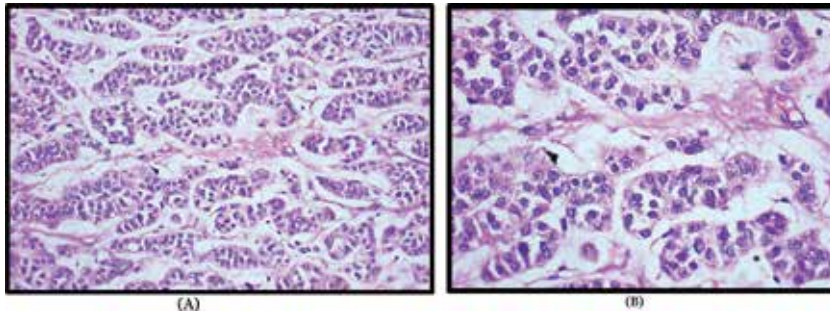


Figure 2.

(A) Section from a 45-year-old patient, presented with a 13 cm mass in the left adrenal gland. Histology reveals clusters of cells having anisocytosis and enlarged nuclei with prominent nucleoli. The fair number of darkly stained atypical mitosis is also evident (hematoxylin and eosin stain, $\times 200$). (B) Histology reveals clusters of cells having anisocytosis and enlarged nuclei with prominent nucleoli. The cells are separated by myxoid stroma. The fair number of darkly stained atypical mitosis is also evident (hematoxylin and eosin stain, $\times 400$).

Histologic criteria for malignancy in adrenal cortical tumors are assessed as follows [2, 3, 6, 9, 35, 43–45]:

1. High nuclear grade (grades III and IV according to the criteria of Fuhrman)
2. Mitotic rate > 5 per 50 HPF (10 HPF in each of the five areas that are most suspicious to be malignant)
3. Atypical mitotic figures (abnormal distribution of chromosomes or an excessive number of mitotic spindles)
4. Eosinophilic tumor cell cytoplasm ($>75\%$ of tumor cells or $<25\%$ clear vacuolated cells resembling the normal fasciculata)
5. Diffuse architecture ($>33\%$ of the tumor forming patternless sheets of cells)
6. Necrosis (occurring in confluent nests of cells)
7. Venous invasion (endothelial-lined vessel with smooth muscle as a component of the wall)
8. Sinusoidal invasion (endothelial-lined vessel in the adrenal with little supportive tissue)
9. Capsular invasion (nests or cords of tumor extended into or through the capsule with the corresponding stromal reaction)

Weiss et al. proposed a scoring system which was further modified and is widely accepted to report adrenal cortex carcinomas. These criteria include [35, 43–45, 54] (Table 3).

6.2.5 Interpretation

Adrenal cortical adenoma: total score < 3 .

Adrenal cortical carcinoma: total score ≥ 3 .

Thus if the modified Weiss score is ≥ 3 , then a diagnosis of adrenocortical carcinoma is given.

Criteria	Score	
	Absent	Present
Mitotic rate (≥ 6 mitotic figures/50 HPF)	0	1
Cytoplasm characteristics [clear vs. compact (compact >75% of cells)]	0	1
Abnormal mitoses	0	1
Tumor necrosis	0	1
Invasion of the capsule	0	1

Overall score = 2 × mitotic rate + 2 × cytoplasm + abnormal mitoses + necrosis + capsular invasion.

Table 3.
Weiss scoring for adrenocortical carcinoma.

Characteristics	Adrenocortical adenoma	Adrenocortical carcinoma
Macroscopy		
Weight	Usually less than 100 g	More than 100 g
Hemorrhage	+/-	+++
Necrosis	+/-	+++
Cystic degeneration	+/-	+++
IHC		
MIB-1	Negative	Positive
Vimentin	Negative	Positive
Inhibin	Positive	Positive
Melanin	Positive	Positive
Calretinin	Positive	Positive
BCL-2	Positive	Positive
C-kit	Negative	Positive
EMA	Negative	Negative
Cytokeratin	Negative	Negative
NSE	—	Positive
Synaptophysin	—	Positive
Chromogranin	—	Negative

Table 4.
Differentiating features between adrenocortical adenoma and adrenocortical carcinoma.

However there are other features that may help in differentiating between adenomas and carcinoma. These are listed in **Table 4** [35, 43–45].

7. Conclusion

Adrenal glands have an essential role in maintaining the normal hemostasis. However the three layers of adrenal cortex, the zona glomerulosa, zona fasciculata, and zona reticularis, secrete essential hormones that are involved in fluid and electrolyte balance, regulating renin-angiotensin-aldosterone system, production of glucocorticoids, and synthesis of sex hormones. These hormones play an important

role in maintaining the normal homeostasis of the body. Various lesions in adrenal, benign as well as malignant, are known to cause disturbances in the internal milieu of our body. It is therefore essential to know the physiology as well as various types of disorders that can be encountered so as to define proper management of the patient. Also lesions of adrenal gland are attributed to various genetic abnormalities, knowledge of which can be implicated to study the pathogenesis and in applying this knowledge in prognosis as well as developing targeted therapy for these lesions.

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Section 3

Mitochondrial Imaging

PET Imaging of Mitochondrial Function in the Living Brain

Hideo Tsukada

Abstract

In the last two and half decades, we have conducted research on brain functional imaging in nonhuman primates using animal positron emission tomography (PET) scanners with high spatial resolution. We recently designed and synthesized the novel PET probe [^{18}F]BCPP-EF to quantitatively image mitochondria complex-I (MC-I) activity in the living brain. Brain MC-I activity, measured using [^{18}F]BCPP-EF, was significantly lower in aged monkeys than that in young animals, while no significant reduction was observed in SV2A activity, a synaptic-specific parameter that was measured using [^{11}C]UCB-J. Some aged monkeys exhibited increased amyloid- β deposition in the brain, measured using [^{11}C]PiB, which induced neuroinflammation. A positive correlation was noted with neuroinflammation, measured using [^{11}C]DPA-713 and a negative correlation with MC-I activity. Furthermore, a monkey model of Parkinson's disease prepared by the chronic administration of MPTP revealed suppressed MC-I activity not only in the nigrostriatal dopamine pathway, measured using [^{11}C]PE2I and [^{11}C]6MemTyr, but also in cortical serotonergic neurons, measured using [^{11}C]DASB. This review introduces the translational application of a novel PET probe for noninvasive MC-I imaging from preclinical to clinical PET measurements.

Keywords: brain, mitochondria, aging, neurodegeneration, PET

1. Introduction

The brain has the most complex system among all human organs and plays a central role in physiological, neurological, and metabolic regulation throughout the body. The brain is very metabolically active in proportion to its volume, consuming 20% of total oxygen and 25% of total glucose, and each cell relies on mitochondria to produce energy as adenosine triphosphate (ATP). The brain consists of neuronal and glial cells, each of which uses the different metabolic pathways to produce ATP; astrocytes are highly glycolytic, while neurons depend on oxidative phosphorylation (OXPHOS) in the mitochondria [1]. The electron transport chain (ETC) for OXPHOS in mitochondria consists of five types of complexes from I to V, with complex-I (MC-I; NADH-ubiquinone oxidoreductase, EC 1.6.5.3) forming the first and rate-limiting steps of overall respiratory and OXPHOS.

Neuronal death is regarded the dominant cause of brain aging. The “Mitochondrial Free Radical Theory of Aging” considers mitochondria to be the main drivers of aging due to the generation of reactive oxygen (ROS) and nitrogen (RNS) species through the ETC, and the cumulated impairment of mitochondria by oxidative stress in mitochondrial lipids, proteins, and DNA leads to neuronal

death in the brain [2]. Neuronal damage related to impaired mitochondrial function is a hallmark for several neurodegenerative diseases, including Alzheimer's (AD), Parkinson's (PD), Huntington's diseases (HD), as well as amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS).

The etiology of AD, the most prominent age-related neurodegenerative disease, is multifactorial and associated with various environmental and genetic factors that play a role in its pathogenesis. The deposition of amyloid- β ($A\beta$) in the brain has been suggested to enhance neurodegenerative damage. Structural disruption and loss of neuronal cells induced by $A\beta$, leading to neuronal network dysfunction and synaptic loss in the hippocampus and cerebral cortex, is associated with cognitive impairment in AD patient [3]. Although the molecular mechanisms underlying synaptic dysfunction induced by $A\beta$ have not been fully elucidated, mitochondrial dysfunction caused by $A\beta$ is associated with synaptic functional alterations in the living brain [3].

PD is the second most prominent neurodegenerative disease and is characterized by the progressive degradation of the nigrostriatal pathway with the selective loss of dopamine (DA) neurons, resulting in movement abnormalities such as resting tremor, rigidity, akinesia, and impaired postural reflexes. A hallmark of idiopathic PD is the deposition of Lewy bodies containing insoluble and aggregated α -synuclein in the cytoplasmic fraction of nigrostriatal neurons in the DA pathway [4]. The loss of MC-I catalytic activity in the ETC has been reported in tissues obtained from sporadic PD patients [5] with increased oxidative stress [6].

Regarding the noninvasive assessment of mitochondrial function in the living brain, we recently developed a novel probe, [^{18}F]2-tert-butyl-4-chloro-5-{6-[2-(2-fluoroethyl)-ethoxyl-pyridin-3-yl]methoxy}-2H-pyrisazin-3-one ([^{18}F]BCPP-EF), for positron emission tomography (PET) [7]. The effects of physiological aging, aging-related $A\beta$ deposition, and chronic MPTP treatments on cortical MC-I function were assessed in conscious monkeys using [^{18}F]BCPP-EF, with the aim of translating clinical PET research to AD and PD patients.

2. Effects of aging on MC-I and SV2A function in the living brain

Mitochondria from aged brains consume less oxygen and, thus, produce lower amounts of ATP [8]. Different types of neurons show differences in topologies with various numbers of synaptic connections. Since synaptic and non-synaptic mitochondria have different protein compositions and respiratory and ROS production rates, synaptic mitochondria are more vulnerable to oxidative damage during the aging process than non-synaptic mitochondria [9]. In quantitative assessments of the effects of physiological aging on mitochondria and synaptic function in the living brain, the activities of MC-I and synaptic vesicle glycoprotein 2A (SV2A), an ideal biomarker for synaptic density, were comparatively evaluated in the living brains of young and aged rhesus monkeys.

2.1 Methods

Six young male (3–5 years old) and eight aged male (20–24 years old) rhesus monkeys (*Macaca mulatta*) were examined in the PET study using [^{18}F]BCPP-EF for MC-I and (*R*)-1-((3-[^{11}C]methyl-[^{11}C]pyridine-4-yl)methyl)-4-(3,4,5-trifluorophenyl)pyrrolidin-2-one ([^{11}C]UCB-J) for SV2A [10]. MRI was performed on each monkey using a 3.0 T MR imager (Singna Excite HDxt 3.0 T, GE Healthcare) using a 3D spoiled gradient echo (SPGR) sequence under pentobarbital anesthesia. To avoid anesthetic effects on brain function as well as PET probe kinetics, PET scans were

conducted under conscious condition using high-resolution animal PET scanners (SHR-7700 and 38,000, Hamamatsu Photonics) as reported previously [11–14].

[¹⁸F]BCPP-EF was prepared by the nucleophilic [¹⁸F] fluorination of the corresponding precursor, as reported previously [9]. Radiochemical purity was more than 99%, and specific radioactivity was 47.8 ± 12.7 GBq/ μ mol. [¹¹C]UCB-J was labeled via the Suzuki cross-coupling of [¹¹C]methyl iodide with a boronate precursor, as reported previously [10]. Radiochemical purity was more than 98%, and specific radioactivity was 62.3 ± 25.1 GBq/ μ mol.

After overnight fast, a venous cannula for the PET ligand injection and an arterial cannula for blood sampling were inserted into both inferior limbs. The animal's head was rigidly fixed to a monkey chair using an acrylic head-restraining device surgically attached on the skull. The monkey sitting in the chair was placed at a position in the PET gantry with stereotactic coordinates aligned parallel to the orbito-metal (OM) plane. After a transmission scan for 30 min using a [⁶⁸Ge]-[⁶⁸Ga] rotation rod source, a dynamic emission scan with [¹⁸F]BCPP-EF or [¹¹C]UCB-J was conducted for 90 min after the PET probe injection as a bolus.

To assess the specificity of [¹⁸F]BCPP-EF binding to MC-I, which was previously examined in rats [15], rotenone, an MC-I inhibitor, at a dose of 0.1 mg/kg was infused into young monkeys through a vein cannula for 1 h, followed by [¹⁸F]BCPP-EF injection.

PET data were reconstructed by the dynamic row action maximum likelihood algorithm (DRAMA) method using a 2.0-mm Gaussian post filter [16], with attenuation correction using transmission scan data. Individual PET and MRI images were co-registered. Volumes of interest (VOIs) in brain regions were drawn manually on MRI, and the time activity curve (TAC) of each PET probe was taken for kinetic analyses. These imaging analytical processes were performed using PMOD software (PMOD Technologies Ltd.).

In a quantitative analyses of [¹⁸F]BCPP-EF and [¹¹C]UCB-J, arterial blood samples were frequently obtain after the PET probe injection, centrifuged to separate plasma, and ethanol was added to some plasma samples, followed by centrifugation. The supernatants obtained were developed with thin layer chromatography with a mobile phase of ethyl acetate for [¹⁸F]BCPP-EF and chloroform/methanol = 9:1 for [¹¹C]UCB-J, respectively. The ratio of the unmetabolized fraction was assessed using a phosphoimaging plate analyzed by a bioimaging analyzer (FLA-7000, GE Healthcare). The arterial input function of unmetabolized [¹⁸F]BCPP-EF or [¹¹C]UCB-J was calculated using data obtained by a correction of the radioactivity ratio in the unmetabolized fraction to total fraction. A kinetic analysis of [¹⁸F]BCPP-EF or [¹¹C]UCB-J was performed to calculate distribution volume (V_T) using a Logan graphical analysis [17] with PMOD software (PMOD Technologies Ltd.).

2.2 Results

Brain TACs of [¹⁸F]BCPP-EF in young animals peaked at approximately 15 min after the injection, except in the occipital cortex, which peaked at 40 min, and TACs then were gradually eliminated with time under conscious condition. The pre-administration of rotenone resulted in a faster elimination rate of [¹⁸F]BCPP-EF from the brain than that in normal. In aged animals, the peak time of TACs shifted to slightly later after the injection and showed significant lower [¹⁸F]BCPP-EF uptake levels than those in young animals.

The washout of [¹⁸F]BCPP-EF-related radioactivity and its metabolites rate in plasma were very rapid, with only 10% of the non-metabolized parent probe remaining 60 min after the injection. These kinetic patterns of [¹⁸F]BCPP-EF were almost identical among control young, rotenone-treated young, and aged animals.

A metabolic analysis confirmed that its metabolites were very polar, indicating no uptake of metabolites into the brain through the blood–brain barrier. These results confirmed that this PET probe has ideal properties for the quantitative analysis of MC-I activity using a metabolite-corrected arterial input function.

As shown in the upper panel of **Figure 1**, V_T -PET images of young brains indicated that the binding of [^{18}F]BCPP-EF was highest in the occipital cortex, higher in the striatum, intermediate in the frontal and temporal cortices and cerebellum, and lowest in the hippocampus. The pre-administration of rotenone at a dose of 0.1 mg/kg/h significantly reduced the uptake of [^{18}F]BCPP-EF into the young monkey brain, specifically in the frontal and temporal cortices and striatum. When aging effects on V_T -PET images were assessed, the binding of [^{18}F]BCPP-EF throughout the brain was significantly lowered in aged animals than in young animals, as shown in the lower panel of **Figure 1**.

The TAC of [^{11}C]UCB-J in young animals exhibited slower kinetics than [^{18}F]BCPP-EF throughout the brain regions, peaking approximately 30 min after the injection, and TACs gradually decreased with time under conscious conditions. TACs in aged animals peaked 30 min after the injection and showed slightly lower [^{11}C]UCB-J uptake levels than those in young animals. The washout of [^{11}C]UCB-J-related radioactivity and metabolic rates in plasma were slower than those of [^{18}F]BCPP-EF, with 25% of the non-metabolized parent probe remaining 60 min after the injection. These kinetic patterns of [^{18}F]BCPP-EF were not significantly different between young and aged animals. A metabolic analysis confirmed that its metabolites were very polar, suggesting no uptake of the metabolites into the brain through the blood–brain barrier. These results confirmed that this PET probe is useful for the quantitative analysis of SV2A activity using a metabolite-corrected arterial input function.

As shown in the upper panel of **Figure 2**, V_T -PET images of the young brain revealed the highest binding of [^{11}C]UCB-J in the frontal, temporal, and occipital

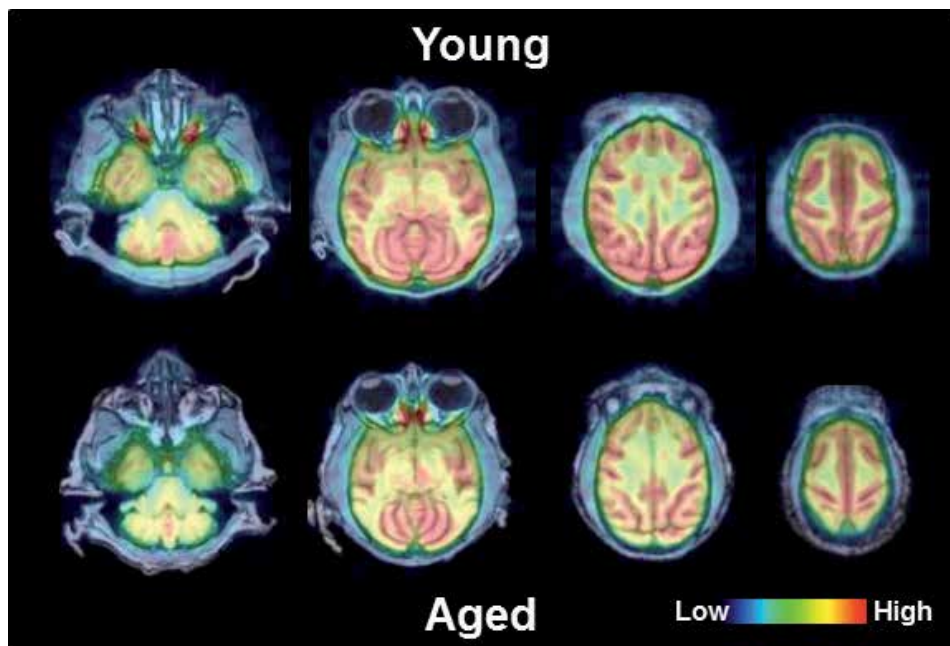


Figure 1.

Effects of aging on PET images of MC-I in living brains of conscious monkeys. PET scans were conducted for 91 min using [^{18}F]BCPP-EF for MC-I, and V_T images of [^{18}F]BCPP-EF were created using a Logan graphical plot analysis with metabolite-corrected plasma input.

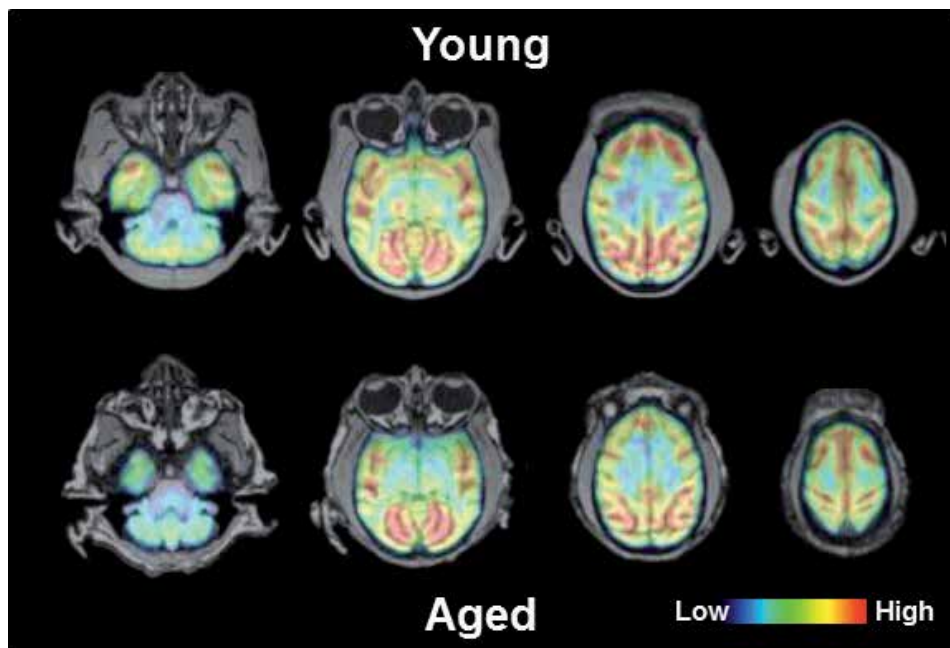


Figure 2. Effects of aging on PET images of SV2A in living brains of conscious monkeys. PET scans were conducted for 91 min using [^{11}C]UCB-J for SV2A, and V_T images of [^{11}C]UCB-J were created using a Logan graphical plot analysis with metabolite-corrected plasma input.

cortices, higher in the striatum, intermediate in the hippocampus, and the lowest in the cerebellum. Thus, the distribution pattern of [^{11}C]UCB-J resembled to that of [^{18}F]BCPP-EF throughout the brain of young animal.

However, in contrast to [^{18}F]BCPP-EF, when the V_T of [^{11}C]UCB-J was compared between young and aged monkeys, no significant age-related changes in cerebral SV2A activity were detected except the olfactory bulb, as shown in the lower panel of **Figure 2**.

2.3 Discussion

The present results showed age-related reduction in mitochondria function assessed by MC-I activity using [^{18}F]BCPP-EF, as reported previously [11], while no significant alterations in synaptic density were observed based on SV2A activity using [^{11}C]UCB-J.

We evaluated [^{18}F]BCPP-EF by in vitro (living brain slices), ex vivo (dissected tissues), and in vivo (living rat brains) assessments in comparison with the conventional MC-I PET probe, [^{18}F]BMS-747158-02 ([^{18}F]BMS), which was developed as a myocardial perfusion PET imaging probe [18], and the results obtained suggested that [^{18}F]BCPP-EF is a more suitable PET probe than [^{18}F]BMS for MCI assessments in the living brain [7, 15]. [^{18}F]BCPP-EF was characterized by (1) high specific binding with moderate affinity, (2) a proper $\log D_{7.4}$ value, (3) long metabolic stability with fast clearance in plasma, (4) sufficient brain uptake with a proper elimination rate from the brain, and (5) low dependency to cerebral blood flow changes [19]. As a PET probe, these properties of [^{18}F]BCPP-EF may contribute for a noninvasive and quantitative analysis of MC-I in the living brain [11], thereby allowing for the high detectability of age-related reductions in MC-I activity in the living monkey brain. Although several postmortem in vitro studies previously reported that (1)

age-related brain MC-I impairments were associated with a decline in mitochondria respiration [20, 21] and (2) the activities of MC-I and -IV, but not MC-II, III, or V, decreased with aging in the brains of rodents [22] and humans [20, 21], this study was the first to successfully confirm age-related reductions in MC-I activity in the living brain of nonhuman primates [11].

As described above, [¹⁸F]BCPP-EF detected age-related reduction in MC-I activity; however, this PET probe was unable to discriminate the subdomain (synapse, dendrites, or soma) of MC-I that was exclusively damaged due to the limitation of the spatial resolution of PET. In attempts to answer this question, [¹¹C]UCB-J, a novel PET probe for SV2A [10], was applied to the same subject groups on the assumption that if this MC-I reduction mainly reflected the presynaptic domain, the binding of [¹¹C]UCB-J to SV2A will also show a similar age-related reduction pattern in the brain. However, contrary to this assumption, no significant changes in [¹¹C]UCB-J binding to SV2A were observed, except the olfactory bulb, from those in the same young and aged animals applied in the assessment using [¹⁸F]BCPP-EF.

Several postmortem *in vitro* studies have suggested age-related reductions in spine density; however, these findings remain controversial. One study using rat brains revealed that the synaptic vesicle density in axospinous synapse, a major population of synapses, rapidly increased until 3 weeks old and then decreased to the adult level, followed by no changes in senescence [23]. However, another study on mouse brain showed that age-related deficits in sensory perception were not associated with synaptic loss in the somatosensory cortex but were related to alterations in the size and stability of spine buttons [24], which might not be detected as the changes in SV2A activity.

The discrepancy in age-related effects between [¹¹C]UCB-J and [¹⁸F]BCPP-EF suggest that MC-I dysfunction detected by [¹⁸F]BCPP-EF mainly reflect neuronal damage in dendrite and soma domains and are not specific to presynaptic domain. Although difficulties are associated with assessing mitochondrial distribution in neuronal cells, it was demonstrated in the cultured neuron that more than 90% of mitochondria were confirmed to be in the dendrite shaft overlapping in soma and large-diameter proximal dendrites [25]. This localized distribution pattern of the mitochondria suggests that even if mitochondria in the presynaptic domain are specifically impaired, difficulties may be associated with detecting changes in the minor subdomain using [¹⁸F]BCPP-EF. The present results also suggested that in the early stage of the aging process, at least, in nonhuman primates, neuronal damage in the synaptic domain, if any, may be too subtle to be detected as decreased [¹¹C]UCB-J binding to SV2A.

3. Effects of amyloid- β deposition on MC-I function in the living brain

Patients of AD, the most prominent age-related neurodegenerative disease, are characterized by the deposition of fibrillary A β into senile plaques and hyperphosphorylated tau (P-tau) into neurofibrillary tangle (NFT) in the brain. Monomer A β has been implicated in normal developmental synaptic plasticity, for example, in the olfactory bulb under physiological conditions [26]. However, the aggregation of A β switches its physiological role into a pathologically toxic function; thus dense plaques damage the surrounding brain tissues [27], causing synaptic elimination and impaired synaptic function [28]. Although the molecular mechanisms underlying neuronal damage induced by A β have not been fully elucidated, mitochondrial dysfunction may be associated with A β -induced synaptic dysfunction [3] and also with neuroinflammation [29], resulting in cognitive impairment in AD patients. In order to clarify the relationship between A β deposition, neuroinflammation, and mitochondrial function, [¹¹C]PiB for A β [30], [¹¹C]DPA-713 for translocator protein

(TSPO) [31], and [^{18}F]BCPP-EF for MC-I were comparatively evaluated in the living brains of aged rhesus monkeys. Furthermore, as a gold standard parameter of cerebral metabolism, [^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) was also applied to compare the diagnostic and prognostic usefulness with [^{18}F]BCPP-EF.

3.1 Methods

Twenty aged male (20–24 years old) rhesus monkeys (*Macaca mulatta*) were investigated in the PET study using [^{11}C]PiB for A β , [^{11}C]DPA-713 for TSPO, and [^{18}F]BCPP-EF for MC-I under conscious conditions using a high-resolution PET scanner as described in 2.1.

[^{11}C]PiB was synthesized by the *N*-methylation of nor-compound *N*-desmethyl-PiB with [^{11}C]methyl triflate [30]. Radiochemical purity was more than 99%, and specific radioactivity was 36.7 ± 10.1 GBq/ μmol . [^{11}C]DPA-713 was synthesized by the *N*-methylation of nor-compound *N*-desmethyl-DPA with [^{11}C]methyl triflate [31]. Radiochemical purity was more than 99%, and specific radioactivity was 99.3 ± 32.2 GBq/ μmol . [^{18}F]BCPP-EF was radiolabeled as shown in 2.1. Radiochemical purity was more than 99%, and specific radioactivity was 139.6 ± 37.0 GBq/ μmol . [^{18}F]FDG was produced by the nucleophilic [^{18}F]fluorination of mannose triflate following the basic hydrolysis of 2- [^{18}F]fluoro-1.3.4.6-tetra-*O*-acetyl-D-glucose.

In the analysis of [^{11}C]PiB, standard uptake value (SUV) images from 60 to 90 min were created, VOIs were set on each SUV images, and the SUV ratios (SUVR) of each region (SUVR_{reg}) against SUV in the cerebellum (SUVR_{cereb}) were calculated [32]. In the analysis of [^{11}C]DPA-713, SUV images from 40 to 60 min were created, and VOIs were set on each SUV image. Since any cerebral regions were not applicable as the reference region with negligible TSPO levels [33], the SUV, not SUVR, was assessed. A quantitative analysis of [^{18}F]BCPP-EF was performed as described in 2.1. During PET scanning with [^{18}F]FDG, continuous arterial sampling was conducted, and PET images from 40 to 60 min after the injection were obtained to calculate the regional cerebral metabolic rate of glucose (rCMR_{glc}) using an autoradiographic method. The rCMR_{glc} ratios of each region (rCMR_{glc}-region) against rCMR_{glc} in the cerebellum (rCMR_{glc}-cere) were calculated [34].

3.2 Results

Since the cortical V_T values of [^{18}F]BCPP-EF in aged monkeys were previously shown to have a higher CV value of ca. 25% than those in young ones of ca. 7% [11], the reasons for the larger variation of MC-I activity were elucidated using PET imaging with [^{11}C]PiB for A β deposition and [^{11}C]DPA-713 for TSPO in 20 aged monkeys. **Figure 3** shows the PET/MRI images of aged monkeys with the lowest (A) and highest (E) [^{11}C]PiB binding, with high SUV not only being detected in cortical but also subcortical regions. PET results obtained with [^{11}C]PiB were supported by an immunohistochemical assessment conducted after PET assessments [12]. The images of [^{11}C]DPA-713 (B and F) corresponding to each monkey (A and E) revealed that monkeys with the lowest (A) and highest (E) [^{11}C]PiB binding showed the lower (B) and higher (F) [^{11}C]DPA-713 binding. In contrast, the images of [^{18}F]BCPP-EF corresponding to each monkey with the lowest (A) [^{11}C]PiB binding exhibited higher [^{18}F]BCPP-EF uptake (C), while those with the highest [^{11}C]PiB binding (E) showed the lower [^{18}F]BCPP-EF uptake (G).

As shown in **Figure 4A**, when the SUV values of [^{11}C]DPA-713 were plotted against the SUVR of [^{11}C]PiB in the cortical VOIs of all animals, the results

obtained revealed a significant positive correlation between each parameter, indicating that A β deposition-induced neuroinflammation in the living brains of aged monkeys. In contrast, the plotting of V_T of [18 F]BCPP-EF against SUVR of [11 C]PiB showed a reverse correlation in the cortical VOIs of all animals, suggesting that A β deposition-induced MC-I impairments in the brains of aged monkeys (**Figure 4B**).

Based on **Figures 3H** and **4C**, it is important to note that a correlation was not observed between glucose metabolism assessed as the SUVR of [18 F]FDG and A β deposition measured as the SUVR of [11 C]PiB, and this may have been due to [18 F]FDG uptake into the neuroinflammation-related activated microglial cells.

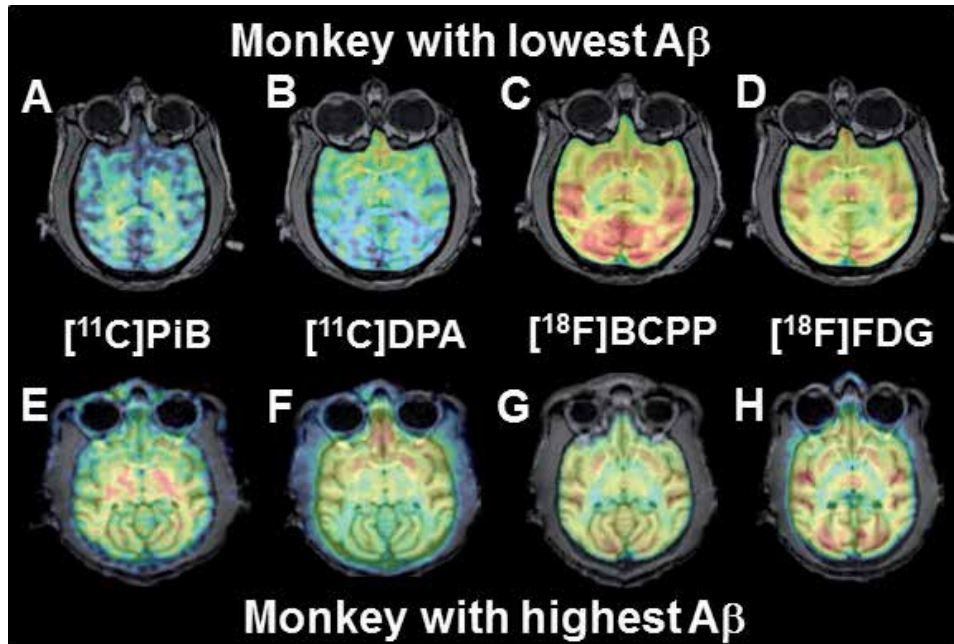


Figure 3. Effects of A β deposition on PET images of [11 C]PiB (A and E), [11 C]DPA-713 (B and F), [18 F]BCPP-EF (C and G), and [18 F]FDG (D and H) in living brains of conscious monkeys. PET scans were conducted in monkey with the lowest (A–D) and highest (E–H) A β deposition among 20 aged monkeys.

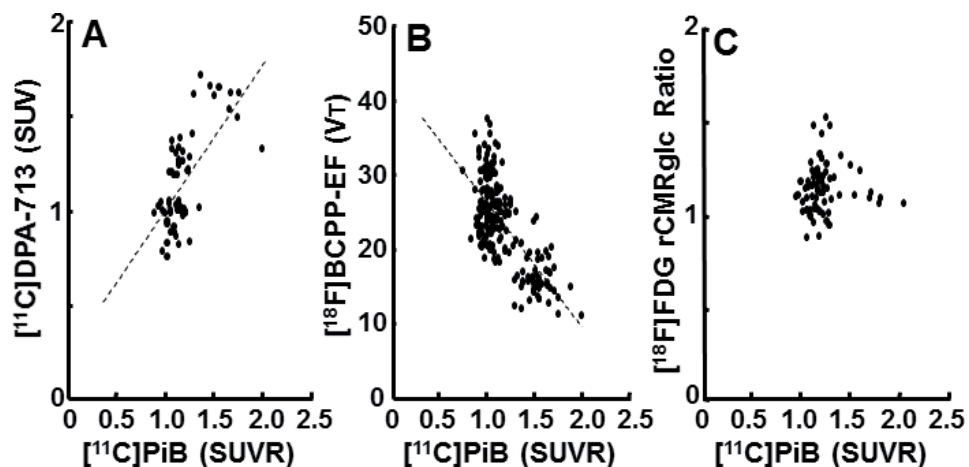


Figure 4. Correlations between the SUV of [11 C]DPA-713 (A), V_T of [18 F]BCPP-EF (B), and rCMRglc ratio of [18 F]FDG (C) against the SUVR of [11 C]PiB in cortical and hippocampal regions of living brains of aged monkeys.

3.3 Discussion

The present results demonstrated the potential of MC-I impairment assessed using [^{18}F]BCPP-EF as a useful biomarker for A β deposition-related neurodegeneration, measured using [^{11}C]PiB, and neuroinflammation, measured using [^{11}C]DPA-713. In contrast, the assessment of rCMRglc using conventional [^{18}F]FDG was not as sensitive for detecting the neurodegenerative damage associated with inflammation in the early stage of disease onset because of [^{18}F]FDG uptake into not only normal neuronal cells but also into inflammatory cells, such as activated microglia in the brain [15, 19, 29]. Since activated microglia dominantly facilitate glycolysis, not ORPHOS system through mitochondria, to produce ATP, more glucose is required in microglia than in normal neuron, resulting in higher [^{18}F]FDG uptake than in normal cells. Thus, MC-I assessed using [^{18}F]BCPP-EF has a potential as a biomarker to assess neurodegeneration more accurately without being affected by inflammation in the living brain.

The present PET images obtained using [^{11}C]PiB did not reveal as prominent A β deposition in aged monkey brains as that in the brains of PD patients. Previous studies suggested that no species other than humans exhibited marked neuron loss or cognitive impairment observed as clinical grade AD in humans, and aged monkeys did not exhibit as high A β deposition as seen in AD patients [35] or [^{11}C]PiB binding [36] as that in AD patients. Therefore, we assumed that the aged monkey model exhibited a similar mild cognitive impairment (MCI)-like, not AD-like, state to that in humans. In the AD state after a lag period of MCI, A β deposition level reached a plateau; therefore no correlation was observed between [^{11}C]PiB uptake and cognitive levels or metabolic dysfunction assessed using [^{18}F]FDG [37]. In contrast, [^{11}C]PiB may function as a quantitative and sensitive biomarker for A β -related neuronal damage in aged monkey brains resembling the MCI-like state in humans [38]. Furthermore, a recent study reported the primary role of non-deposited, non-fibrillar assemblies of A β peptides, and, thus, they may become precursors for fibrillogenesis, which lead to oxidative neurotoxicity by ROS in the brains of very early stage of AD patients [39]. ROS-related mitochondrial OXPHOS failure in the brain has been implicated in neurodegenerative disorders [40]. Mitochondria are the main intercellular source of ROS and also the main target of oxidative damage, leading to the significant disruption of brain function. A recent study demonstrated the direct effects of A β on mitochondria; thus, in addition to extracellular deposition, A β was detected in cytoplasmic mitochondrial compartments [41], leading to dysfunction with the suppressed availability of nucleus-encoded proteins, a decreased rate of NABH reduction, and enhanced ROS generation [42]. Since we previously confirmed a positive correlation between the V_T of [^{18}F]BCPP-EF and rCMRO $_2$, a gold standard for brain activation [19], the A β deposition-related MC-I functional impairment observed in the present study may reflect diminished activity and/or the loss of neurons with neuroinflammation. The present study revealed a correlation between [^{11}C]PiB binding and [^{18}F]BCPP-EF uptake, demonstrating the usefulness of an assessment of MC-I for the diagnostic staging of MCI and early stage AD.

In the present study, TSPO activity evaluated using [^{11}C]DPA-713, an index of neuroinflammation, was stronger in the aged monkey brain with higher [^{11}C]PiB binding to A β . Furthermore, [^{11}C]DPA-713 binding to TSPO and rCMRglc measured using [^{18}F]FDG revealed a correlation (data not shown) [12]. These results suggest A β deposition-induced neuroinflammation with the activation of microglial cells.

Therefore, the detectability of MC-I impairment using [^{18}F]BCPP-EF for neuronal damage in AD appears to be superior to the assessment of glucose metabolism measured using conventional [^{18}F]FDG.

4. Effects of MPTP-induced parkinsonism on MC-I in the living brain

Patients with PD show the progressive degradation of the nigrostriatal pathway with the selective loss of DA neurons in the substantia nigra pars compacta (SNc), resulting in movement disorders, which are induced after the loss of ca. 50% of neurons in the SNc and a reduction in DA to ca. 20% of normal levels in the striatum [43]. A pathological hallmark of PD is Lewy bodies and Lewy neurites, containing intracytoplasmic insoluble and aggregated protein of α -synuclein [4]. The spread of these pathologies closely correlates with disease progression. Furthermore, an intracerebral injection of insoluble α -synuclein converted normal α -synuclein into an abnormal form, which then propagated throughout the brains of monkeys [44] and common marmosets [45]. The soluble, β sheet-rich oligomers of α -synuclein induced mitochondrial dysfunction by inhibiting MC-I, enhancing ROS production, and activating the mitochondrial permeability transition pore (PTP), leading to mitochondrial swelling and neuronal death [46].

Since exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a syndrome mimicking PD symptoms accompanied by selective DA damage in the nigrostriatal pathway [47], we developed a PD model of monkeys by systemic and repeated MPTP administration [48, 49] and evaluated serotonin (5-HT) transporter (SERT) availability using [^{11}C]-3-amino-4-(2-dimethylamino-methylphenylsulfanyl)-benzonitrile ([^{11}C]DASB) [50] 5-HT 1A receptor (5-HT_{1A}R) binding using 4-(2'-methoxyphenyl)-1-[2'-(*N*-2"-pyridinyl)-*p*-[^{18}F]fluoro-benzamido]ethylpiperazine ([^{18}F]MPPF) [51] in parallel with DA parameters for presynaptic DA synthesis (DAS) using 6-[^{11}C]methyl-*m*-tyrosine ([^{11}C]6MemTyr) [52, 53], DA transporter (DAT) using [^{11}C]N-(3-iodoprop-2E-enyl)-2 β -carboxymethoxy-3 β -(4-methyl-phenyl) nortropine ([^{11}C]PE2I) [54], postsynaptic DA D₂R using [^{11}C]Raclopride, and MC-I activity using [^{18}F]BCPP-EF in the living brains of MPTP-treated monkeys [13].

4.1 Methods

Young adult male rhesus monkeys (*Macaca mulatta*) were used to prepare the PD model, as reported previously [48, 49]. MPTP at doses ranging between 0.2 and 0.4 mg/kg in phosphate-buffered saline was injected intravenously over a 4-month period until stable Parkinsonian syndrome developed. MPTP-treated monkeys were subjected to PET scans using specific PET probes for DA, 5-HT neuronal systems, and MC-I under conscious condition using a high-resolution PET scanner, as described in 2.1. In order to avoid the potential for spontaneous recovery as well as direct inhibition of [^{18}F]BCPP-EF binding to MC-I by MPTP, all PET measurements were started at least after 2 months after the last treatment with MPTP.

[^{11}C]PE2I was radiolabeled by the *O*-[^{11}C]methylation of its nor-compound with [^{11}C]methyl triflate. Radiochemical purity was more than 98% and specific radioactivity of 117.1 ± 42.9 GBq/ μmol . [^{11}C]6MemTyr was developed in our laboratory using rapid Pd(0)-mediated cross-coupling between [^{11}C]methyl iodide and its boronate precursor [52, 53], showing radiochemical purity of more than 99% and specific radioactivity of 71.6 ± 37.4 GBq/ μmol . [^{11}C]Raclopride was radiolabeled by *N*-[^{11}C]methylation of its nor-compound with [^{11}C]methyl triflate. Radiochemical purity was more than 98% and specific radioactivity of 65.4 ± 15.8 GBq/ μmol . [^{18}F]BCPP-EF was radiolabeled as shown in 2.1. Radiochemical purity was more than 99%, and specific radioactivity was 58.9 ± 7.9 GBq/ μmol . [^{11}C]DASB was prepared by the *N*-[^{11}C]methylation of its nor-compound with [^{11}C]methyl triflate [50] with radiochemical purity of more than 99% and specific radioactivity of

47.6 ± 11.1 GBq/ μmol . [^{18}F]MPPF was synthesized by the nucleophilic [^{18}F]fluorination of its nitro precursor [51] with radiochemical purity of more than 99% and specific radioactivity of 90.4 ± 25.6 GBq/ μmol .

Quantitative analyses of [^{11}C]PE2I, [^{11}C]Raclopride, [^{11}C]DASB, and [^{18}F]MPPF were performed with a simplified reference tissue model to calculate non-displaceable binding potential (BP_{ND}) [55] using PMOD software (PMOD Technologies Ltd., Zurich, Switzerland). As an indirect input function, TAC in the cerebellum was applied. A quantitative analysis of [^{18}F]BCPP-EF was performed as described in 2.1. A quantitative analysis of [^{11}C]6MemTyr to calculate the K_i value was performed using a multiple-time graphical analysis [56] using PMOD software (PMOD Technologies Ltd., Zurich, Switzerland) applying the TAC in the occipital cortex as an input function.

4.2 Results

The effects of chronic MPTP treatments on the DA neuronal system in the brain were revealed in **Figure 5**. PET measurements using [^{11}C]PE2I for DAT (A), [^{11}C]6MemTyr for DAS (B), and [^{11}C]Raclopride for D_2R (C) clearly imaged the striatum of the normal monkey brain. After the repeated treatment with MPTP, presynaptic DAT availability assessed using [^{11}C]PE2I was significantly lower in the caudate (4.0% of normal), putamen (4.9% of normal), and SNc (18.6% of normal) (E) of the MPTP-treated monkey brain than in the normal monkey (A). Another presynaptic parameter of DAS assessed using [^{11}C]6MemTyr was also markedly lower in the caudate (13.6% of normal), putamen (12.3% of normal), and SNc (41.1% of normal) (F) of MPTP monkey brain than in normal animal monkey brain (B). In contrast, no significant changes in [^{11}C]Raclopride binding to postsynaptic D_2R were observed in the striatum or SNc between normal (C) and MPTP-treated animals (G).

The effects of the MPTP treatments on PET images of MC-I in the brain are shown in **Figure 5D** and **H**. The cerebral uptake of [^{18}F]BCPP-EF in normal monkeys showed homogeneous and symmetric patterns in the both hemispheres with clear separation of the cortical and basal ganglion regions (D). After the repeated treatment with MPTP, the V_T of [^{18}F]BCPP-EF exhibited significant reductions ranging between 60 and 70% of normal levels not only in the nigrostriatal pathway with abundant DA neurons but also in extra-striatal non-DA regions such as the cortex (H). No significant decreases in the V_T of [^{18}F]BCPP-EF were observed in the cerebellum.

Since the chronic treatment with MPTP, known as a DA specific toxin, unexpectedly induced decreases in MC-I activity in extra-striatal non-DA regions, another monoaminergic neuronal system of 5-HT was further evaluated. In the normal monkey brain, [^{11}C]DASB binding to SERT was high in the striatal and mid-brain regions (**Figure 6A**), while [^{18}F]MPPF binding to 5-HT $_{1A}$ R was high in the cortical regions (B). The quantitative analysis of [^{11}C]DASB revealed significantly lower SERT availability throughout the brain in MPTP-treated monkey, except the raphe nucleus (**Figure 6C**), than in normal monkeys (A). The reduction in [^{11}C]DASB availability to SERT was the greatest in the occipital cortex (19.7% of normal); intermediate in the frontal, parietal, and temporal cortices (50.0, 40.7, and 51.6% of normal); smaller in the caudate (60.3% of normal); and the smallest in the putamen and SNc (66.0 and 67.7% of normal). In contrast, no significant changes were observed in [^{18}F]MPPF binding to 5-HT $_{1A}$ R throughout the brain between normal (**Figure 3B**) and MPTP-treated monkeys (D).

In order to assess the relationship between MC-I and DA and 5-HT neuronal systems in MPTP-treated monkey brains, the degrees of reductions in DAT measured using [^{11}C]PE2I, DAS measured using [^{11}C]6MemTyr in the nigrostriatal pathway (caudate, putamen, and SNc), and SERT measured using [^{11}C]DASB in

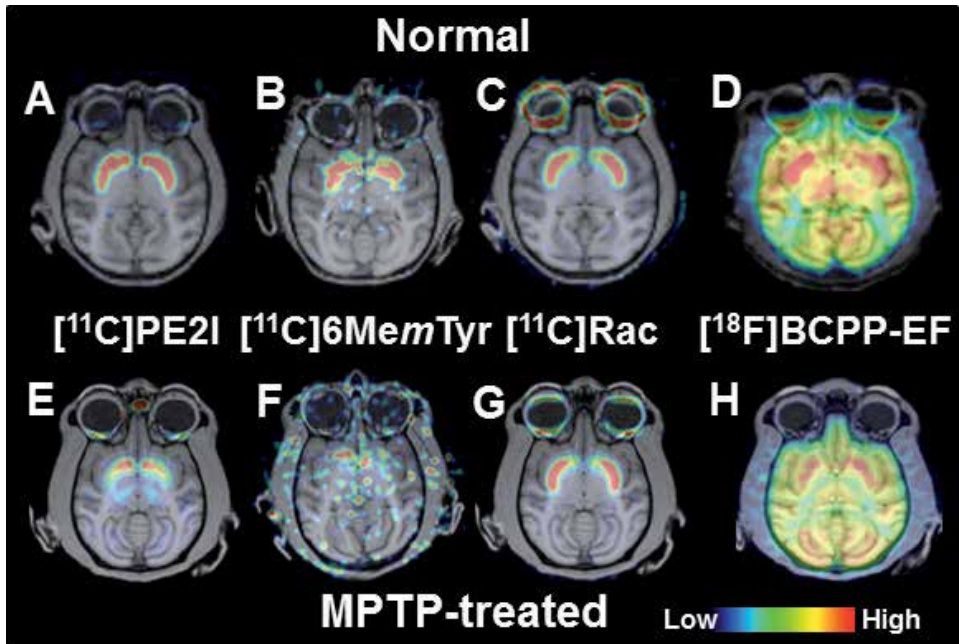


Figure 5. Effects of the MPTP treatment on PET images of DA and MC-I in living brains of conscious monkeys. PET scans were conducted for 91 min using $[^{11}\text{C}]\text{PE}2\text{I}$ for DAT (A and E) and $[^{18}\text{F}]\text{BCPP-EF}$ for MC-I (D and H) and for 60 min using $[^{11}\text{C}]\text{6MemTyr}$ for DAS (B and F) and $[^{11}\text{C}]\text{Raclopride}$ for D_2R (C and G). V_T images of $[^{18}\text{F}]\text{BCPP-EF}$ (D and H) were created using a Logan graphical plot analysis with metabolite-corrected plasma input. BP_{ND} images of $[^{11}\text{C}]\text{PE}2\text{I}$ (A and E) and ^{11}C -Raclopride (C and G) and multiple-time graphical analysis Ki images of $[^{11}\text{C}]\text{6MemTyr}$ (B and F) were created using the corresponding TACs in the cerebellum as input functions.

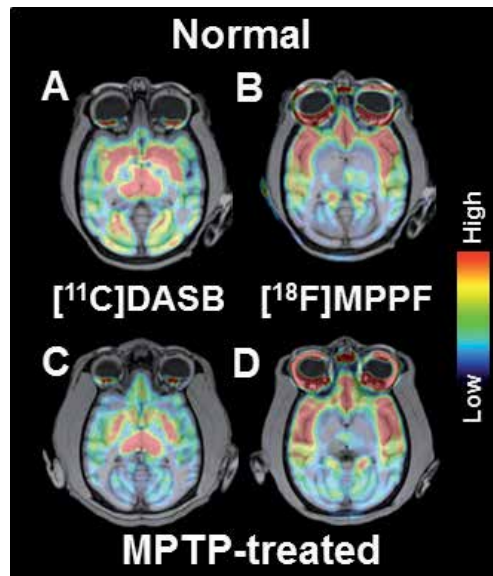


Figure 6. Effects of the MPTP treatment on PET images of 5-HT in living brains of conscious monkeys. PET scans were conducted for 91 min with $[^{11}\text{C}]\text{DASB}$ for SERT (A and C) and $[^{18}\text{F}]\text{FMPPF}$ for $5\text{-HT}_{1\text{A}}\text{R}$ (B and D). BP_{ND} images of $[^{11}\text{C}]\text{DASB}$ (A and C) and $[^{18}\text{F}]\text{MPPF}$ (B and D) were created using the corresponding TACs in the cerebellum as input functions.

the nigrostriatal and cortical (frontal, occipital, temporal, and parietal cortices) regions were plotted against those in MC-I measured using [^{18}F]BCPP-EF. Positive correlations were observed between the $\Delta\text{BP}_{\text{ND}}$ of [^{11}C]PE2I and $\Delta\text{V}_{\text{T}}$ of [^{18}F]BCPP-EF and between ΔKi of [^{11}C]6MemTyr and $\Delta\text{V}_{\text{T}}$ of [^{18}F]BCPP-EF in the nigrostriatal regions. In addition to the DA system, a positive correlation was noted between the $\Delta\text{BP}_{\text{ND}}$ of [^{11}C]DASB and $\Delta\text{V}_{\text{T}}$ of [^{18}F]BCPP-EF in the cortical regions, while no correlation was found between $\Delta\text{BP}_{\text{ND}}$ of [^{11}C]DASB and $\Delta\text{V}_{\text{T}}$ of [^{18}F]BCPP-EF in the nigrostriatal regions.

4.3 Discussion

The administration of MPTP causes the slow progressive loss of DA neurons over a period of several months, and the decrease in nigrostriatal DA levels is responsible for the motor symptoms of MPTP-treated animals, resembling clinical symptoms in PD patient. Thus, impaired ETC for OXPHOS due to a MC-I deficiency may account for neuronal cell death in PD. Alternatively, the MC-I deficiency in nigrostriatal DA system of PD patients may be secondary to mitochondrial damage due to oxidative stress. MC-I is a site of ROS production and is particularly vulnerable to oxidative damage [57]. The results of the present study showing a deficiency in presynaptic DA activity in the nigrostriatal pathway were consistent with the conventional theory shown above.

In contrast, the present study applying [^{18}F]BCPP-EF, a novel PET probe for MC-I, demonstrated that systemic and chronic MPTP treatments induced neuronal damage not only in the nigrostriatal DA pathway but also in the 5-HT neuronal system in the cortex of the monkey brain. Recent clinical studies suggested that in addition to motor symptoms, which are exclusively related to the nigrostriatal DA system, PD is a disease associated with non-motor symptoms, such as depression and cognitive deficits, which may be related to changes in other monoamines (noradrenaline and serotonin) in extra-striatal regions [58]. Lewy body and neurite deposition, a pathological hallmark of PD, occurs within the raphe nucleus containing 5-HT neurons of the caudal brainstem [59]. Decreases in 5-HT concentrations have been reported in the cortical regions of the postmortem brains of PD patients [60]. Furthermore, PET imaging on non-depressed PD patients revealed diffuse reductions in SERT availability throughout the brain [61] and decreased 5-HT $_{1A}$ R binding in the brains of non-depressed and depressed PD patients [62]. The present results obtained in MPTP-treated monkeys were consistent with the recent clinical observations on 5-HT abnormalities in PD patients, which cannot be diagnosed by PET measurements of dopaminergic parameters only.

5. Conclusion

This chapter introduced the potential of [^{18}F]BCPP-EF as a PET probe for the noninvasive and quantitative imaging of MC-I activity in the living brain. The detectability of MC-I impairments using [^{18}F]BCPP-EF for neuronal damage in AD appears to be superior to the assessment of glucose metabolism measured using conventional [^{18}F]FDG. Furthermore, impaired serotonergic neuronal function in cortical regions suggests a relationship with depressive syndrome in PD patients. PET imaging of mitochondria function using [^{18}F]BCPP-EF will provide novel insights into the pathology, diagnosis, and treatment efficacy assessments of a wide range of neurodegenerative diseases, including AD, PD, HD, ALS, and MS.

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Conflict of interest


All authors are employees of Hamamatsu Photonics K.K., and this research was supported by the company's budget. The authors declare that they have no competing interests.

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Section 4

Mitochondrial Function
and Dysfunction

Mitochondrial Proteomic and Molecular Network Alterations in Human Ovarian Cancers

Xianquan Zhan and Na Li

Abstract

Mitochondrion is a multi-functional organelle, which plays important role in human ovarian cancers. Mitochondrial quantitative proteomics was used to detect, identify, and quantify proteins from mitochondrial samples prepared from ovarian cancer and normal control ovary tissues. A total of 5115 mitochondrial proteins and 1198 mitochondrial differentially expressed proteins (mtDEPs) were identified in human ovarian cancer compared to control tissues. Pathway network analysis revealed multiple pathway network changes to involve those mitochondrial proteins and mtDEPs. These findings provide the scientific data about the role of mitochondria plays in ovarian cancer, and offer the source for discovery of mitochondrial biomarker for ovarian cancers.

Keywords: mitochondrial proteome, proteomics, molecular networks, biomarker, ovarian cancer

1. Introduction

Mitochondrion is a multi-functional organelle, which is the center of cell energy metabolism, cell signaling, and oxidative stress [1, 2]. Mitochondrial dysfunction is a hallmark in human ovarian cancers, and plays important roles in ovarian carcinogenesis, which has been looked as the cause, biomarker, and therapeutic target for ovarian cancers [3–5]. First, a study finds mitochondrial morphology is significantly changed in ovarian cancers compared to controls. Electron microscopy morphology study shows that mitochondria are abundant and large volume in ovarian cancer cells and tissues [6, 7]. Second, mitochondrial ribosomal protein-encoding genes might be the anti-oncogenes to serve as new biomarkers and therapeutic targets. For example, bcl-2-interacting mitochondrial ribosomal protein L41 (MRPL41) is differentially expressed in carcinomas to associate with various epigenetic states [8]. Mitochondrial ribosomal protein S23 (MRPS23) is involved in cancer cell proliferation, which might serve as the therapeutic target [9]. MRPS15 is significantly upregulated in epithelial breast cells and tissues [10]. Mitochondrial COX1 is expressed abnormally in multiple cancers [11–13]. Many cancer-relevant communication signaling pathways are linked to mitochondrial proteins. Third, mitochondria are the center of oxidative stress, which might be the ‘fuel’ center for a cancer metabolism [10]. The abnormal energy metabolism, namely the Warburg and reverse-Warburg effects, is the important characteristics in cancers [14].

Therefore, mitochondria play important roles in tumorigenesis, proliferation, angiogenesis, invasiveness, and metastasis of cancer cells [14, 15]. Proteins are the important performer in maintaining mitochondrial morphology and functions. It emphasizes the important scientific merits of mitochondrial proteomics in ovarian cancer research and clinical practice [16–22]. Mitochondrial proteins function in mutually interacted molecular pathway network system, which fits the real situation of ovarian cancer that is a multi-cause, multi-process, and multi-result disease [23–25]. It is very difficult to use single-parameter biomarker to predict, diagnose, and prognostic assess ovarian cancer, thus multi-parameter biomarkers or molecule pattern biomarker is necessary for ovarian cancer prediction, prevention, and treatment [26, 27]. Mitochondrial proteomics is an effective approach to systematically investigate the role of mitochondria in ovarian cancer for discovery of reliable mitochondrial protein biomarkers to insight into the molecular mechanism and determination of therapeutic target to mitochondria for ovarian cancers. Quantitative proteomic methods commonly include two-dimensional gel electrophoresis (2DGE) [28, 29] or two-dimensional difference in-gel electrophoresis (2D DIGE) [30] comparative proteomics, and gel-free-based quantitative proteomics [14, 15], for example, isobaric tags for relative and absolute quantification (iTRAQ) [31, 32], tandem mass tag (TMT) [33], or label-free-based quantitative proteomics [34, 35], with different advantages and disadvantages, respectively. Those quantitative proteomic methods can achieve a high-throughput and high-sensitive identification of mitochondrial proteins and post-translational modifications. Currently, stable isotopic labeled large-scale 2DGE coupled with high-sensitivity liquid chromatography-tandem mass spectrometry (LC-MS/MS) is able to detect, identify, and quantify up to least 500,000 protein proteoforms in human tissue proteoforms [36, 37]. iTRAQ, TMT, or label-free is commonly coupled with two-dimensional LC-MS/MS (2DLC-MS/MS), which enables detect, identify, and quantify up to several thousands of proteins and PTMs, even though these gel-free methods are unable to discriminate proteoforms and homolog proteins [38].

Ovarian cancer is a malignant cancer with high morbidity and mortality [39, 40] and without clear molecular mechanisms and effectively reliable biomarkers for its early-stage diagnosis to improve its prognosis. This book chapter used iTRAQ-labeled strong cation exchange chromatography (SCX)-LC-MS/MS method to detect, identify, and quantify mitochondrial proteins and mitochondrial differentially expressed proteins (mtDEPs) between human ovarian cancer and control ovary tissues. The identified mitochondrial proteins and differentially expressed proteins were subject to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway network analysis for revealing pathway network alteration in ovarian cancers compared to controls. Those findings provide the scientific data to establish mitochondrial proteomic reference map of ovarian cancer, mtDEP profile and the corresponding pathway network alterations to link with ovarian cancer pathogenesis, which is the resource for discovery of potential biomarkers and mitochondria-targeting drug targets for ovarian cancers.

2. Methods

2.1 Ovarian cancer tissues and preparation of mitochondria protein samples

Seven ovarian cancer tissues and eleven control ovaries with benign gynecologic disease were used in this study. Mitochondria were isolated and purified from ovarian cancer and control tissues with differential-speed centrifugation and

Nycodenz density gradient centrifugation. The purified mitochondria were verified with electron microscopy, and Western blot with different antibodies specific to different subcellular organelles, including COX4I1 (mitochondrion), flotillin-1 (cytomembrane), GM130 (Golgi apparatus), catalase (peroxisomes), cathepsin B (lysosome), and lamin B (cell nucleus). The proteins were extracted from purified mitochondrial samples for iTRAQ-labeled quantitative proteomic analysis. The detailed procedure was described in our previous publications [14, 15].

2.2 iTRAQ-based quantitative proteomics analysis

The prepared mitochondrial proteins (200 µg/each sample) were treated with *N*-hydroxysuccinimide (SDT), followed by reduction, alkylation, digestion with trypsin, and desalination. The tryptic peptide (100 µg/each sample) was labeled with iTRAQ reagent, and each sample was labeled three times. The six labeled tryptic peptide samples were mixed, followed by peptide fractionation with strong cation exchange (SCX) chromatography. Each SCX-fractionated sample was subject to LC-MS/MS analysis on a Q Exactive mass spectrometer (Thermo Scientific) within a 60-min LC separation gradient to obtain MS/MS data. The MS/MS data were used for identity of proteins with MASCOT search engine. The iTRAQ reporter-ion intensities were used to quantify each protein and determine each mtDEPs. The detailed procedure was described in our previous publications [14, 15].

2.3 Bioinformatics and pathway network analysis

The identified proteins and DEPs in mitochondrial samples were subject to GO and KEGG pathway enrichment analysis with Cytoscape, and DAVID online software (<https://david.ncifcrf.gov/home.jsp>). Multiple Experiment Viewer (<https://sourceforge.net/projects/mev-tm4/files/mev-tm4/>) was used to make heat map. GO analysis included cellular component (CC), molecular function (MF), and biological process (BP). PANTHER (<http://www.pantherdb.org/>) was used to further enrich GO CC.

2.4 Validation of mtDEPs and molecular networks in cell models and mitochondrial tissues

Ovarian cancer cells TOV-21G and control cells IOSE80 were used to extract RNAs and proteins. Quantitative real-time PCR (qRT-PCR) was used to measure the mRNA expression levels of GLDC, PCK2, IDH2, CPT2 and HMGCS2 in TOV-21G cells compared to IOSE80 cells. Western blot was used to measure the protein expression levels of GLDC, PCK2, IDH2, CPT2 and HMGCS2 in TOV-21G cells compared to IOSE80 cells, and in ovarian cancer mitochondrial samples compared to control mitochondrial samples; and β -actin was used as internal standard for Western blot analysis.

2.5 Statistical analysis

For GO and KEGG enrichment analyses, *p* values were corrected with Benjamini-Hochberg (FDR) for multiple testing. For qRT-PCR and Western blot, the student's *t*-test was used to measure between-group difference with SPSS software 13.0, and data was presented as the mean \pm SD with *p* < 0.05. Each experiment was repeated at least three times.

3. Results and discussion

3.1 Mitochondrial proteomic profile in human ovarian cancer tissue

iTRAQ-labeling coupled with SCX-LC-MS/MS identified 5115 proteins in mitochondrial samples prepared from human ovarian cancer and control ovary tissues, with at least one peptide sequence matches (PSMs). All of identified proteins was collected in the supplemental Table 1 in our previous publication [15]. Those 5115 proteins mainly distributed within pI 3.81–12.25 and molecular weight (MW) 2.6–1158.2 kDa, and in multiple cell components including cell junction (0.8%), cell part (42.7%), extracellular matrix (0.6%), macromolecular complex (17.8%), organelle (28.2%), and synapse (0.3%) (**Figure 1**). Of them, 2565 (50.14%) were increased, and 2550 (49.86%) were decreased in the abundance in ovarian cancers compared to control ovaries. Furthermore, statistical significance analysis revealed 1198 mtDEPs in human ovarian cancers compared to control ovaries, including 523 (43.66%) upregulated proteins and 675 (56.34%) downregulated proteins, with fold-change ≥ 1.5 or ≤ -1.5 , and $p < 0.05$. Those 1198 mtDEPs were collected in the supplemental Table 1 in our previous publication [14]. Those mtDEPs might be directly linked to ovarian cancer pathogenesis, and the potential resource for biomarkers. From a systemic molecular network angle, one must realize that those non-significant difference proteins might be also important in ovarian cancer pathogenesis because they might be the hub-molecule in a network, because some studies have found that some hub-molecules changed smaller than those boundary molecules in a molecular network in a given condition.

3.2 Pathway networks involved in mitochondrial proteins in ovarian cancer

KEGG pathway network analysis revealed 52 statistically significant pathways to involve mitochondrial proteins including mtDEPs in ovarian cancers compared to

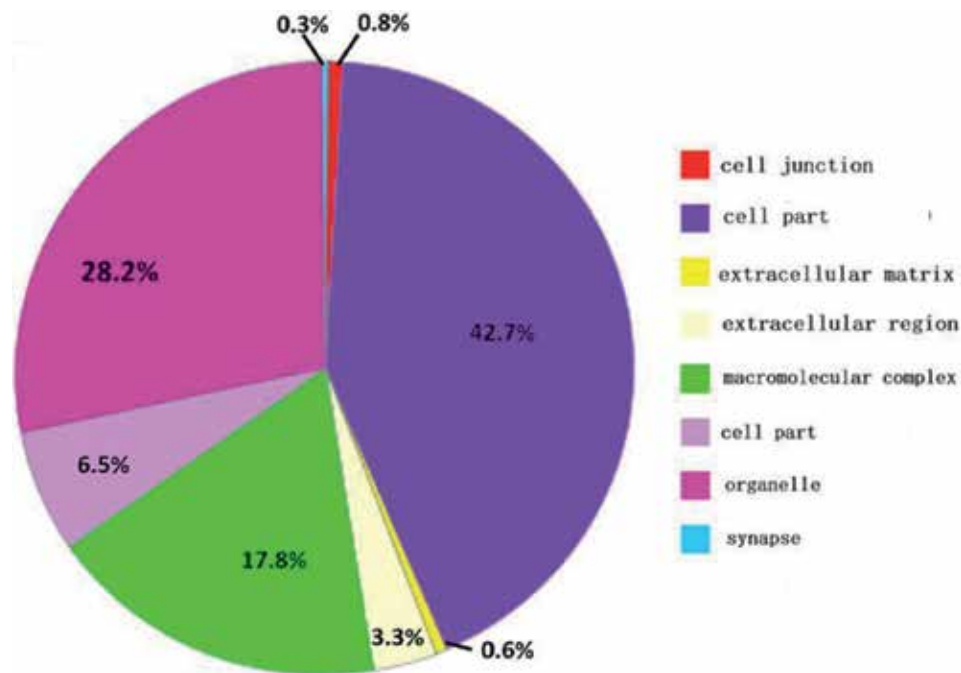


Figure 1. Subcellular location of 5115 proteins analyzed with PANTHER. Modified from Li et al. [15], with permission from Bioscientifica Ltd., copyright 2018.

Category	Term	RT	Count	%	P-value	Benjamini
KEGG_PATHWAY	Lysosome	RT	52	1.3	3.70E-02	2.00E-01
KEGG_PATHWAY	Peroxisome	RT	53	1.5	8.00E-08	4.60E-06
KEGG_PATHWAY	Valine, leucine and isoleucine degradation	RT	41	1.0	1.10E-07	5.50E-06
KEGG_PATHWAY	Phagosome	RT	77	2.1	1.20E-05	2.90E-04
KEGG_PATHWAY	Citrate cycle (TCA cycle)	RT	19	0.8	1.80E-07	7.50E-06
KEGG_PATHWAY	Oxidative phosphorylation	RT	94	2.0	3.40E-07	1.10E-05
KEGG_PATHWAY	Glycolysis/ Gluconeogenesis	RT	33	0.8	1.60E-02	1.20E-01
KEGG_PATHWAY	Fatty acid metabolism	RT	29	0.8	1.90E-03	2.20E-02
KEGG_PATHWAY	Prion diseases	RT	14	0.6	2.20E-03	2.40E-02
KEGG_PATHWAY	Propanoate metabolism	RT	13	0.5	1.40E-03	1.60E-02
KEGG_PATHWAY	Sulfur metabolism	RT	7	0.3	2.90E-03	3.10E-02
KEGG_PATHWAY	Pyruvate metabolism	RT	15	0.6	5.30E-03	4.90E-02
KEGG_PATHWAY	beta-Alanine metabolism	RT	11	0.5	3.10E-02	2.00E-01
KEGG_PATHWAY	Butanoate metabolism	RT	10	0.4	3.30E-02	2.00E-01
KEGG_PATHWAY	Tryptophan metabolism	RT	13	0.5	3.30E-02	2.00E-01
KEGG_PATHWAY	Arginine and proline metabolism	RT	15	0.6	4.00E-02	2.10E-01
KEGG_PATHWAY	Metabolic pathways	RT	524	12.6	1.30E-12	1.80E-10
KEGG_PATHWAY	Carbon metabolism	RT	75	2.2	3.80E-12	3.70E-10
KEGG_PATHWAY	2-Oxocarboxylic acid metabolism	RT	9	0.4	4.30E-03	4.30E-02
KEGG_PATHWAY	Glutathione metabolism	RT	33	0.9	4.90E-05	1.00E-03
KEGG_PATHWAY	Glyoxylate and dicarboxylate metabolism	RT	15	0.6	4.20E-05	9.40E-04
KEGG_PATHWAY	Porphyrin and chlorophyll metabolism	RT	14	0.6	2.10E-02	1.50E-01
KEGG_PATHWAY	Ribosome	RT	110	3.0	3.00E-20	8.80E-18
KEGG_PATHWAY	Biosynthesis of antibiotics	RT	124	3.2	3.50E-11	2.60E-09
KEGG_PATHWAY	Aminoacyl-tRNA biosynthesis	RT	24	1.0	4.30E-04	6.60E-03

Category	Term	RT	Count	%	P-value	Benjamini
KEGG_PATHWAY	Biosynthesis of amino acids	RT	41	1.0	1.10E-03	1.50E-02
KEGG_PATHWAY	Terpenoid backbone biosynthesis	RT	10	0.4	7.80E-03	6.60E-02
KEGG_PATHWAY	Proteasome	RT	30	0.6	3.10E-02	2.00E-01
KEGG_PATHWAY	Protein digestion and absorption	RT	24	1.0	2.30E-02	1.60E-01
KEGG_PATHWAY	Fatty acid degradation	RT	27	0.7	5.30E-03	5.00E-02
KEGG_PATHWAY	Protein processing in endoplasmic reticulum	RT	86	2.4	3.20E-07	1.10E-05
KEGG_PATHWAY	PPAR signaling pathway	RT	20	0.8	1.60E-02	1.20E-01
KEGG_PATHWAY	ECM-receptor interaction	RT	46	1.3	2.00E-04	3.20E-03
KEGG_PATHWAY	Pentose phosphate pathway	RT	11	0.5	1.90E-02	1.40E-01
KEGG_PATHWAY	Focal adhesion	RT	88	2.3	1.30E-03	1.70E-02
KEGG_PATHWAY	Protein export	RT	19	0.5	3.00E-03	3.10E-02
KEGG_PATHWAY	Parkinson's disease	RT	97	2.1	1.20E-06	3.30E-05
KEGG_PATHWAY	Alzheimer's disease	RT	99	2.3	3.60E-06	9.40E-05
KEGG_PATHWAY	Huntington's disease	RT	101	2.3	5.30E-05	1.00E-03
KEGG_PATHWAY	Amoebiasis	RT	36	1.5	5.60E-05	1.00E-03
KEGG_PATHWAY	Complement and coagulation cascades	RT	26	1.1	1.20E-04	2.10E-03
KEGG_PATHWAY	Viral myocarditis	RT	21	0.9	9.10E-04	1.30E-02
KEGG_PATHWAY	Cardiac muscle contraction	RT	25	1.0	1.30E-03	1.60E-02
KEGG_PATHWAY	<i>Staphylococcus aureus</i> infection	RT	18	0.8	7.60E-03	6.70E-02
KEGG_PATHWAY	Bacterial invasion of epithelial cells	RT	38	1.0	1.10E-02	9.00E-02
KEGG_PATHWAY	Vasopressin-regulated water reabsorption	RT	15	0.6	1.30E-02	1.10E-01
KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	RT	21	0.9	1.50E-02	1.10E-01
KEGG_PATHWAY	Platelet activation	RT	58	1.3	3.40E-02	2.00E-01

Category	Term	RT	Count	%	P-value	Benjamini
KEGG_PATHWAY	Regulation of actin cytoskeleton	RT	87	2.0	3.50E-02	2.00E-01
KEGG_PATHWAY	Legionellosis	RT	16	0.7	3.70E-02	2.10E-01
KEGG_PATHWAY	Toxoplasmosis	RT	29	1.2	4.50E-02	2.30E-01
KEGG_PATHWAY	Systemic lupus erythematosus	RT	32	1.3	4.90E-02	2.50E-01

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Table 1.
 52 statistically significant KEGG pathways enriched from 5115 proteins in ovarian cancers.

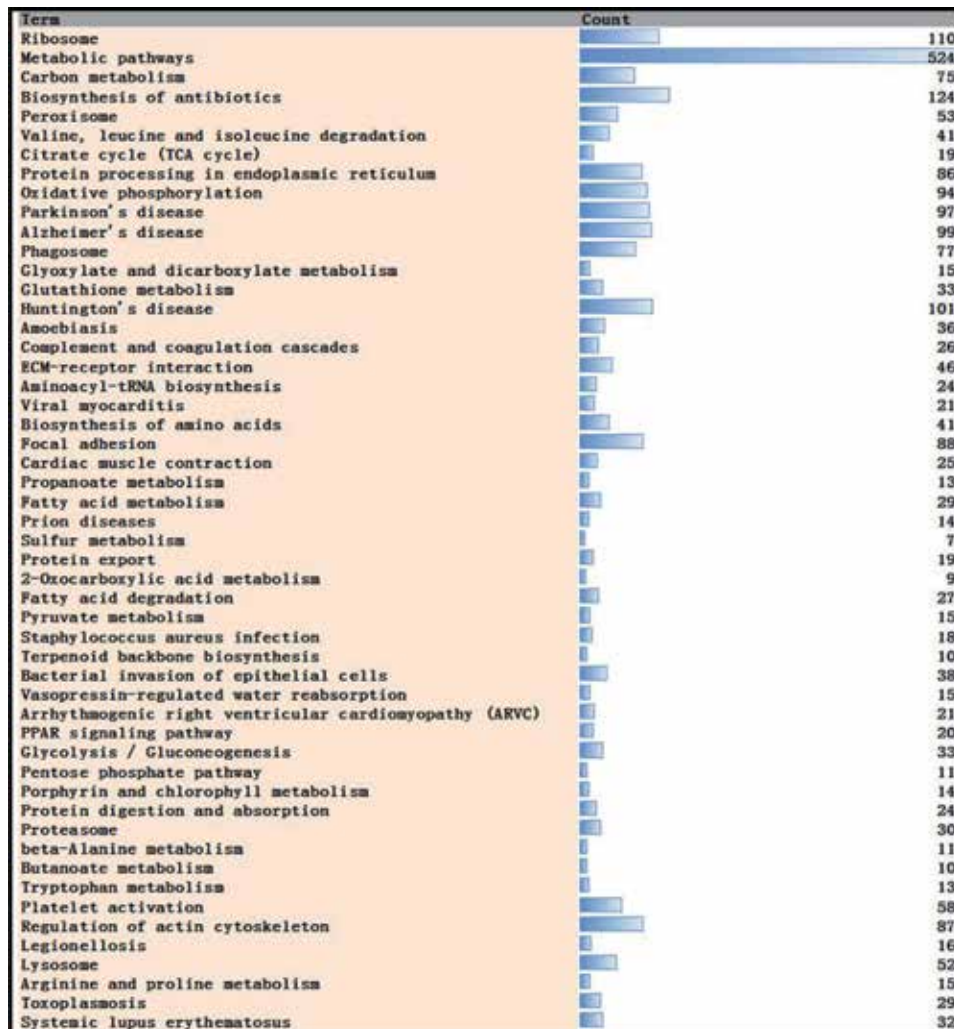


Figure 2.
 52 statistically significant KEGG pathways enriched from 5115 proteins in ovarian cancers. Modified from Li et al. [15], with permission from Bioscientifica Ltd., copyright 2018.

control ovaries (Table 1 and Figure 2), including phagosome, peroxisome, valine, leucine and isoleucine degradation, lysosome, fatty acid metabolism, citrate cycle (TCA cycle), oxidative phosphorylation, glycolysis/gluconeogenesis, metabolic

Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	Ratio (T/N)	t-test p-value
Q8IVP5	FUN14 domain-containing protein 1	FUNDC1	10.97	1	1	1.16	4.82E-2
B4E164	cDNA FLJ56613, highly similar to Serine/threonine-protein kinase TBK1 (EC 2.7.11.1)	TBK1	2.42	1	1	1.25	1.12E-2
O60313	Dynamin-like 120 kDa protein, mitochondrial	OPA1	51.15	44	130	1.19	3.72E-4
Q99623	Prohibitin-2	PHB2	81.61	24	220	1.26	4.44E-4
B4E3V2	cDNA FLJ52854, highly similar to Sequestosome-1	p62	10.47	1	1	1.10	1.96E-1
H0YBC7	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (Fragment)	BNIP3L (NIX)	9.19	1	2	0.77	2.31E-3
A0A0S2Z5I6	Optineurin isoform 3	OPTN	7.94	2	2	0.62	1.01E-2
E7EU96	Casein kinase II subunit alpha	CSNK2A1 (CK)	25.45	6	8	0.84	1.20E-2
Q96HS1	Serine/threonine-protein phosphatase PGAM5, mitochondrial	PGAM5	32.53	10	37	1.49	3.32E-3
B7Z737	cDNA FLJ52784, highly similar to Bcl-2-like 13 protein	Bcl2-L13	13.17	2	2	0.81	3.99E-2

PSMs = peptide sequence matches; MW = molecular weight; Ratio (T/N) = ratio of tumors to normal controls. Reproduced from Li et al. [15], with permission from Bioscientifica Ltd., copyright 2018.

Table 2.

Mitophagy adaptors and regulatory molecules involved the identified proteins in ovarian cancer biological system.

pathways, carbon metabolism, glyoxylate and dicarboxylate metabolism, glutathione metabolism, propanoate metabolism, sulfur metabolism, 2-oxocarboxylic acid metabolism, pyruvate metabolism, porphyrin and chlorophyll metabolism, beta-alanine metabolism, butanoate metabolism, tryptophan metabolism, arginine and proline metabolism, ribosome, protein processing in endoplasmic reticulum, biosynthesis of amino acids, aminoacyl-tRNA biosynthesis, proteasome, protein

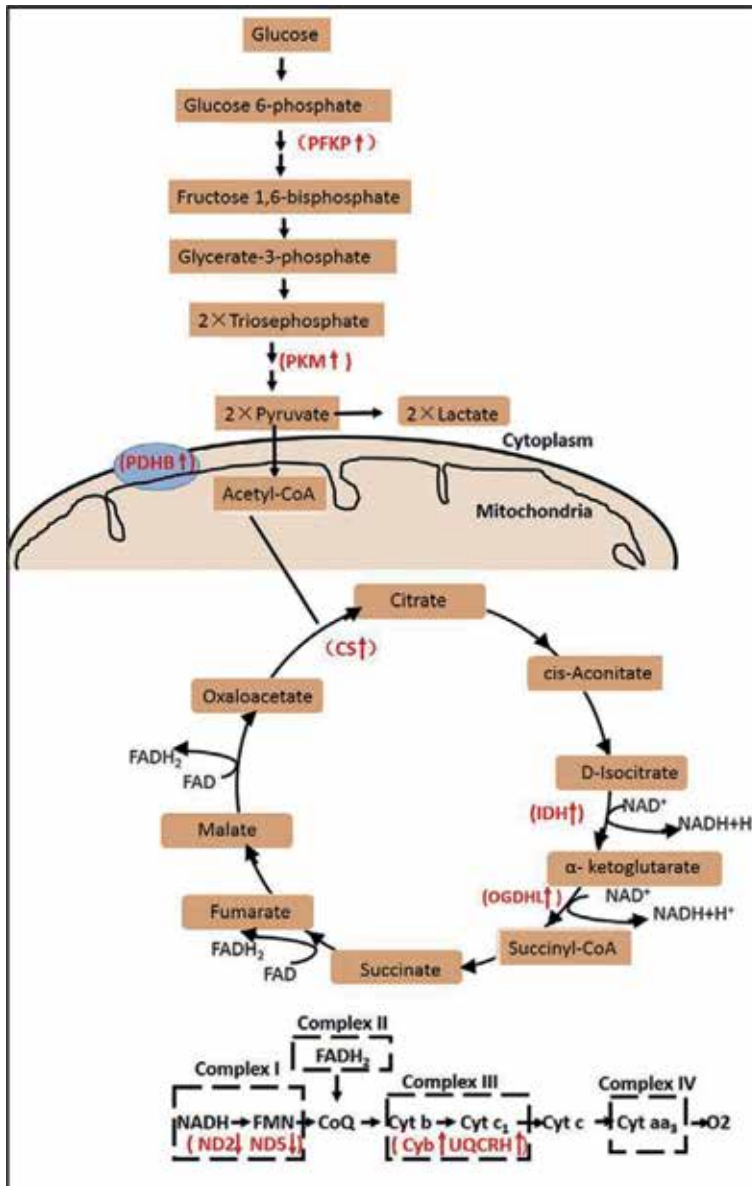


Figure 3. Energy metabolism pathway changed in ovarian cancer. Reproduced from Li et al. [14], with permission from Elsevier Inc., copyright 2018.

digestion and absorption, ECM-receptor interaction, focal adhesion, protein export, signaling pathway, complement and coagulation cascades, platelet activation, PPAR pentose phosphate pathway, fatty acid degradation, vasopressin-regulated water reabsorption, and regulation of actin cytoskeleton. Those pathway systems provided an overall molecular network changes in ovarian cancers, which might be important in ovarian cancer pathogenesis.

Among those altered pathway systems, especially interested is that mitophagy pathway and energy metabolism pathway were significantly changed in ovarian cancers compared to controls. The changed mitophagy pathway in ovarian cancer included phagosome, peroxisome, valine, leucine and isoleucine degradation, lysosome, and fatty acid metabolism pathways [15]. Mitophagy is to engulf any

Accession no.	Protein	Unique peptide	Coverage (%)	PSMs	Ratio (T/N)	p value (t test)
Q01813	Phosphofructokinase, platelet (PFKP)				1.90	2.28E-02
P11177	Pyruvate dehydrogenase E1 component subunit beta (PDHB)	14	52.92	79	1.51	3.25E-03
A0A024R5Z9	Pyruvate kinase (PKM)				2.38	1.50E-04
O43837	Isocitrate dehydrogenase [NAD] subunit beta (IDH3B)	13	41.56	43	1.75	8.69E-03
B4DJV2	Citrate synthase (CS)	13	26.93	73	1.59	4.65E-03
P50213	Isocitrate dehydrogenase [NAD] subunit alpha (IDH3A)	18	47.81	53	1.60	2.27E-02
P48735	Isocitrate dehydrogenase [NADP] (IDH2)	27	56.64	355	2.02	2.07E-03
A0A0A0QN99	Cytochrome b reductase 1 (CYB)	14	4.21	4	1.71	7.60E-03
Q9ULD0	2-oxoglutarate dehydrogenase-like (OGDHL)	13	26.83	58	1.55	1.25E-03
A0A096WB60	NADH-ubiquinone oxidoreductase chain 5 (MT-ND5)	1	5.14	6	0.38	3.34E-04
P07919	Cytochrome b-c1 complex subunit 6 (QCR 6)	5	51.65	18	1.59	1.63E-02
A0A059T3A1	NADH-ubiquinone oxidoreductase chain 2 (MT-ND2)	1	4.61	2	0.38	6.03E-04
P38919	Eukaryotic initiation factor 4A-III (EIF4AIII)	4	11.92	9	0.71	1.48E-02

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Table 3. Differentially expressed glycolysis/Kreb's cycle/mitochondrial respiratory chain/RNA binding proteins in EOC.

material in autophagosome, and subsequently fuses with lysosomes to release high-energy substance such as fatty acid and amino acid. Autophagosome also commonly contains mitochondria, proteins, or peroxisome. Mitophagy processes are involved in autophagy machinery, mitophagy adaptors, and regulatory molecules such as Bcl2-L12, p62, OPTN, prohibitin 2, OPA1, CK, PGAM5, BNIP3L(NIX), and FUNDC1 (Table 2). These findings were consistent with previous studies. The changed energy metabolism pathway in ovarian cancers included citrate cycle (TCA cycle), oxidative phosphorylation, and glycolysis (Figure 3) [14], and the important molecules were significantly changed in three energy metabolism pathways, including PFKM, PKM, PDHB, CS, and IDH2 (Table 3). It clearly demonstrated the Warburg and reverse-Warburg effects coexisted in ovarian cancers.

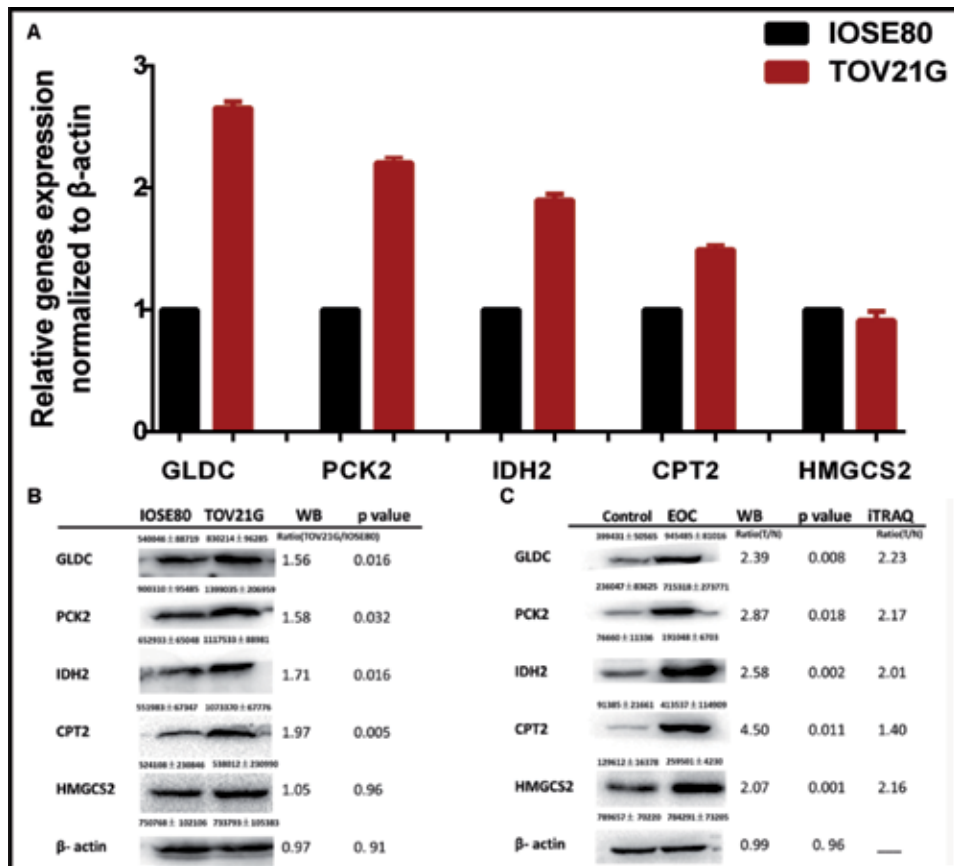


Figure 4. Validation of potential biomarkers (GLDC, PCK2, IDH2, CPT2 and HMGCS2) in ovarian cancer cell model with qRT-PCR (A) and Western blot (B), and in human mitochondrial samples with Western blot (C). β -actin was used as internal standard. Reproduced from Li et al. [15], with permission from Bioscientifica Ltd., copyright 2018.

3.3 Potential biomarkers for ovarian cancers

Those 5115 mitochondrial proteins including 1198 mtDEPs were the resource of potential biomarkers for ovarian cancers. For example, mtDEPs in mitophagy pathway and energy metabolism pathway might be effective biomarkers and therapeutic targets for ovarian cancer. Five mtDEPs, including GLDC, PCK2, and IDH2 in peroxisome pathway, CPT2 in fatty acid degradation pathway, and HMGCS2 in the valine, leucine and isoleucine degradation pathway were further validated by qRT-PCR and Western blot in ovarian cancer cells compared to normal control cells (Figure 4A and B), and by Western blot in the ovarian cancer tissue mitochondrial samples (Figure 4C). These results also confirmed the results of iTRAQ quantitative proteomics.

4. Conclusions

iTRAQ-labeled SCX-LC-MS/MS quantitative proteomics was an effective method to detect, identify, and quantify mitochondrial proteins and mtDEPs in mitochondrial samples prepared from human ovarian cancer and control ovary tissues. Totally 5115 mitochondrial proteins including 1198 mtDEPs were identified in ovarian

cancers, and 52 statistically significant pathways were identified to involve those mtDEPs. More interesting is that this study found mitophagy pathway (phagosome, peroxisome, valine, leucine and isoleucine degradation, lysosome, and fatty acid metabolism), and energy metabolism pathways (citrate cycle, oxidative phosphorylation, and glycolysis) were significantly changed in ovarian cancers. The important molecules Bcl2-L12, p62, OPTN, prohibitin 2, OPA1, CK, PGAM5, BNIP3L(NIX), and FUNDC1 in mitophagy pathway, and PFKM, PKM, PDHB, CS, and IDH2 in energy metabolism pathways were significantly changed. It clearly demonstrated the changed mitophagy and energy metabolism pathways played important roles in ovarian cancers. These findings provide the large-scale proteomic variation profiles and molecular network alterations for ovarian cancer, which are the important scientific data to insight into the roles of mitochondrial dysfunction in ovarian cancer.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Author’s contributions

X.Z. conceived the concept, designed the manuscript, wrote and critically revised the manuscript, coordinated and was responsible for the correspondence work and financial support. N.L. participated in the literature analysis, data analysis, and prepared figures.

Acronyms and abbreviations

GO	gene ontology
iTRAQ	isobaric tags for relative and absolute quantification
KEGG	kyoto encyclopedia of genes and genomes
LC	liquid chromatography
MRPL41	Bcl-2-interacting mitochondrial ribosomal protein L41
MRPS23	mitochondrial ribosomal protein S23
MS/MS	tandem mass spectrometry
PSMs	peptide sequence matches
SCX	strong cation exchange
TMT	tandem mass tag
2DGE	two-dimensional gel electrophoresis
2D DIGE	two-dimensional difference in-gel electrophoresis
2DLC	two-dimensional liquid chromatography


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Mitochondrial Dysfunction as a Key Event during Aging: From Synaptic Failure to Memory Loss

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Abstract

Mitochondria are important cellular organelles with key regulatory functions in energy production, oxidative balance, and calcium homeostasis. This is especially important in the brain, since neurons require a large number of functional mitochondria to supply their high energy requirement, mainly for synaptic processes. A decrease in the activity and quality of mitochondria in the brain, particularly in the hippocampus, is associated with normal aging and a large number of neurodegenerative diseases compromising memory function. Although synaptic and cognitive dysfunction is multifactorial, growing evidence demonstrates that mitochondria play a key role in these processes and suggests that maintaining mitochondrial function could prevent these age-dependent alterations. In this chapter, we will discuss the hippocampal mitochondrial dysfunction present in aging and how these defects promote age-associated synaptic damage and cognitive impairment. We will summarize evidence that shows how neurodegeneration can be accelerated or attenuated during aging by modulating mitochondrial function.

Keywords: aging, mitochondria, oxidative stress, synapses, memory

1. Introduction

Aging is an extensively studied process, identifying a growing interest in how and why cognitive processes are affected from a neurobiological approach [1]. Aging is a multifactorial biological process, characterized by deterioration of physiological and cellular functions including brain function [2], where age is the main risk factor for the development of pathologies such as cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases [3]. Cognitive deterioration occurs during aging, where reasoning, attention, and memory, among other processes, decrease gradually with the age [4]. Cellular senescence and alterations to mitochondria and in proteolytic systems are considered hallmarks of aging [3], where one of the most studied is the mitochondria [5]. In fact, mitochondrial dysfunction has been directly associated with the aging phenotype and the majority of diseases that lead to cognitive damage.

Over the last decades, a great interest has arisen regarding mitochondrial structure and function due to its relation with the aging brain [5]. Mitochondria are organelles essential for energy production, whose size is usually 0.5–1 μm , composed

by two membranes, forming the intermembranous space and the mitochondrial matrix [6]. The outer membrane contains many copies of the transport protein porin (or voltage-dependent anion-selective channels (VDAC)), which allows the passage of molecules with a maximum weight of 5 KDalton (KDa), and the inner membrane forms numerous invaginations, tubular structures, called cristae [6]. Mitochondria are capable of remodeling their architecture through fission and fusion processes, allowing morphological adaptation to different situations [6]. Fission is essential for mitochondrial duplication and is necessary for mitophagy, allowing dysfunctional mitochondrial sections to be recycled. Fusion allows mitochondria to interconnect, allowing damaged mitochondria to maintain their function. However, fission-fusion processes are interrupted during aging, generating damaged mitochondria [7].

Mitochondria have a small circular genome called mtDNA, which encodes 22 tRNAs, 2 mitochondrial rRNAs, and 13 subunits of the electron transport chain (ETC) [8]. mtDNA can be damaged by exposure to reactive oxygen species (ROS), chemical carcinogens, and ionizing radiation affecting the mitochondrial function; changes are also observed during aging [9]. The internal mitochondrial membrane contains the ETC, responsible for generating ATP. ETC is formed by five protein complexes; complex I (NADH dehydrogenase) receives electrons of NADH which pass through the ETC via oxidation-reduction reactions forming an electrochemical gradient that allows the formation of ATP. In addition, FADH₂ donates its electrons to complex II (succinate dehydrogenase) performing the same action for ATP generation but at lower production levels [10]. As a secondary product, the ETC forms ROS, specifically by complexes I and III, but its production is controlled by antioxidant enzymes [11]. Therefore, in normal conditions ROS production is moderate, providing certain physiological roles [11]; however, during aging ROS accumulation causes biological damage known as “oxidative stress” [12].

In the past, mitochondria have always been highlighted for its role in ATP production; however, another key function is to maintain intracellular calcium homeostasis [13]. The outer mitochondrial membrane is permeable to ions and ~5 KDa metabolites because its lipid bilayer has transmembrane proteins that form the mitochondrial permeability transition pore (mPTP). mPTP opening and closing dynamics regulates the concentration of calcium [13]. However, in conditions of high calcium concentrations, permanent mPTP opening generates massive transport of ions and small molecules <1.5 KDa through the membrane, causing increased ROS production, inhibition of the ETC, and mitochondrial swelling, which finally results in the release of pro-apoptotic factors and cell death [14].

In this chapter we will discuss the mitochondrial alterations observed in the brain during aging, focusing on mitochondrial functions including redox balance, bioenergetics, and calcium homeostasis, and its implications in the aging process. In addition, we will discuss the contribution of mitochondrial dysfunction to synaptic failure and cognitive impairment. Finally, we will summarize potential treatments that have been proposed to prevent or attenuate the loss of mitochondrial function that could be used as potential antiaging treatment.

2. Oxidative stress: the main characteristic of normal aging

Aging is a complex process that involves both intrinsic and extrinsic factors [3]. Several researches showed that the reduction of synaptic function during aging could be related to increased oxidative stress and mitochondrial dysfunction [15, 16]. The latter involves decreased production of energy and redox balance, activation of nitric oxide synthase, and an abundant generation of free radicals; meanwhile increased ROS production impairs neuronal function at advanced ages [17].

Mitochondria have a pivotal role in ROS production; they are the main organelle producer of species such as hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and hydroxyl radicals (OH^-) [18]. ROS comprise all molecules derived from oxygen, can exist independently, and contain one or more unpaired electrons in their orbitals [19]. Normally, mitochondria generate ROS as a result of adequate function of the ETC by complexes I and III in OXPHOS to produce ATP. Likewise, electrons that escape mitochondrial ETC can reduce oxygen to form O^{2-} [20]. Additionally, H_2O_2 is more stable than O^{2-} and can diffuse freely through the membranes to the cytosol or nucleus, causing oxidative damage to many cellular compartments [21].

The mitochondrial ROS produced in normal conditions have important physiological roles in maintaining cell homeostasis, participate as signaling molecules, and are also related to the regulation of cell survival [22]. In contrast, excessive ROS production promotes cellular damage [23]. For example, recent evidence suggests that higher mitochondrion-derived ROS result in enhanced formation of $\text{A}\beta$, an effect that is prevented with the use of antioxidants that rescue mitochondrial function in cellular and animal models of Alzheimer's disease (AD) [24]. This suggests that higher ROS generation mediated by mitochondria is involved in early stages of age-associated diseases and during aging [25].

Cells maintain a balance between free radicals by the action of antioxidant molecules, which neutralize or remove them [22]. Cells are equipped with a variety of defense mechanisms to remove ROS, including antioxidant enzymes that facilitate antioxidant reactions and decompose ROS [26]. Among the antioxidant enzymes are glutathione reductase (GR), glutathione peroxidase (GPx), and catalase (CAT). In addition, superoxide dismutases (SODs), such as copper-zinc-superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD), help the dismutation of superoxide radicals to generate H_2O_2 , which is further removed by CAT and GPx enzymes [26]. Altogether, these antioxidant defenses regulate the amount of ROS, preventing accumulation and oxidative stress [27].

Oxidative stress occurs when the antioxidant defense mechanisms are unable to neutralize free radicals in the cell. This imbalance between the production of oxidative molecules and the antioxidant defense leads to an accumulation of ROS, which oxidize and produce damage to lipids, proteins, and DNA molecules. Similarly, ROS could alter many cell compartments, for example, promoting peroxidation of lipid membranes and inactivation of enzymes by oxidation [28].

In oxidative stress conditions, the concentration of ROS increases transiently or chronically, altering the cellular metabolism and its regulation [23]. Interestingly, these elements are implicated in the aging process, the mitochondrial free radical theory of aging (MFRTA) being the most accepted theory to explain the age-associated degeneration [29]. This theory exposed by Harman proposes that mitochondria play a central role in aging and indicates that aging is the product of accumulated damage caused by mitochondrial ROS in the cells and tissues of organisms [30, 31]. Nevertheless, this theory has also been questioned, since aging is a multifactorial biological process and not just the consequence of a unique factor [32]. Thus, the mitochondrial theory of aging is relevant since these organelles are energy sources for cells and coordinate important processes such as apoptosis. During aging, accumulation of mtDNA mutations is increased, the mitochondrial genes related to energy production become progressively less active, and the mitochondria are observed as fragmented, producing less energy [33]. The brain is particularly susceptible to oxidative damage being the most aerobically active organ in the body due to its high metabolism [34]. The brain is generally in a redox balance; however, the high production and accumulation of ROS accompanied by a reduction in the antioxidant defense system plays a key role in aging, causing damaging effects due to the large number of potential harmful intermediates that cause neuronal

dysfunction [3, 35]. In turn, increased oxygen radical-induced oxidative damage during aging leads to significant changes in brain mitochondrial function [29]. Therefore, oxidative stress is implicated in aging and a wide range of age-related pathologies, such as AD and Parkinson's disease (PD), among others [3, 16, 36].

In the aged brain, a reduction in normal antioxidant defense machineries is observed, which increases the brain's susceptibility to the harmful effects of oxidative molecules [27, 37]. In addition to this, mitochondrial dysfunction contributes to ROS overproduction. It is important to emphasize that a decrease in the activity of oxidative enzymes accompanied by excessive production of oxidant molecules during aging is the main toxic mechanism that explains the neurodegeneration observed at an advanced age [27, 37]. Since mitochondria are the main source of ROS production, they are in turn more exposed to oxidative damage at a faster and stronger rate than other organelles and cell compartments [16]. Moreover, the mitochondrial antioxidant system is less active than the antioxidant systems of other organelles, a feature that increases with age [5]. These mitochondrial "defects" can greatly affect several cellular processes that contribute to the aging phenotype [38]. Therefore, age is an important risk factor that increases the susceptibility of mitochondria, making them more vulnerable to oxidative stress, resulting in a vicious cycle of mitochondrial dysfunction and more oxidative damage [5, 33]. Mitochondria should be considered as a key factor in the development of age-related neurodegeneration, and therefore therapeutic strategies such as mitochondrial protectors or antioxidants that improve mitochondrial function could be used to prevent or delay aging.

3. Bioenergetic failure during aging

One of the main functions of mitochondria is energy production in the form of ATP through OXPHOS [39]. The main substrate of neurons is glucose. Through the glycolytic pathway, the cell generates only two ATP molecules per glucose molecule; however in this pathway two pyruvate molecules are produced. These molecules then enter the mitochondria to be oxidized in the tricarboxylic acid (TCA) cycle producing NADH and FADH₂, which in turn enter the ETC to produce high amounts of ATP by OXPHOS [40]. Electrons from NADH and FADH₂ are transferred through four complexes to molecular oxygen, pumping protons to the inter-membrane space, which form a proton gradient that generates the mitochondrial membrane potential ($\Delta\psi_m$). This $\Delta\psi_m$ is fundamental for adequate mitochondrial function, mainly for ATP synthesis by the ATPase complex [39].

Due to the importance of mitochondrial energy production, failures in mitochondrial bioenergetics are related to several neurological diseases such as amyotrophic lateral sclerosis (ALS) [41] and several age-associated neurodegenerative disorders such as AD [42]. A mitochondrial failure can be caused by either a dysfunction in the OXPHOS complexes or by mutations in the mtDNA, which encodes for 13 proteins that makes the different subunits of the ETC complexes. Interestingly, mtDNA mutations produce a group of pathologies known as primary mitochondrial disorders, characterized by neurological alterations. Thus, neurodegeneration is often related to mitochondrial dysfunction as a primary or secondary target, mediating the pathogenic events [43]. Since the main energy source in brain cells is glucose oxidation, the energy obtained from the mitochondrial OXPHOS system is vital to fulfilling their high basal energy requirement, including maintenance of the membrane potential for the propagation of electric signals, reestablishment of the ion balance after the action potential (Na⁺/K⁺ ATPase activity), vesicle recycling, and neurotransmitter release [44].

Therefore, a deficit in ATP production can lead to neuronal damage and finally cell death, producing diverse defects in brain functions as occurs during normal aging, considered an important risk to the development of neurodegenerative disease [45]. An example of this is the glutamate-glutamine cycle, an essential process for the release of glutamate from the presynaptic terminal. Glutamate uptake occurs in astrocytes, where it is converted to glutamine and then transferred back to glutamatergic neurons. For these processes to occur, the neuron requires ATP [46], as well as for the accumulation of glutamate in synaptic vesicles [47]. Therefore lower ATP levels will result in reduced glutamate release leading to decreased excitatory synapsis and consequently decreased synaptic plasticity as a result of altered long-term potentiation (LTP) and long-term depression (LTD) [48].

In addition, AD is a neurodegenerative pathology where mitochondrial deficiency is observed in oxidative phosphorylation, with defective OXPHOS enzymes [49]. Several studies showed decreased cytochrome c oxidase activity but an increase in mitochondrial mRNA for complex IV, which may be a compensatory response for the reduced cytochrome c oxidase activity [50, 51]. Also, there is a decrease in NADH dehydrogenase expression and an increase in complex III mRNA in AD patients [51]. These defects in the OXPHOS complex impede correct ATP production and increase ROS production [52], which could generate damage to mitochondrial proteins, activate the mPTP, and mutagenize the mtDNA, leading to defective OXPHOS. All these mitochondrial defects can ultimately contribute to the characteristic synapse loss in the neocortex and hippocampus of AD patients [53], which correlates with cognitive impairment and memory loss. Similarly, critical mitochondrial dysfunction has been associated with PD. Several mutations in proteins that can directly or indirectly regulate mitochondrial activity and morphology have been described. Examples of this are PTEN-induced kinase 1 (PINK1) which induced mitochondrial autophagy during stress [54] and protein deglycase (DJ-1) which is a multifunctional protein that reacts against anti-oxidative stress [55]. These two proteins are localized in the mitochondria, while parkin, another protein that degrades dysfunctional mitochondria, translocates to damaged mitochondria [54]. The first evidence that mitochondria could be related to PD was published by Langston et al. in 1983 where they showed that the mitochondrial complex I inhibitor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause acute and irreversible parkinsonian symptoms in humans [56]. Later, other mitochondrial toxins such as rotenone, which induces similar symptoms to MPTP, were described, leading to the development of a rotenone rodent model of Parkinson's disease [57]. Other studies of postmortem tissue from PD patients showed defects in complex I, NADH dehydrogenase in the substantia nigra [58].

Interestingly, similar bioenergetic deficits present in neurodegenerative disease are also seen in normal aging. In this natural process, it is well known that a large number of mutations accumulate in the mtDNA in different tissues, such as the brain and the muscle, the tissues with most accumulation of mutations, possibly due to the higher energetic demand [59]. For example, in a study performed with humans between 49 and 92 years old, they showed an increase by 25% in the muscle fibers that exhibit abnormalities in mitochondrial ETC in 92-year-old individuals compared to 49 year olds [60]. Another study showed that a mouse with a deficiency for mitochondrial DNA polymerase γ (POLG) had an impaired proof-reading ability, accumulated mtDNA mutations, and presented a premature-aged phenotype (hair loss, graying, kyphosis, reduced survival percentage, loss of bone mass, etc.) at the age of 9 months [61]. These mutations can lead to mitochondrial dysfunction associated with a deficient respiratory chain and a decrease in ATP production [62]. There is evidence that the brain of aged mice (26 months old) contains mutations in protein-coding regions that result in significant changes

in the complex I subunit ND5 and complex III subunit CytB [62]. These mutations may limit correct assembly of these complexes, which correlates with their decreased activity during aging [62]. Other studies also showed downregulation of several genes coding for mitochondrial proteins in heart tissue, which correlated with a significant decrease in the respiratory capacity of mitochondria to oxidize substrates [63]. In liver tissue, there is a decrease in the respiratory control ratio and in ADP/oxygen (an index of ATP synthesis efficiency) in senescence-accelerated mice (SAMP8) mitochondria [64], suggesting that at 18 months of age, there is insufficient ATP for normal cell metabolism, which may be due to a dysfunctional energy transfer mechanism.

In the brain, it is widely known that with aging there is a decrease in the electron transfer activity accompanied by a decrease mainly in complexes I and IV [65, 66]. Several studies showed that complex IV activity is decreased in substantia nigra, hippocampal dentate gyrus, frontal cortex, and cerebellum during aging [67, 68]. A study performed with aged CD1 Swiss mice showed that NADH-cytochrome c reductase (complexes I and III) activity is the most affected during aging, decreasing by 48% in old mice (18 months), while succinate-cytochrome c reductase (complexes II and III) remain unmodified with age, indicating selective impairment of NADH dehydrogenase activity (complex I) during normal aging [69]. Likewise, cytochrome oxidase (complex IV) activity is decreased by 13% in old animals [69]. Additionally, there is evidence of increased expression of mitochondrial genes for complexes I, III, IV, and V in 18-month-old mice in the hippocampus, medial prefrontal cortex, and striatum [70], suggesting a compensatory mechanism that could induce overproduction of ETC proteins. However, this increased mRNA expression is not sustained over time, since 24-month-old mice have decreased expression of ETC complexes [70].

Another parameter that is altered during aging is the $\Delta\psi_m$ due to increased H^+ permeability of the inner mitochondrial membrane and a consequent failure in maintaining the H^+ electrochemical gradient [71]. There is evidence that there is a decrease in the membrane potential in the cortical and striatal mitochondria of 33-month-old rats [72]. In the same way, a study in primary cultures of glial cells from the brain of young (4–6 months) and old (26–29 months) mice shows a decay in the $\Delta\psi_m$ in astrocytes [73].

Thus, mitochondrial bioenergetic failure is a hallmark of different diseases including neurodegenerative disorders. Interestingly, these same patterns of decreased ATP production, OXPHOS failure, and depolarization of the mitochondrial membrane are seen during aging, a natural process of everybody's life. This makes the mitochondria an important player in all neurological degeneration related with aging, such as synaptic failure and cognitive impairment.

4. Age-associated calcium dysregulation

Calcium (Ca^{+2}) is an ion that participates in a wide variety of functions in the cells of organisms, being an intracellular regulator of many physiological processes [74]. Intracellular calcium signals participate in the regulation of a large number of processes, which include gene expression, cell cycle stages, control of muscle contraction, autophagy, and cell death, among other functions, being a second intracellular messenger [74, 75].

In the central nervous system (CNS), Ca^{+2} plays a very important role in the neuronal synapse, mainly promoting exocytosis of the synaptic vesicles in the presynaptic region, meanwhile in the postsynaptic site is important for regulating the morphology of dendritic spines and spinogenesis [76, 77]. Calcium homeostasis

is fundamental for correct cellular function, and the mitochondria are structures important for maintaining the intracellular calcium concentrations [78]. They participate in the local regulation of cellular Ca^{+2} homeostasis, since it captures Ca^{+2} from the cytosol in response to ion fluxes through channels in both the inner and outer plasma membranes or by release of Ca^{+2} from the endoplasmic reticulum (ER) [79]. Thus, when the cytosolic concentration of calcium increases, mitochondria capture and accumulate large amounts of the ion in order to control intracellular concentrations. Therefore, for appropriate neuronal functioning, adequate parameters of intracellular calcium concentrations must be maintained [80]. Interestingly, aged brain neurons are incapable of regulating intracellular calcium, mainly due to dysfunctional mitochondria and increased oxidative stress [78, 81].

Mitochondrial dysfunction substantially contributes to biological aging [78]. In aging, oxidative stress affects mitochondrial function and, therefore, its role in Ca^{+2} homeostasis [78, 82]. When calcium homeostasis is altered, it has a detrimental role on the aging brain and is also associated with the development of neurodegenerative diseases [83]. Several studies showed that an increase of the Ca^{+2} affects synaptic communication, neurotransmitter release, and signal transduction, all this generating excitotoxicity and neuronal loss [84]. In addition, these alterations could also contribute to memory impairment [16, 84].

In this way, since the aging is associated with a marked cognitive decline, the calcium imbalance hypothesis proposed by Khachaturian turns out to be well accepted [85, 86]. This hypothesis proposes that changes in calcium regulation gradually modulate normal brain aging and, at the same time, increase their vulnerability to neurodegenerative diseases such as AD [87]. Calcium signaling depends of the transient elevation of its intracellular concentration. In brain cells, reduced regulation of calcium homeostasis is an early event during aging, altering multiple signaling pathways and affecting various molecular and cellular functions [88].

Due to their high buffering capacity, mitochondria are an essential component for maintaining calcium homeostasis, due to their involvement in the regulation of intracellular calcium signaling [89]. Also, other mitochondrial characteristics that facilitate its role in the regulation of calcium signaling is its structural plasticity produced by fusion and fission processes in the mitochondrial network, as well as its distribution within the neuron [90]. Aging affects mitochondrial dynamics leading to mitochondrial fragmentation and alterations to these functions [90]. Studies in mitochondria isolated from the cortex of aged animals exhibited more ROS production and mitochondrial swelling after increased Ca^{+2} loading than that of young animals [78]. Therefore, these findings suggest that the aging increased the sensitivity of the mitochondria to calcium overload, generating mitochondrial swelling [81]. Mitochondrial swelling results in the opening of the mPTP [91], and in aged animals, mPTP opening occurs prematurely, indicating reduced Ca^{+2} buffering during aging [78, 81].

The mPTP is a large nonselective channel located in the inner mitochondrial membrane and communicates the mitochondrial matrix directly with the cytoplasm [81]. Their opening is activated by Ca^{+2} , phosphate, ROS, increased pH, and magnesium (Mg^{+2}) [92]. Transitory opening of mPTP allows the release of excessive calcium ions that accumulate in the mitochondria, but prolonged opening leads to the movement of ions and small molecules generating depolarization of the mitochondrial membrane and in turn releasing pro-apoptotic factors, which results in a reduction of ATP and finally causes cell death [81, 91].

The structure of mPTP is not completely clear. Experimental approaches have distinguished several protein components such as VDAC, the adenine nucleotide translocase protein (ANT), and the mitochondrial matrix protein cyclophilin D (Cyp-D) [91]. Recent research incorporates the F1FO subunits of ATP synthase, a

key enzyme of the OXPHOS complex, which participates in ATP production and maintenance of the membrane potential [91]. Interestingly, deregulation of this enzyme associated with aging has been reported, showing decreased expression of OSCP and in F1FO ATP synthase activity [81, 91]. These changes have also been observed in imaged brains that present age-related neurodegenerative pathologies [81, 91]. Cyp-D is a specific mitochondrial protein and generally considered to be a critical component of mPTP formation [91]. Several studies indicate that Cyp-D is the most important component facilitating mPTP formation, thus leading to decreased ATP production, increasing ROS generation, and eventually causing cell death [81, 91], although it is not yet completely clear how Cyp-D triggers mPTP formation [92]. The opening of mPTP dissipates $\Delta\psi_m$, uncoupling the mitochondria and causing swelling [91]. The expression of Cyp-D increases with age and is related to several age-associated neurological diseases such as AD [91, 93]. For example, Gauba et al. have reported that Cyp-D promotes the dysfunction of ATP synthase F1FO, in the mitochondria of aged brains, observing a significant increase in the expression of this protein with age [91]. In contrast, it has been observed that deletion of Cyp-D improves cognitive and mitochondrial functions in both aging and in neurodegenerative diseases [91, 93].

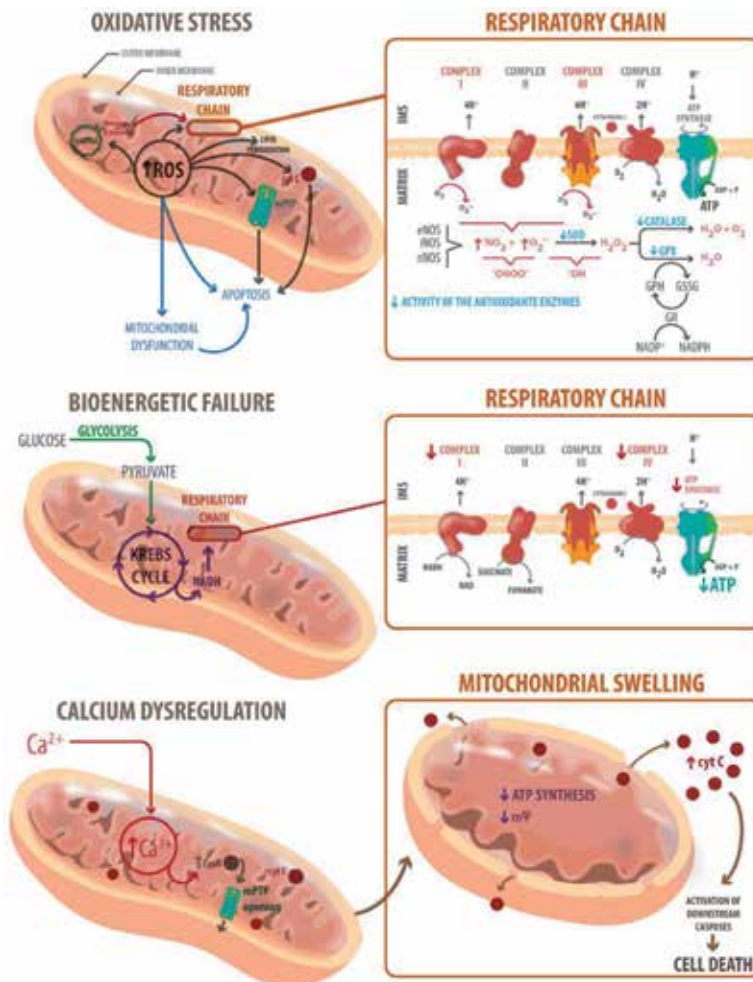


Figure 1.
The mitochondrial functions are impaired during aging.

The increased life expectancy and the high incidence of neurodegenerative diseases require a better understanding of the aging processes and the mechanisms associated with it. Thus, comprehension of the interactions between calcium homeostasis and calcium-dependent processes during aging can help in the design of more effective therapeutic strategies. Maintaining calcium homeostasis and controlling the opening of mPTP are important factors that can be considered as a potential therapeutic objective to maintain the quality of life during aging and prevent mitochondrial damage and progressive cognitive deterioration associated with age that contribute to the development of neurodegenerative diseases.

Figure 1 is a schematic representation of the main mitochondrial functions affected during normal aging. It shows increased oxidative stress as a result of a REDOX imbalance due to decreased activity of antioxidant enzymes and increased reactive oxygen species. It also shows the characteristic bioenergetics failure, as a consequence of diminished OXPHOS functioning, specifically by decreased activity of complexes I and IV of the ETC, which lead to reduced ATP production and, finally, calcium dysregulation, which leads to mitochondrial swelling due to a permanent opening of the mitochondrial permeability transition pore.

5. Mitochondrial dysfunction and cognitive impairment

Synaptic plasticity in the adult nervous system is a response to changes in the environment and synaptic activity, involving dendritic spine growth or retraction and synaptogenesis, which are believed to be responsible for learning and memory [94]. In the hippocampus, one form of synapse plasticity is LTP, which produces a stronger transmission for consolidation of long-term memory [95]. For this process, neurons need to synthesize proteins *de novo* at the dendritic spines where different neurotrophic factors play a key role [96]. An impairment in this process leads to neurodegeneration, as a result of the initial loss of synaptic structure and function and finally cell death [96].

The previously described mitochondrial dysfunction could be an important factor in synapse loss associated with cognitive decline observed during aging and in neurodegenerative disease [97]. Since mitochondria are present in axonal terminals and dendrite spines playing a critical role in calcium flux, ROS homeostasis, and ATP production in the synapses, this organelle is a key element for neuronal plasticity [94]. In addition, mitochondrial transport to the synaptic regions is essential for the correct function of this neuronal network [98].

Multiple pathological conditions present cognitive impairment related with a mitochondrial dysfunction. For example, chronic kidney disease (CKD) patients with cognitive damage have increased oxidative stress and decreased antioxidant enzymes (SOD, CAT, GPx, and GSH levels in plasma) compared to CKD patients without cognitive impairment [99]. Also, patients with hypoxia, ischemia induced by a traumatic brain injury (TBI), and diabetes showed cognitive decline and different signs of mitochondrial impairment such as glutamate excitotoxicity, calcium overload, opening of mPTP (which dissipates the mitochondrial electrochemical gradient leading to cell death), and increased ROS levels [100–102]. Interestingly, pyramidal neurons in the cerebral cortex and hippocampus are more susceptible to this type of injury [103], suggesting that these cellular defects may affect mainly synaptic plasticity, learning, and memory.

In the context of neurodegenerative disease, mitochondrial impairment and oxidative stress are the target of A β neurotoxicity, promoting cognitive impairment in AD [104]. The degree of cognitive impairment in AD has been related to the amount of A β accumulated in mitochondria [105], resulting in a loss of the $\Delta\psi_m$ in

synaptic regions and ultimately leading to the characteristic synaptic loss observed in AD [106]. It has been suggested that Cyp-D can interact with A β contributing to synaptic perturbations. A Cyp-D deficiency can notably improve synaptic function and therefore improve learning and memory in an AD mouse model [107]. It was recently proposed that tau protein can regulate synaptic activity, affecting mitochondrial function and axonal transport [108], and post-transductionally modified tau can induce mitochondrial damage, leading to synaptic dysfunction [109]. In fact, hyper-phosphorylated tau impairs mitochondrial respiratory chain function, increases ROS levels, decreases the activity of detoxifying enzymes, and produces $\Delta\psi_m$ dissipation [108]. Thus, the accumulation of tau can lead to synaptic deficits and cognitive impairment [110].

Interestingly, cognitive decline is not only characteristic of disease and injury, since cognitive impairment is also observed during aging. During normal aging, it is well established that there is a reduction in the surface area and cortical thickness, resulting in a volume loss in the whole brain, being the non-cortical regions, such as the hippocampus and striatum, more vulnerable to this age-related atrophy [111]. In this context, a study performed with Sprague-Dawley rats of 14, 18, 23, and 27 months of age showed changes in the volume of different brain parts using magnetic resonance imaging (MRI) [112]. In that study they showed enlargement of lateral ventricles and a decrease in the volume of the medial prefrontal cortex, hippocampus, and striatum in 27-month-old rats, which correlates with cognitive deficiency. Twenty-three- and twenty-seven-month-old rats have decreased recognition memory and decreased spatial learning and memory [112]. Another common symptom of aging is cognitive fatigue (CF) characterized by an increase in the facility of becoming tired, lack of energy, and failure to sustain attention when performing cognitively demanding tasks with a high mental effort [113]. There is evidence of a correlation between the decreased connectivity strength of the neuronal network established between the cortical and the striatum areas and a higher CF at an advanced age, suggesting that the cortical-striatal network plays a crucial role in the CF phenomenon [114].

In humans, similar cognitive decline is observed during aging, present as a deficit in episodic or declarative memory, spatial learning, working memory, and attention [115]. These processes are mainly dependent on an adequate function of the hippocampus. Structural and functional changes in the hippocampus are related to the severity and development of neurodegenerative disorders associated with cognitive decline. In fact, many of the cognitive deficits seen with aging can be replicated in animal models with bilateral hippocampal damage [116]. The connection between dentate gyrus (DG) and the CA3 area of the hippocampus is responsible for the formation of new memories, and this is naturally decreased in the aged brain [117], with different biochemical modifications that affect its ability to generate and consolidate LTP [117]. Diverse studies have shown that during aging, the auto-associative network of CA3 is strengthened, and the processing of new information coming in from the entorhinal cortex is weaker [118]. Thus, the stored information becomes dominant in contrast to the ability to encode new information [118]. Also, there is a decrease in gray matter volume, where age-related changes in the temporal lobe involve mainly changes in the hippocampus [4].

Also, different studies show high levels of tau in cerebrospinal fluid (CSF) during aging [119, 120]. For example, the characteristic hearing loss (HL) present in aging influences neurodegeneration by promoting tau pathology in CSF [120], which produces cognitive impairment via synapsis dysfunction and neuronal loss [110]. Other studies evidence the age-related impairment of executive functions, verbal and nonverbal cognitive switching (independent of gender, education, and IQ), and the ability to focus attention and/or multitask [121, 122]. Studies have

shown that during aging, there is a reduction in inhibitory mechanisms in the CA3, where short-term plasticity and LTP are compromised [123]. Interestingly, mitochondria play a central role in LTP, enhancing mitochondrial gene expression [124], satisfying the ATP demand by producing changes in mitochondrial energy production, and regulating calcium homeostasis by increasing calcium pump activity [125]. Also, there is evidence that mitochondrial dysfunction can lead to failure in connectivity of brain cortex producing cognitive impairment [126]. Thus, mitochondrial dysfunction during aging can be related with degeneration of synapses, triggering cell death.

In this mitochondrial context of aging, it is interesting that in brain regions highly associated with cognitive function, such as the hippocampus and cortex, there is a high amount of impaired mitochondria, with dysfunctional respiration, excessive ROS production, loss of $\Delta\psi_m$, and decreased cytochrome c oxidase activity. Meanwhile mitochondria are less affected in areas of the brain that are less involved in cognitive abilities [105]. Therefore, mitochondrial function is a key component in cognition. It allows proper information processing through the brain network, being an important player in synaptic transmission. Mitochondrial dysfunction generates deficits in synapses that trigger cognitive impairment in

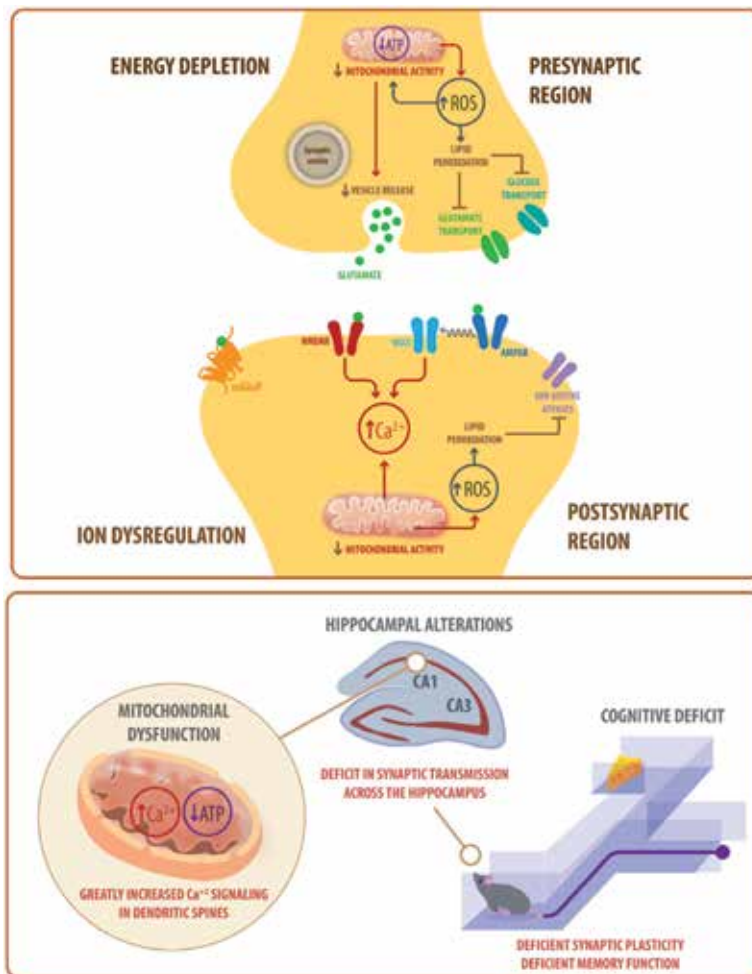


Figure 2.
A synaptic failure leads to cognitive impairment in aging.

neurodegenerative disease but also in natural aging. Thus, the understanding of these processes may be critical in these times where the aged population is increasing; therefore, improving their quality of life is a priority.

Figure 2 above shows the synaptic effects of mitochondrial failure. In the presynaptic region, decreased mitochondrial activity diminishes ATP content, altering the exocytosis of synaptic vesicles. Also, increased ROS production induces lipid peroxidation, affecting glutamate and glucose transport. In the postsynaptic region, decreased mitochondrial activity disrupts calcium homeostasis, altering postsynaptic signaling. Besides, the increased ROS production and consequent lipid peroxidation impaired ion-motive ATPases. **Figure 2** below schematizes that mitochondrial dysfunction at the CA1 of the hippocampus impaired synaptic transmission resulting in cognitive impairment.

6. Mitochondrial therapies as an antiaging treatment

Since mitochondrial dysfunction is a key event promoting aging, interventions that focus on maintaining or restoring the correct functioning of the mitochondria seem fundamental. For this purpose, two different experimental strategies could be used [127], physiological approaches or pharmacological approximations, which will be briefly summarized in this section.

From the physiological point of view, maintaining a lifestyle that includes recurrent physical exercise preserves mitochondrial function [127]. During aging a loss of age-associated muscle mass is directly related with decreased mitochondria-dependent metabolic capacity, as well as with reduced mitochondrial biogenesis [128]. Biogenesis of new mitochondria is regulated by the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), which also regulates redox balance and energetic function [129]. Interestingly, PGC-1 α expression is decreased in aging reducing its signaling pathway and gene target [129], an effect that could be counteracted by exercise, demonstrating that exercise also increases mtDNA content in the muscle of aged rats [130]. In addition, exercise increases the expression of the CAT enzyme, reducing ROS levels [131]. Similarly, exercise promotes both fission and fusion events as indicated by upregulated levels of Fis1 and Mfn1 protein in the muscle tissue of old animals and by increased Mfn2 and Drp1 mRNA in the skeletal muscle of older women [132]. Finally, exercise could contribute to restoration of mitochondrial Ca⁺² homeostasis, increasing the protein levels of mitochondrial Ca⁺² uniporter (MCU) [132]. Thus, exercise during aging could promote the genesis of new mitochondria or could attenuate the mitochondrial dysfunction observed at an advanced age.

A second physiological approximation important for simulating mitochondrial function is caloric restriction, which has been demonstrated in different models that are able to reduce the age-related phenotype and to increase lifespan [133]. The beneficial effects of caloric restriction are directly associated with the bioenergetic defects observed in aging, activating ATP production through fatty acid metabolism [134]. Mechanistically, caloric restriction increases the activity of complexes I, III, and IV of the ETC, as well as MnSOD, which results in increased ATP and reduced ROS levels [135]. Likewise, caloric restriction enhances Ca⁺² mitochondrial buffering, decreasing Cyp-D levels [135]. Therefore, regulating caloric ingestion is possible for maintaining mitochondrial activity during aging.

Mitochondrial function can also be regulated pharmacologically, for example, through the administration of polyphenols such as resveratrol, green tea, and red

wine [127, 136]. Specifically, they act by promoting mitochondrial OXPHOS and activating cellular antioxidant mechanisms [137]. Another possibility is the use of antioxidant compounds such as MitoQ, an electron scavenger that prevents the formation of mitochondrial free radicals [138]. Similarly, α -tocopherol (MitoVitE), α -phenyl-tert-butyl nitrone (MitoPBN), the piperidine nitroxide MitoTEMPOL, the antioxidant SkQ1, and elamipretide (SS-31) enter and accumulate in the mitochondria preventing oxidative stress and preserving mitochondrial function [139, 140]. The numerous studies probing the clinical efficacy of these compounds validate the importance of mitochondria in aging [139]. To promote the natural antioxidant effect in the cell, treatment with N-acetylcysteine, vitamin C and other physiological antioxidant molecules have also been shown to be effective as palliative treatment of senescence [141, 142].

It is also important to highlight the positive effects induced by the direct administration of fatty acids including omega-3 fatty acid α -linolenic acid, due to studies in vivo that have shown its capacity to extend lifespan [143]. This could be a consequence of increased β -oxidation, which results in higher mitochondrial energy production, by increasing mitochondrial biogenesis or by reducing oxidative stress [144]. Finally, we will mention the effects of Metformin, a drug commonly used for the treatment of type 2 diabetes. Metformin has a hypoglycemic effect in the plasma and promotes increased insulin sensibility by a mechanism that remains unclear [145]. However, favorable effects have been observed, where DNA damage and inflammation are prevented, impeding cellular damage by reducing ROS production [146].

Therefore, these approaches highlight the key role that mitochondrial function play during aging, where correct mitochondrial activity could extend lifespan, whereas metabolic alterations could compromise mitochondrial function, accelerating the aging phenotype.

7. Future directions: importance of synaptic mitochondrial dysfunction in aging

It is now known that the mitochondria have a fundamental role during the aging process. In neurons, the mitochondria are classified into two groups according to their localization, such as synaptic and non-synaptic mitochondria. Non-synaptic mitochondria are distributed throughout the cell body and in the neural prolongations, meanwhile synaptic mitochondria are exclusively found in synapses, both at the pre- and postsynaptic level [147]. Thus, it is not surprising that synaptic mitochondria, which have a higher energy requirement in order to sustain synaptic activity, present functional differences compared to non-synaptic mitochondria. For example, synaptic mitochondria have higher peroxide production than non-synaptic ones [148]. During aging, it seems that these differences are accentuated between these two mitochondrial populations. Aged cortical synaptic mitochondria present decreased oxidative capacity and higher susceptibility to calcium overload, in contrast to non-synaptic mitochondria that preserve their respiratory capacity [16]. Similarly, we observed that hippocampal synaptic mitochondria fail previous to non-synaptic mitochondria during aging and suffer premature mitochondrial swelling with age, contributing hippocampus-dependent memory loss (manuscript in preparation). Thus, maintaining adequate function of synaptic mitochondria seems to be the new challenge in order to attenuate the aging phenotype, reducing the synaptic and cognitive failure characteristics of older individuals.

8. Conclusions

Taken together, the evidence presented in this chapter strongly suggests a close relationship between mitochondrial function and a wide range of processes associated with aging. In general, it is possible to propose an age-dependent decline observed in several organs such as the brain correlated with a loss of mitochondrial activity, generating a bioenergetic deficit and redox imbalance that promote oxidative stress. This promotes additional mitochondrial fail, affecting cellular calcium homeostasis, critical for neurons due to its important roles in the synapses. Thus, synaptic defects conduce to cognitive impairment. Finally, we propose that the synaptic mitochondria are a critical mitochondrial pool to preserve synaptic communication despite the passing of the years.

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Coenzyme Q₁₀ and L-Carnitine Disturbances in Children with Mitochondrial Diseases

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Abstract

Coenzyme Q10 (CoQ10) and L-carnitine are very important biologically active compounds involved in energy metabolism. L-carnitine and coenzyme Q10 disturbances in mitochondrial diseases require the correction. Patients and methods: The levels of coenzyme Q10 and L-carnitine (total carnitine, free carnitine, and acyl-carnitines) were determined in children with mitochondrial diseases (25 children and 16 children, respectively). High-performance liquid chromatography with UV detection (chromatograph Shimadzu Nexera LC-30) and chromatography-mass spectrometry (Agilent 6410 QQQ, USA) were used. As an additional parameter of possible coenzyme Q10 and carnitine insufficiency, the coenzyme Q10/cholesterol ratio and acylcarnitines/free carnitine ratio were calculated. Results: A significantly low ratio of coenzyme Q10/cholesterol in children with mitochondrial diseases was revealed— 0.10 ± 0.01 vs. 0.19 ± 0.01 in the control group ($p < 0.001$). A lower absolute level of coenzyme Q10 and tendency toward a more pronounced decrease in the Q10/cholesterol ratio in older patients (6–16 years) were shown. The free carnitine blood level was within the normal range and averaged at $29.8 \pm 2.6 \mu\text{mol/l}$; however, the level was lower than that in the control group ($44 \pm 5.2 \mu\text{mol/l}$, $p < 0.05$). A pronounced significant increase in the acylcarnitines/free carnitine ratio was determined— 1.5 ± 0.05 (the normal range < 0.6).

Keywords: children, mitochondrial diseases, coenzyme Q10, carnitine, treatment

1. Introduction

Mitochondrial diseases are a large heterogeneous group of pathological conditions caused by genetically determined defects in the mitochondria's structure and function in the release of the energy of organic substances and its accumulation in the form of macroergic phosphate compounds by the generation of adenosine triphosphate [1]. These disorders can be due to mutations in mitochondrial DNA or due to mutations in nuclear DNA. Some mitochondrial diseases are rare. However, in general, mitochondrial encephalopathy is the most common neurometabolic disorder [2]. Defects in the respiratory chain and oxidative phosphorylation are the basis of the pathogenesis of these severe diseases.

Mitochondrial diseases have a wide range of clinical presentations with a generally poor prognosis: failure to thrive, encephalopathy, respiratory insufficiency,

hypotonia, ataxia, seizures, cardiac involvement, hepatopathy and nephropathy, sensorineural hearing loss, retinopathy, lesions of the basal ganglia, and others. The common laboratory signs are lactic acidosis, hypo- or hyperglycemia (diabetes), elevated creatine kinase and aminotransferases, and urine organic acid/amino acid abnormalities [3].

Current therapies are frequently inefficient and mostly palliative. The treatment strategy for mitochondrial diseases is to improve the efficiency of biological processes in the respiratory chain and oxidative phosphorylation. Patients are prescribed complex treatment, including drugs that affect different stages of energy metabolism. This treatment approach shows a higher positive effect than monotherapy [4]. Coenzyme Q10 and L-carnitine are very important biologically active substances involved in energy metabolism. So, coenzyme Q10 and L-carnitine are often recommended for the treatment of mitochondrial diseases [5]. However, some authors acknowledge the lack of rationale behind these recommendations since the data from randomized clinical trials are still lacking.

2. Functions of CoQ10 and its biological role

Coenzyme Q10 is the most common ubiquinone in the human body. Its structure contains a quinoid ring and 10 isoprenyl groups. Coenzyme Q10 is structurally similar to vitamins E and K. Coenzyme Q10 exists in oxidized (ubiquinone) and reduced (ubiquinol) forms and is known to be a constituent of the biological membranes [6]. Coenzyme Q10 is one of the main components of the electron transport chain of mitochondria. In the form of ubiquinone, it acts as an electron transporter from Complex I and Complex II to Complex III. In this process, the formation of the reduced form—ubiquinol—occurs. Ubiquinol is a powerful antioxidant, which has a protective effect on biological membranes, regulates their permeability, inhibits peroxidation of plasma lipoproteins, and provides a recovery of tocopherol activity [6, 7]. According to the recent data, coenzyme Q10 is reported to be involved in the regulation of some gene expression and inflammatory mediators, in particular, by influencing the transcription factor NFkappaB1; its participation in DNA replication and repair was shown [8, 9].

In mammals, the largest amount of coenzyme Q10 is found in the heart and skeletal muscles. In the peripheral blood, coenzyme Q10 is bound to lipoproteins, and its level is positively correlated with total cholesterol [10, 11].

Most of the body's daily coenzyme Q10 requirement is derived from endogenous synthesis; small amounts of coenzyme Q10 are obtained from foods such as meat, fish, and nuts. Biosynthesis is a multi-step process, taking place on the inner mitochondrial membrane. Vitamins B2, B3, B6, B12, and C and folic and pantothenic acids are known to participate in the coenzyme Q10 biosynthetic pathway under the control of a dozen genes. The intensity of biosynthesis declines substantially with age [12, 13].

3. Coenzyme Q10 deficiency in disorders: the possibility of diagnosis

Primary coenzyme Q10 deficiency is due to a defect in its biosynthesis. These diseases form a separate group of mitochondrial diseases and are associated with mutations in multiple genes including PDSS1, PDSS2, CoQ2, CoQ6, CoQ9, and ADCK3. These diseases are characterized by a decrease in the level of coenzyme Q10 in tissues and in fibroblasts whereas the blood levels can be normal [11, 14, 15]. Secondary coenzyme deficiency with low plasma and tissue coenzyme Q10 levels

can occur in patients taking anticancer agents and statins. The hypocholesterolemic effect of statins is due to the inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase and a disruption of the synthesis of mevalonic acid, which is one of the precursors of not only cholesterol but also ubiquinone. Approximately a half of patients receiving statins show a decrease in coenzyme Q₁₀ in the muscle tissue; myalgia and myoglobinuria may also be observed [16, 17].

Secondary coenzyme Q₁₀ deficiency and low levels of coenzyme Q₁₀ in plasma and tissues are found in certain diseases of older age (Parkinson's and Alzheimer's disease, atherosclerosis, diabetes mellitus, etc.) [13, 18, 19], in some hereditary diseases, including inborn errors of metabolism— mevalonic aciduria, phenylketonuria, glutaric acidemia II, ataxia-oculomotor apraxia 1, and cardiofaciocutaneous syndrome [11, 20]. Coenzyme Q₁₀ deficiency in mevalonic aciduria and in phenylketonuria can be explained by insufficient cholesterol production: the decrease in the activity of mevalonate kinase and the inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase with high levels of phenylalanine, respectively. In phenylketonuria, ubiquinone deficiency may well be exogenous due to dietary restrictions such as avoidance of animal products [10].

A decrease in coenzyme Q₁₀ was reported in the peripheral blood and muscles in some (20–40%) patients with mitochondrial pathology associated with mutations and depletion of mitochondrial DNA [21, 22]. Of interest, in children with myopathy due to other causes, there were no changes in the content of coenzyme Q₁₀ in the muscles, except for the patients with Duchenne muscular dystrophy [23].

The coenzyme Q₁₀ deficiency can be detected in biological fluids (plasma or serum), fibroblasts, and muscle tissue. However, the blood level of coenzyme Q₁₀ is not considered as a reliable indicator of its state in the body. There is no clear correlation between the levels of ubiquinone in plasma and muscle tissue. This parameter is influenced by the lipid intake from foods and the blood levels of cholesterol and low-density lipoproteins [7, 10, 11]. Therefore, the ratio of coenzyme Q₁₀ to cholesterol and low-density lipoproteins is proposed for clinical use. Apparently, the measurement of coenzyme Q₁₀ in the peripheral blood mononuclear cells appears to be a promising detection method.

4. Low blood level of coenzyme Q₁₀ as a diagnostic marker of mitochondrial encephalomyopathy and the rationale for therapy

In the Research and Clinical Institute of Pediatrics, an examination of 16 children (group 1) aged 1–16 years (average age 8.3 ± 1.5 years) with mitochondrial diseases was performed.

In nine children, the disease was caused by deletions or point mutations of mitochondrial DNA: Kearns-Sayre syndrome (common deletion of mitochondrial DNA) in three; mitochondrial encephalomyopathy with pyramidal-extrapyramidal syndrome (*MTND1* mutation) in two; mitochondrial encephalomyopathy with cardiomyopathy (*MTTK* mutations) in two; mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (*MTTL1* mutation) in 1; and maternal inherited Leigh syndrome (*MTND3* mutation) in 1.

Seven children were diagnosed with mitochondrial diseases of nuclear origin: leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (*DARS2* mutations) in four and Leigh syndrome (*SURF1* mutations) in three.

The comparison group (group 2) consisted of 13 children with neurodegenerative diseases aged 1–15 years (mean age 6.4 ± 1.3 y): 6 children with neuronal ceroid

lipofuscinosis 2 (*TPP1* mutations), 2 with sialidosis type 2 (*NEU1* mutations), and 5 with progressive ataxia not genetically confirmed. The control group (group 3) consisted of 29 healthy children aged 1–16 years (mean age 7.4 ± 0.8 y) who underwent a routine examination at the health center (an informed consent of the parents was obtained) [24]. The ratio of boys:girls is 16:13. There was no statistically significant difference in the average age between the three groups.

A detailed work-up of the patients of the first two groups included clinical (pedigree, neurological, cardiological, and other examinations) examination and biochemical tests. In all children, the plasma level of coenzyme Q10 was determined by high-performance liquid chromatography with UV detection (chromatograph Shimadzu Nexera LC-30). The blood cholesterol level was determined photometrically (analyzer Konelab Prime 60i), followed by the calculation of the coenzyme Q10/cholesterol ratio. The DNA diagnosis of diseases was carried out in the laboratory of inborn errors of metabolism of Research Centre for Medical Genetics (Moscow, Russia).

Statistical data processing was carried out by methods of variation statistics and correlation analysis (Statistica, Excel 7.0). Student's t-test was used to assess the statistical significance of the data, and the differences were considered statistically significant at $p < 0.05$.

The blood levels of coenzyme Q10 in children with mitochondrial diseases (group 1) were 0.56 ± 0.05 $\mu\text{mol/l}$ (Table 1) and did not differ from that in the control group but was significantly lower ($p < 0.01$) than that in children with neurodegenerative diseases (group 2). The blood level of coenzyme Q10 in children with neurodegenerative diseases was 1.53 ± 0.23 $\mu\text{mol/l}$ and significantly exceeded that in healthy children ($p < 0.01$).

The blood cholesterol level in patients of the first and second groups was significantly higher than that in healthy children ($p < 0.01$). The ratio of coenzyme Q10/cholesterol was severely impaired compared with that in healthy children (see Table 1). This parameter was significantly decreased in patients with mitochondrial diseases (0.10 ± 0.01 vs. 0.19 ± 0.01 , $p < 0.001$) but increased in those with neurodegenerative diseases (0.31 ± 0.04 , $p < 0.002$).

For the subgroup analysis, children aged 1–5 and 6–16 years were analyzed separately within each group (Table 2). In healthy children (group 3), there were no age-related differences.

In the group of older patients with mitochondrial diseases, the blood level of coenzyme Q10 was significantly lower than that in the younger subgroup ($p < 0.05$) and was significantly different from its level in children in the second

Groups of children	Levels in the blood		
	Coenzyme Q10, $\mu\text{mol/l}$	Cholesterol, $\mu\text{mol/l}$	Coenzyme Q10/cholesterol
Group 1, n = 16	0.56 ± 0.05	5.7 ± 0.32	0.10 ± 0.01
Group 2, n = 13	1.53 ± 0.23	4.8 ± 0.31	0.31 ± 0.04
Group 3, n = 29	0.67 ± 0.04	3.5 ± 0.12	0.19 ± 0.01
p	$^{1-2}p < 0.001$ $^{2-3}p < 0.001$	$^{1-3}p < 0.001$ $^{2-3}p < 0.001$	$^{1-2}p < 0.001$ $^{1-3}p < 0.001$ $^{2-3}p < 0.002$

^{1-2}p , ^{1-3}p , and ^{2-3}p - significance of differences between groups (1-2, 1-3, and 1-3)

Table 1. Coenzyme Q10 and cholesterol levels ($M \pm m$) in the blood of the examined children.

Groups of children	Levels in the blood		
	Coenzyme Q10, μmol/l	Cholesterol, μmol/l	Coenzyme Q10/ cholesterol
Group 1, n = 16			
Age 1–5 years, n = 6	0.73 ± 0.15*	6.4 ± 0.81 ¹⁻² p < 0.05 ¹⁻³ p < 0.001	0.12 ± 0.03 ¹⁻² p < 0.001 ¹⁻³ p < 0.05
Age 6–16 years, n = 10	0.47 ± 0.04 ¹⁻² p < 0.001 ¹⁻³ p < 0.05	5.5 ± 0.38 ¹⁻³ p < 0.001	0.09 ± 0.01 ¹⁻² p < 0.001 ¹⁻³ p < 0.001
Group 2, n = 13			
Age 1–5 years, n = 7	1.18 ± 0.26 ²⁻³ p < 0.001	4.4 ± 0.44 ²⁻³ p < 0.05	0.26 ± 0.03 ²⁻³ p < 0.05
Age 6–16 years, n = 6	1.93 ± 0.37 ²⁻³ p < 0.001	5.3 ± 0.38 ²⁻³ p < 0.001	0.37 ± 0.07 ²⁻³ p < 0.05
Group 3, n = 29			
Age 1–5 years, n = 15	0.64 ± 0.04	3.5 ± 0.16	0.19 ± 0.02
Age 6–16 years, n = 14	0.69 ± 0.07	3.6 ± 0.18	0.20 ± 0.02

*Significant difference from the older age subgroup; p < 0.05. ¹⁻²p, ¹⁻³p, and ²⁻³p - significance of differences between groups (1-2, 1-3, and 1-3)

Table 2.
 Coenzyme Q₁₀ and cholesterol levels (M ± m) in the blood in children of different age subgroups.

(p < 0.001) and third (p < 0.05) groups of the same age. In addition, older patients (6–16 years) tend to have lower cholesterol levels and lower coenzyme Q10/cholesterol ratios than younger patients (1–5 years). In the group of children with mitochondrial encephalomyopathies, there was a negative correlation between the blood level of coenzyme Q10 and cholesterol and the patient's age (r = -0.54 and r = -0.48, respectively; p < 0.05). There were no differences in the studied parameters between children with mitochondrial diseases caused by mutations of mitochondrial (n = 9) and nuclear (n = 6) DNA. By age, these subgroups were not different.

In the group of children with neurodegenerative diseases, the opposite tendency was observed toward higher rates of coenzyme Q10, cholesterol, and their ratios in patients of the older subgroup (see **Table 2**); unreliability of differences seems to be associated with a small number of patients. A positive correlation between the blood levels of coenzyme Q10 and cholesterol (r = 0.66; p < 0.05) was established. These parameters correlated positively with the age of the patients (r = 0.37 and r = 0.41, respectively; p < 0.05).

To sum up, our study demonstrated that the average level of coenzyme Q10 in patients with mitochondrial diseases did not differ from that in healthy children. However, these patients had a higher cholesterol level and, as a result, a reduced ratio of coenzyme Q10/cholesterol. In patients of the older subgroup (6–16 years), the changes were more pronounced: significantly lower levels of coenzyme Q10 and a tendency toward a lower Q10/cholesterol ratio than the younger subgroup (1–5 years).

In children with neurodegenerative diseases (not caused by primary mitochondrial dysfunction), the level of cholesterol (as well as in the first group) was higher than that in healthy children. Other test results differed considerably from those in patients with mitochondrial pathology. High blood level of coenzyme Q10 and an

increase in the coenzyme Q10/cholesterol ratio were revealed, and these changes increased with age.

Due to a small sample size, the obtained data were considered as preliminary. Nevertheless, our results revealed a coenzyme Q10 deficiency in children with mitochondrial diseases and emphasized the difference in the pathogenesis of primary mitochondrial diseases and neurodegenerative diseases of non-mitochondrial origin. In both patient groups, the age-related aggravation of these changes was noted to correspond to a progressive disease course. The similarity of clinical manifestations underlies the difficulties in the differential diagnosis between mitochondrial encephalomyopathies and neurodegenerative diseases. The detection of the plasma coenzyme Q10 level can be helpful in the differential diagnosis between these conditions.

In addition, a low coenzyme Q10/cholesterol ratio and a tendency toward a decrease in the coenzyme Q10 levels with age suggest its insufficiency in patients with progressive mitochondrial encephalomyopathy. This provides a rationale for the use of coenzyme Q10 in the treatment of patients with mitochondrial diseases.

5. Functions of carnitine and its biological role

Carnitine (β -hydroxy- γ -trimethylaminobutyric acid) is a low molecular weight compound, which is crucial for energy metabolism in the human body. As an L-stereoisomer, carnitine is present in various tissues. Endogenous carnitine formation occurs in the liver, kidney, and brain cells through the transformation of the amino acids lysine and methionine, with a glycine involvement. Vitamins C, B6, and B3 and iron ions are the cofactors for carnitine biosynthesis. The biosynthesis steps are under the control of mitochondrial enzymes (trimethyl-lysine deoxygenase, 3-OH-trimethyl-lysine aldolase, 4-trimethylaminobutanal dehydrogenase, butyrobetaine dioxygenase) [25]. However, biosynthesis provides only a part of carnitine daily requirements, and the main source of its intake is animal foods including red meat, fish, and dairy products. Carnitine absorption in the gastrointestinal tract, tubular reabsorption, and delivery to the tissue are provided by transport proteins, with OCTN2 being the main carnitine transporter. Carnitine is transported to the skeletal muscles and myocardium—these tissues contain the main reserves of carnitine, due to their high activity of lipid metabolism [26]. The importance of carnitine for the body is evidenced by its almost complete reabsorption in the renal tubules.

Studies have established the value of carnitine for the processes of biological oxidation and maintenance of mitochondrial functions in the human body. Carnitine is crucial in conditions of a high energy consumption. These conditions such as intercurrent diseases, increased physical activity, starvation, etc. are characterized by increased catabolism. After depletion of carbohydrate reserves, lipids become the main sources of the ATP synthesis in the body.

One of the main vital functions of carnitine is bioenergetics. Carnitine is involved in lipid catabolism, providing its initial stages—an activation and a transfer of long-chain fatty acids from the cytoplasm through the outer and inner mitochondrial membranes to the mitochondrial matrix, thereby making them available for subsequent β -oxidation to form acetyl-CoA. In addition, fatty acid oxidation is the main pathway of ketogenesis, and ketone bodies are an additional energy substrate for peripheral tissues and the brain [26, 27].

The effect of carnitine on fat metabolism occurs through its participation in the cytoplasmic synthesis of fatty acids. Carnitine provides the reverse transfer of acetyl groups of mitochondrial acetyl-CoA through the mitochondrial membrane into the cytoplasm.

An important function of carnitine is due to its ability to bind acyl radicals. Thus, carnitine regulates tissue energy metabolism, affecting the ratio of acyl-CoA/free CoA in the mitochondria. Likewise, the detoxifying role of carnitine is achieved through binding of organic acid derivatives (intermediates in oxidative processes) and its excretion from the cell. These organic acid derivatives, accumulating in the mitochondria and cytoplasm, have an adverse effect, by inhibiting the enzyme activity.

Carnitine appears to play an important role in the permeability of mitochondrial membranes. The protective impact of carnitine relies on the prevention of a negative membranotropic action of toxic agents, inhibitors of complexes of a mitochondrial respiratory chain (3-nitropropionic acid, methylphenylpyridine, and others), and inducers of apoptosis (long-chain fatty acid radicals). Carnitine supplementation in experimental animals prevents these disorders or significantly reduces their severity and prevents a degenerative damage to the nervous tissue [28–31].

In addition, a favorable impact of carnitine on the cytokine production and on vascular endothelium was revealed. Carnitine is capable of restoring endothelial function and preventing of vascular remodeling caused by a decrease in nitric oxide production [25, 32, 33]. Apparently, the carnitine functions require further research.

6. Carnitine deficiency in diseases

There are primary and secondary carnitine deficiencies. The primary deficiency is due to an autosomal recessive defect of the gene SLC22A5, which is expressed in the skeletal muscles, heart, and kidneys. The gene SLC22A5 encodes a transport protein OCTN2, the sodium-dependent organic cation transporter. The genetic defect disrupts the transport of carnitine into the tissues and reabsorption in the renal tubules. The clinical manifestations of the disease include cardiomyopathy, skeletal myopathy, fatty liver, and kidney dystrophy [34].

The causes of secondary carnitine deficiency are diverse and associated with an interruption of endogenous synthesis and disturbance of absorption from food and of retention in the body, as well as with an increased excretion through the kidneys or gastrointestinal tract [35]. The activity of endogenous carnitine biosynthesis depends on the function of the liver and kidneys. Biosynthesis decreases with malnutrition due to a protein deficiency. Carnitine removal is enhanced in stress, intercurrent diseases, and impaired renal tubular function. Low blood carnitine level is determined in children with epilepsy treated with valproate, in patients with heart failure, and in patients on hemodialysis [36–38].

Secondary insufficiency occurs in inborn errors of metabolism. In particular, secondary carnitine insufficiency is characteristic for a large group of hereditary diseases of organic and fatty acid metabolism. In these diseases, low levels of carnitine in the peripheral blood and tissues result from the accumulation of acylcarnitines and their enhanced renal excretion [34, 39].

7. The carnitine insufficiency in children with mitochondrial encephalomyopathies

Some patients with mitochondrial diseases (about one-fourth patients) were reported to have a decrease in carnitine levels in the peripheral blood [40, 41]. Our study was designed to diagnose and treat carnitine insufficiency in patients with mitochondrial diseases; to achieve this, we analyzed the clinical parameters and

laboratory findings of 40 children aged 2–15 years: 25 with mitochondrial encephalomyopathies (group 1) and 15 with congenital myopathies (group 2). Group 1 consisted of 10 children with Kearns-Sayre syndrome (common deletion of mitochondrial DNA); 2 with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MTTL1 mutation); 1 with myoclonic epilepsy associated with ragged-red fibers (MTTK mutation); 5 with leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (DARS2 mutations); 1 with Barth syndrome (TAZ mutation); 3 with Leigh syndrome (SURF1 mutations); and 3 with POLG-related diseases (mitochondrial recessive ataxia syndrome in 2 and autosomal recessive progressive external ophthalmoplegia in 1). Group 2 consisted of 10 children with central core disease and 5 with minicore myopathy. The control group included 10 children without mitochondrial diseases or congenital myopathies, who attended the clinic.

The clinical examination included pedigree, neurological, cardiological, and other examinations. In all children, acid–base balance of the blood and the level of lactic and pyruvic acids were determined. The level of L-carnitine—total carnitine, free carnitine (C0), and acylcarnitines (AC)—in dry blood spots was measured by chromatography–mass spectrometry (Agilent 6410 QQQ, USA). As an additional parameter of a possible carnitine insufficiency, the ratio AC/C0 was calculated. The DNA diagnosis of mitochondrial diseases was carried out in the laboratory of inborn errors of metabolism of the Research Centre for Medical Genetics (Moscow, Russia). Structural myopathy was diagnosed using a morphological study of muscle tissue (light and electronic microscopy, histochemical methods).

Statistical data processing was carried out by methods of variation statistics and correlation analysis (Statistica, Excel 7.0). Student's t-test was used to assess the statistical significance of the data, and the differences were considered statistically significant at $p < 0.05$.

In children with mitochondrial diseases, the clinical presentations of the nervous and muscular system involvement prevailed: fast fatigability, low exercise tolerance, muscle weakness and hypotension, and a development delay. Severe psychomotor retardation was noted in three children with Leigh syndrome. Most patients had ataxia, ophthalmoplegia, headaches and vomiting, and heart damage (atrioventricular blockade, cardiomyopathy). Some patients had a short stature, seizures, retinitis pigmentosa, a hearing loss, and an impaired liver function with a moderate increase in blood aspartate and alanine aminotransferase.

Compensated metabolic acidosis was detected in 12 of 25 children. Elevated blood lactate levels were noted within the range 2.9–6.7 mmol/l in 20 children (the normal range 1.0–1.7 mmol/l). In 11 patients, the pyruvate level was elevated (0.19–0.39 mmol/l, norm 0.09–0.12 $\mu\text{mol/l}$).

In children with mitochondrial diseases, the total carnitine blood levels (**Figure 1**) ranged from 39.1 to 95.3 $\mu\text{mol/l}$, averaging ($M \pm m$) $75.8 \pm 6.2 \mu\text{mol/l}$ (in the control group—from 41.1 to 119.9 $\mu\text{mol/l}$; $73.7 \pm 9.7 \mu\text{mol/l}$). There was a negative correlation between the total carnitine and lactate in the peripheral blood of patients with mitochondrial diseases ($r = -0.65$; $p < 0.05$).

The free carnitine blood level was within the normal range (19–60 $\mu\text{mol/l}$) and averaged at $29.8 \pm 2.6 \mu\text{mol/l}$; however, the level was lower than that in the control group ($44 \pm 5.2 \mu\text{mol/l}$, $p < 0.05$). In six patients, the free carnitine blood level was at the lower limit of the normal range, not exceeding 25 $\mu\text{mol/l}$.

The average acylcarnitine level was $44.5 \pm 3.7 \mu\text{mol/l}$ (see **Figure 1**); 87% of acylcarnitines were represented by acetylcarnitine (C2). Acetylcarnitine level was $38.7 \pm 3.9 \mu\text{mol/l}$ (higher than that in control group; $p < 0.05$); its level in four children went beyond the limit of the normal range (56.3, 60.2, 64.5, 65.7 $\mu\text{mol/l}$ at a rate of up to 50 $\mu\text{mol/l}$).

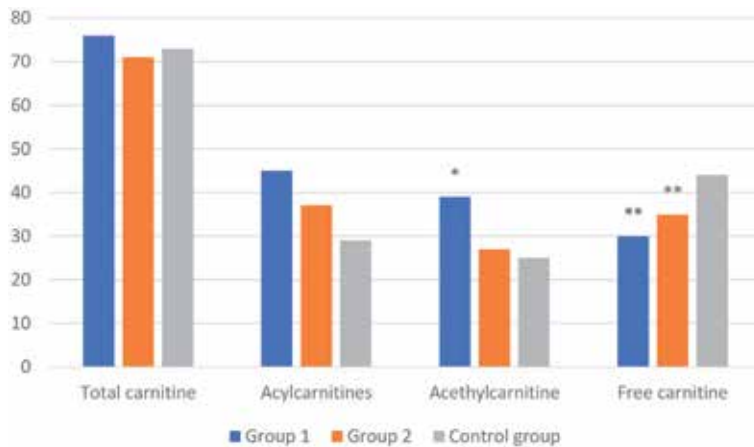


Figure 1.

Comparison of carnitine levels ($\mu\text{mol/l}$) in dry blood spots in children of three groups. * significant difference ($p < 0.05$) with groups 2 and 3. ** significant difference ($p < 0.05$) with group 3.

About a half (13) of the patients had elevated levels of other acylcarnitines: methylmalonyl-(C4DC), tiglyl-(C5: 1), glutaryl-(C5DC), hydroxybutyryl-(C4OH), hydroxyisovaleryl-(C5OH), and hexanoyl-(C6); there was an increase in the blood levels of alanine, glycine, and leucine. These metabolic changes are likely to be associated with the activation of ketogenesis, an impaired metabolism of lactate and pyruvate in mitochondrial diseases.

On average, the proportion of free carnitine was merely 39% of the total blood carnitine (the normal range is 70–80%). The proportion of acylcarnitines was 61%, significantly exceeding that of healthy subjects (20–30%).

The ratio of acylcarnitines and free carnitine AC/C0 was markedly increased to 1.3–1.8, reaching an average of 1.5 ± 0.05 (the normal range < 0.6). The increase in this ratio suggests the relative insufficiency of free carnitine and confirms the accumulation of esterified forms in the total carnitine pool.

In the patients with congenital myopathies, the clinical presentations predominantly included myopathic manifestations, impaired motor development, and increased fatigue. Moderately elevated blood lactate levels were found in eight children (1.9–2.8 mmol/l, the normal range 1.0–1.7 mmol/l). In 10 patients, the pyruvate level was increased (0.17–0.5 mmol/l, norm 0.09–0.12 mmol/l).

The total carnitine blood levels in children ranged from 46.7 to 106.1 $\mu\text{mol/l}$, averaging at ($M \pm m$) $71.4 \pm 3.5 \mu\text{mol/l}$. There was no difference in total carnitine level between the patients with congenital myopathies and the control group.

The mean free carnitine level of $35.0 \pm 1.9 \mu\text{mol/l}$ was within the normal range, but it was lower than that in the control group ($p < 0.05$). In two children with structural myopathy, free carnitine level was at the lower limit of the normal range.

The mean level of acylcarnitines was normal ($36.9 \pm 2.4 \mu\text{mol/l}$). Likewise, the level of acetylcarnitine is not elevated. Of note, levels of other acylcarnitines were moderately elevated in three patients.

The proportion of free carnitine in the total carnitine was reduced—49% (the normal range 70–80%). The proportion of the esterified forms accounted for 51% of the total carnitine, which significantly exceeded the corresponding values of healthy individuals (20–30%). The ratio of acylcarnitines and free carnitine AC/C0 was increased to 0.9–1.7, averaging at 1.1 ± 0.08 (norm < 0.6), which was consistent with carnitine insufficiency. In the control group of conditionally healthy children,

the ratio of AC/C0 was 0.66 ± 0.03 and was significantly different from that in groups 1 and 2 ($p < 0.01$).

The comparison of the carnitine parameters in patients with mitochondrial encephalomyopathies and congenital myopathies showed that in mitochondrial diseases, carnitine deficiency was more pronounced. Although the levels of acyl-carnitines, free and total carnitine, were not significantly different, in the group of children with mitochondrial diseases, there was a tendency for an accumulation of bound carnitine and a lower level of free carnitine. Additionally, an acetylcarnitine level was significantly higher, and the impairment of total carnitine composition was more pronounced in children with mitochondrial diseases than in those with congenital myopathies. This was confirmed by a higher ratio of AC/C0 (1.5 ± 0.05 vs. 1.1 ± 0.08 ; $p < 0.01$).

The detection of carnitine deficiency underscores the need for L-carnitine therapy. L-carnitine per os in a dose of 30–50 mg/kg/day (depending on age) was included in the complex of energy treatment (coenzyme Q10, succinates, vitamin B) of patients with mitochondrial diseases. Within 1 year, three courses of therapy (for 2 months) were prescribed with a period off treatment for 1–2 months.

After 10–12 months of a follow-up of 18 children, a distinct improvement in one third of the patients was demonstrated: a reduction of fatigue, an improvement of exercise tolerance, and a reduction in the frequency of headache and nausea attacks. In one half cases, a stabilization with minimal positive dynamics was observed. These 15 children showed a decrease in the blood lactate level to 1.4–2.3 mmol/l. At the same time, in three children with Leigh syndrome, despite the treatment, a moderate progression of the disease with a persistent lacticidemia was observed.

The total carnitine remained at the same level ($70.5 \pm 5.1 \mu\text{mol/l}$); a tendency ($p > 0.05$) toward an increase in the free carnitine level ($36.2 \pm 2.9 \mu\text{mol/l}$) and a decrease in the acylcarnitine level ($34.3 \pm 4.1 \mu\text{mol/l}$), including acetylcarnitine, was noted. A significant improvement in the AC/C0 ratio was revealed—a decrease to 0.9 ($p < 0.001$); the proportion of free carnitine in total carnitine increased significantly to 52% ($p < 0.01$). In general, the data indicate favorable changes: a reduction in carnitine deficiency, an improvement of its function, and a reduction in ketogenesis and in the severity of lactate metabolism disorders.

8. Conclusion

Coenzyme Q10 and carnitine are important components of energy metabolism that are involved in many biological processes in the human body. Our data suggest that there is an insufficiency of these compounds in patients with mitochondrial diseases. Our studies have not revealed the severe deficiency of these substances, while the evidence for a relative insufficiency were found. According to our laboratory data, coenzyme Q10 deficiency is manifested by a significantly low ratio of coenzyme Q10/cholesterol. Lower absolute level of coenzyme Q10 and tendency toward a more pronounced decrease in the Q10/cholesterol ratio in older patients (6–16 years), in our opinion, are consistent with the progressive course of mitochondrial pathology.

Carnitine deficiency is manifested by a tendency toward a decrease in the free carnitine blood level, a pronounced decrease in its proportion in total carnitine, and a significant increase in the ratio of bound and free carnitines.

The coenzyme Q10 and free carnitine insufficiency certainly adversely affects the course of the disease. The causes for these disorders remain unclear. Perhaps, defective mitochondria are not able to provide adequate biosynthesis of coenzyme Q10. Depletion of free carnitine is likely to occur as a result of the activation of

conjugation of acyl radicals, which accumulate in the disorders of respiratory chain and oxidative phosphorylation.

Given a crucial role of carnitine and coenzyme in mitochondrial energy processes, the insufficiency of these compounds should be treated. Clinical heterogeneity of mitochondrial diseases justifies further research in homogeneous patient groups in order to develop evidence-based recommendations and ensure higher treatment efficacy. Furthermore, a moderate carnitine deficiency in congenital structural myopathies provides indications for carnitine supplementation.

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The mitochondrion is a unique and ubiquitous organelle that contains its own genome, encoding essential proteins that are major components of the respiratory chain and energy production system. Mitochondria play a dominant role in the life and function of eukaryotic cells including neurons and glia, as their survival and activity depend upon mitochondrial energy production and supply. Besides energy production, mitochondria also play a vital role in calcium homeostasis and may induce apoptosis by excitotoxicity. Mitochondrial dysfunction is related to common neurological diseases, such as Parkinson's disease, Alzheimer's disease, Friedreich's ataxia, Huntington's disease, and Multiple Sclerosis. An efficient treatment of mitochondrial dysfunction would open new horizons in the therapeutic perspectives of a substantial number of inflammatory and degenerative neurological disorders.

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