

## Review Article

# Proteomic Profiling of Mitochondrial Enzymes during Skeletal Muscle Aging

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Mitochondria are of central importance for energy generation in skeletal muscles. Expression changes or functional alterations in mitochondrial enzymes play a key role during myogenesis, fibre maturation, and various neuromuscular pathologies, as well as natural fibre aging. Mass spectrometry-based proteomics suggests itself as a convenient large-scale and high-throughput approach to catalogue the mitochondrial protein complement and determine global changes during health and disease. This paper gives a brief overview of the relatively new field of mitochondrial proteomics and discusses the findings from recent proteomic surveys of mitochondrial elements in aged skeletal muscles. Changes in the abundance, biochemical activity, subcellular localization, and/or posttranslational modifications in key mitochondrial enzymes might be useful as novel biomarkers of aging. In the long term, this may advance diagnostic procedures, improve the monitoring of disease progression, help in the testing of side effects due to new drug regimens, and enhance our molecular understanding of age-related muscle degeneration.

## 1. Introduction

The neuromuscular system is severely affected during the natural aging process [1]. Pathophysiological cycles of denervation and impaired reinnervation, the loss of entire motor units, unloading due to prolonged periods of disuse, and excitation-contraction uncoupling may trigger a substantial loss in skeletal muscle mass and function [2]. Although considerable interindividual differences exist in the functional decline of the musculature during aging, most elderly people experience a general loss in skeletal muscle strength [3]. While regular physical activity and a protein-rich diet can partially counteract severe muscle wasting [4], a sedentary life style and certain medical conditions, such as diabetes, cancer, renal failure, chronic obstructive pulmonary disease, or congestive heart failure [5–7], clearly promote muscle degeneration [8]. Skeletal muscle wasting plays a crucial role in physical disability, frailty, and loss of independence in aged people [9]. Skeletal muscle wasting in the elderly has been termed sarcopenia of old age, whereby this muscular impairment is probably due to multiple factors [10], as outlined in Figure 1. On the cellular level, a variety of abnormal

structural, physiological, and biochemical processes have been identified that are directly or indirectly associated with age-dependent muscle wasting. This includes a severe decline in contractile efficiency [11], increased apoptosis [12], denervation-associated atrophy [13], bioenergetic changes [14], impaired ion homeostasis [15], excitation-contraction uncoupling [16], decreased capacity for fibre regeneration [17], a partially diminished cellular stress response [18], and an altered equilibrium of hormones and growth factors crucial for the maintenance of contractile function [19], as well as oxidative stress and mitochondrial abnormalities [20–22]. The general issue of fibre type shifting during aging is still controversial. Although individual muscles in aged humans and animal models of sarcopenia exhibit alterations in the molecular composition of contractile fibres and changes in their glycolytic and aerobic capacity, findings on distinct shifts in fibre types with aging are highly variable [23–26]. However, since mitochondrial functions are clearly impaired in senescent muscle tissues, it was of interest to summarize the impact of recent mass spectrometry-based proteomic studies on the molecular fate of mitochondrial enzymes in senescent fibres. This paper briefly outlines

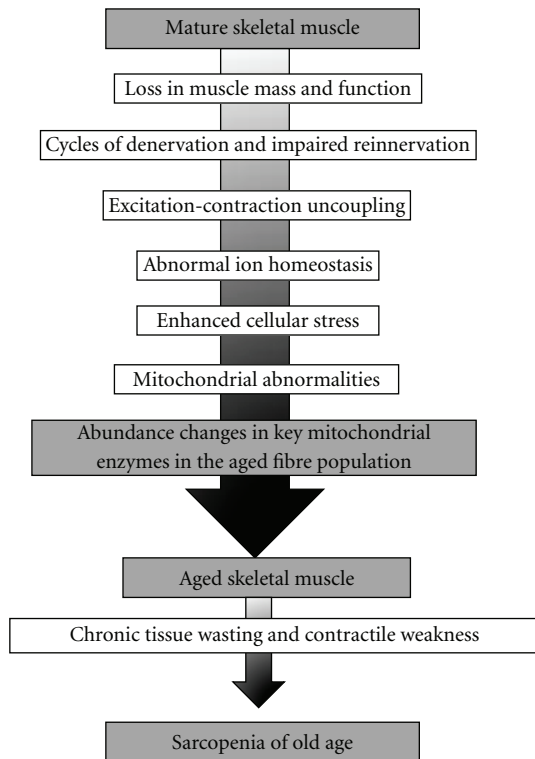


FIGURE 1: Overview of the multifactorial etiology of sarcopenia. Shown are the main physiological and biochemical events that trigger chronic tissue wasting and severe contractile weakness in senescent skeletal muscles. One of the most striking age-related changes is a drastic alteration in the abundance of mitochondrial enzymes.

the proposed role of mitochondria in cellular senescence and recent achievements of mitochondrial proteomics and then focuses on findings from proteomic profiling studies of aged skeletal muscle preparations and the identification of mitochondrial elements as potential markers of fibre aging.

## 2. Mitochondria and Cellular Senescence

Mitochondria are the primary site for energy generation via oxidative phosphorylation and play a key role in protein transport, intermediary metabolism, cell cycle progression, calcium signaling, and the regulation of apoptosis [27]. Proteomic cataloguing studies of this crucial organelle suggest the existence of approximately 1,500 mitochondrial proteins [28–30], whereby altered expression levels within the mitochondrial proteome are critical factors for normal development and numerous diseases [31–33]. Changes in mitochondria have long been associated with playing an integral role during natural aging [34–37], and the pharmacological application of antioxidants for counteracting mitochondria-specific symptoms of senescence is being extensively studied [38]. Interestingly, the mitochondrial theory of aging also encompasses the mechanisms that may lead to cellular senescence in contractile tissues [39–41]. Altered levels of mitochondrial activity in aged muscle tissues

have been well established and extensively reviewed [42–44]. The detrimental accumulation of mitochondrial DNA deletions and mutations on the genetic level and deficiencies in the mitochondrial electron transport chain on the biochemical level are clearly associated with muscle aging. The pathological consequences of an age-related decline in mitochondrial function are the impairment of essential ATP-dependent cellular processes [45] and amplified oxidative stress in senescent tissues due to the increased release of reactive oxygen species from the mitochondrial respiratory chain [46, 47]. In general, senescent muscle tissues are exposed to an enhanced production of mitochondrial reactive oxygen species, increased mitochondrial apoptotic susceptibility, disturbed mitochondrial bioenergetic functions, and a reduced transcriptional drive for mitochondrial biogenesis [22, 48]. Although these functional impairments clearly occur in skeletal muscle mitochondria during aging, biochemical studies have also demonstrated considerable age-related changes in the abundance and posttranslational modifications of key mitochondrial enzymes.

## 3. Profiling of the Mitochondrial Proteome

Proteomics is concerned with the large-scale and high-throughput identification and characterization of the global protein constellation of a given biological entity, such as cells, tissues, or body fluids [49]. Protein complements are separated by standard methods, including gel electrophoresis and liquid chromatography [50–52], and individual protein species are usually identified by mass spectrometry [53–55]. The verification of proteomic data is routinely carried out by biochemical, immunological, cell biological, and physiological assays. Skeletal muscle proteomics, in particular, involves the comprehensive biochemical analysis of protein populations from defined muscle tissues, individual muscles, specific fibre populations, or distinct subcellular fractions [56–58]. Figure 2 outlines the standard workflow for the identification of novel aging-associated biomarkers using gel electrophoresis-based proteomics. Total crude tissue extracts, detergent phase-extracted proteins, or mitochondria-enriched fractions are routinely used as starting material for the determination of new mitochondrial markers. The main analytical steps involved in skeletal muscle proteomics are the extraction of a distinct protein population from crude extracts, subcellular fractions, or affinity-purified protein complexes, the efficient separation of proteins by one-dimensional gel electrophoresis, two-dimensional gel electrophoresis, or liquid chromatography, the densitometric mapping of altered protein concentration levels, the unequivocal identification of protein species by mass spectrometry of protease-generated peptide mixtures, and finally the independent validation of proteomic data by enzyme assays, immunoblot analysis, ligand binding assays, or immunofluorescence microscopy.

Since the concentration range of proteins is not a static entity, but highly dynamic, and because the density of proteins spans several orders of magnitude in complex cellular systems, proteomic studies of crude extracts result

mostly in the cataloguing of abundant and soluble protein species. Thus, conventional gel electrophoresis-based proteomics underestimates certain classes of proteins, such as high-molecular-mass proteins, integral membrane proteins, extremely basic or acid proteins, and low-abundance proteins [50–52]. Over the last few years, proteomic approaches have been refined in order to reduce sample complexity by subcellular fractionation protocols and affinity separation techniques [59–61]. Mass spectrometry-based proteomics suggests itself as an ideal analytical method to determine global changes in the mitochondrial protein complement [62, 63]. Mitochondrial proteomics is concerned with the establishment of the entire organelle-associated protein complement and the dynamic nature of posttranslational modifications in mitochondrial components, as well as differential expression patterns within mitochondrial protein populations due to physiological adaptations or pathological insults [64–66]. Considerable tissue-specific differences exist within the mitochondrial proteome and reflect the diversity of mitochondrial functions in individual organs [67–69]. As listed in Table 1, proteomic maps of mitochondria exist for numerous organs from several different species.

The first comprehensive survey of human mitochondria detected approximately 1,500 spots on a silver-stained reference map and identified 46 mitochondria-associated proteins in placental tissue [70]. Subsequent studies have discovered several hundred mitochondrial proteins by mass spectrometry, using differential centrifugation or density gradients consisting of percoll, metrizamide or nycodenz for prefractionation purposes [71–77]. Proteomic analyses yielded 615, 680 and 940 distinct mitochondrial proteins in human and mouse heart, respectively [71–73], 182 and 192 mitochondrial proteins in mouse and rat liver, respectively [74, 75], 823 mitochondrial proteins in human skeletal muscle [76], and 723 and 1,198 mitochondrial proteins in brown and white fat cell lines, respectively [77]. Several proteomic studies have investigated mitochondrial protein populations in several organ systems in parallel, including liver, muscle, heart, kidney, and brain [67–69]. The most comprehensive comparative report on the mitochondrial proteome has created a compendium of 1,098 genes and their protein expression across 14 mouse tissues [78]. Detailed listings of proteomic studies that have focused on mitochondria in health and disease can be found in recent extensive reviews of this specialized field of subproteomics [28, 30, 65]. These crucial cataloguing exercises form now the basis of detailed comparative investigations into disease-dependent alterations in mitochondria [64], including studying the effects of aging on the mitochondrial proteome [79, 80].

#### 4. Mitochondrial Markers of Skeletal Muscle Aging

Over the last decade, a large number of proteomic studies have identified potential biomarkers of muscle aging [81]. Studies of aged human muscle and the most widely employed animal model of sarcopenia-related abnormalities, the senescent rat [82], have revealed changes in proteins

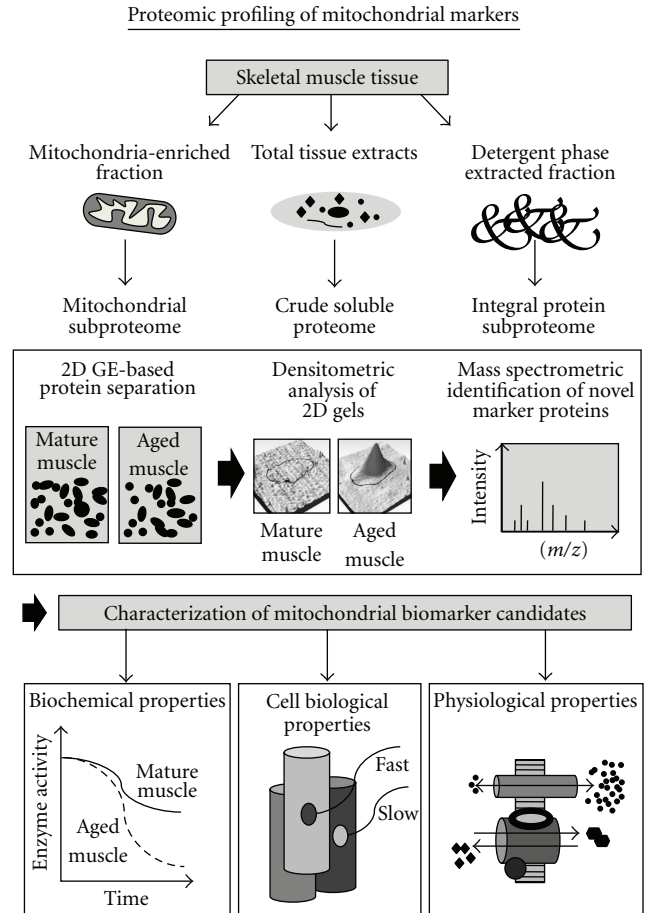


FIGURE 2: Flowchart of the proteomic workflow to identify and characterize novel mitochondrial markers involved in sarcopenia of old age. Shown are the various steps involved in the high-throughput proteomic screening of tissue specimens, including sample preparation, gel electrophoretic separation, densitometric analysis, and mass spectrometric identification of new candidate proteins.

involved in the regulation of excitation-contraction coupling, ion homeostasis, muscle contraction, muscle relaxation, metabolite transportation, energy metabolism, and the cellular stress response [83–99]. Table 2 lists recent proteomic studies that have identified the potential involvement of mitochondrial elements in sarcopenia of old age. The proteomic analysis of total extracts from aged human vastus lateralis muscle has identified numerous aerobic markers with an increased density, including the mitochondrial enzymes ATP synthase, ubiquinol-cytochrome c reductase, and oxoglutarate dehydrogenase during muscle aging [86]. In analogy, elevated levels of mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, ATP synthase, malate dehydrogenase, ubiquinol-cytochrome c reductase, and pyruvate dehydrogenase, were also shown to occur during the aging of rat gastrocnemius muscle [93, 94]. These investigations were performed with the fluorescence difference in-gel electrophoretic technique, one of the most powerful biochemical methods to compare

TABLE 1: List of major profiling studies of the mitochondrial proteome.

| Proteomic studies  | Mitochondrial protein identification   | Reference  |
|--|--|--|
| Proteomic analysis of human placenta   | First comprehensive cataloguing of the mitochondrial proteome, which resulted in the identification of 46 distinct proteins  | Rabilloud et al. [70]  |
| Analysis of human and mouse heart  | Identification of 680 human mitochondrial proteins and 940 mouse mitochondrial proteins in heart muscle  | Taylor et al. [71],<br>Gaucher et al. [72],<br>Zhang et al. [73]                                   |
| Proteomic profiling of mouse and rat liver   | Identification of 182 mouse proteins and 192 rat proteins that are associated with liver mitochondria  | da Cruz et al. [74],<br>Fountoulakis et al. [75]   |
| Proteomic profiling of human skeletal muscle   | Identification of 823 mitochondrial proteins in human vastus lateralis muscle  | Lefort et al. [76]   |
| Proteomic profiling of brown and white adipose cell lines  | Identification of 723 mitochondrial proteins in brown adipose cell line and 1,198 mitochondrial proteins in white adipose cell line  | Forner et al. [77]   |
| Comparative studies for the establishment of the mammalian mitochondrial proteome from various tissues | Identification of tissue-specific expression patterns of mouse and rat mitochondrial proteins from liver, skeletal muscle, kidney, brain, heart, and various other organs. The most comprehensive comparative study established the mitochondrial protein complement in 14 different tissues | Mootha et al. [67],<br>Forner et al. [68],<br>Reifschneider et al. [69],<br>Pagliarini et al. [78] |

concentration changes of distinct protein species in soluble proteomes [100]. The recent proteomic profiling of the detergent phase-extracted protein complement from senescent rat gastrocnemius muscle confirmed a changed concentration of numerous mitochondrial enzymes during aging. The mitochondrial marker enzymes ATP synthase and isocitrate dehydrogenase were found to be significantly increased in aged muscle tissue [99]. In contrast to the highly discriminatory difference in-gel electrophoretic technique used for studying muscle aging [86, 93, 94, 97], proteomic approaches with conventional protein dyes or dyes that cover a limited dynamic range have identified considerably fewer changes in mitochondrial markers [85, 87].

Subproteomic studies of mitochondria-enriched fractions from aged skeletal muscles have shown differential effects on the abundance of mitochondrial enzymes [90, 91, 96–98]. Chang et al. [90] have studied the effect of aging and caloric restriction on the rat mitochondrial proteome. In skeletal muscles, isocitrate dehydrogenase and malate dehydrogenase were shown to be increased in 25-month-old Fisher 344 rats, as compared to 6-month-old rats. Caloric restriction appears to have only a minor effect on age-related changes in the mitochondrial protein complement [90]. Severe metabolic changes in aged skeletal muscle were confirmed by an extensive proteomic survey of mitochondrial preparations from 3-month-old versus 26-month-old rat gastrocnemius muscles [97]. These muscle specimens represent young adult versus senescent contractile tissues, respectively. The fluorescent difference in-gel electrophoretic analysis demonstrated an age-dependent elevation in numerous mitochondrial proteins, including NADH dehydrogenase, ATPase synthase, succinate dehydrogenase, the mitochondrial inner membrane protein mitofilin, peroxiredoxin isoform PRX-III, mitochondrial fission protein Fis1, succinate-coenzyme A ligase, acyl-coenzyme A dehydrogenase, ubiquinol-cytochrome c reductase core I protein, prohibitin, and porin isoform VDAC2 (Figure 3).

Proteomic studies of posttranslational changes in aged skeletal muscle have revealed increased nitration levels in succinate dehydrogenase [83], decreased phosphorylation levels in cytochrome c oxidase and aconitase [92], and altered carbonylation levels in ATP synthase, NADH dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase [91] during muscle aging [101]. Abnormal posttranslational modifications may alter protein stability, subcellular targeting, intra- and intermolecular interactions, as well as coupling efficiency between substrates and active sites in affected mitochondrial enzymes. This might partially explain impaired mitochondrial functioning in senescent fibres. Thus, natural aging of skeletal muscles appears to be associated with distinct changes in posttranslational modifications of important mitochondrial enzymes. Recently, Ferreira et al. [102] compared the proteomes of subsarcolemmal versus intermyofibrillar mitochondria from rat skeletal muscle. A differential expression pattern was established for 38 mitochondrial proteins. In the future, refined proteomic studies might be able to determine whether intermyofibrillar mitochondria are differently affected by muscle aging as compared to subsarcolemmal mitochondria.

## 5. Conclusion

Since improved nutritional intake and exercise intervention can only partially alleviate the symptoms of sarcopenia, there is an urgent need to develop novel pharmacological strategies to prevent age-related muscle wasting [103]. Recent publications by working groups on the etiology, epidemiology, potential interventions, and the clinical assessment of sarcopenia show that a general definition of this common geriatric syndrome is still evolving [104–109]. In the future, it will be crucial to establish reliable sarcopenia-specific biomarkers to develop superior diagnostic tools for the correct classification of this age-dependent muscle pathology [110]. Mass spectrometry-based proteomics suggests itself

TABLE 2: Proteomic identification of mitochondrial proteins during skeletal muscle aging.

| Proteomic study  | Changes in mitochondrial marker proteins  | Reference                     |
|--|---|-------------------------------|
| Analysis of total extracts from aged human vastus lateralis muscle                               | General increase in aerobic markers, including mitochondrial enzymes such as ATP synthase, ubiquinol-cytochrome c reductase, and oxoglutarate dehydrogenase during muscle aging             | Gelfi et al. [86]             |
| Analysis of total extracts from rat gastrocnemius muscle   | Increase in mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, ATP synthase, and malate dehydrogenase during muscle aging                                    | Doran et al. [93]             |
| Analysis of total extracts from rat gastrocnemius muscle   | Differential effect on the abundance of mitochondrial isoforms of aconitase during muscle aging   | O'Connell et al. [87]         |
| Analysis of total extracts from aged rat gastrocnemius muscle                                    | Moderate effect on cytochrome c oxidase and isocitrate dehydrogenase during muscle aging  | Piec et al. [85]              |
| Analysis of total extracts from rat gastrocnemius muscle   | Increase in many enzymes involved in oxidative metabolism, such as ATP synthase, isocitrate dehydrogenase, ubiquinol-cytochrome c reductase, and pyruvate dehydrogenase during muscle aging | Capitanio et al. [94]         |
| Subproteomic study of the effect of aging and caloric restriction on rat muscle mitochondria     | Increased levels of isocitrate dehydrogenase and malate dehydrogenase in aged muscle mitochondria. Caloric restriction appears to have only a marginal effect on the mitochondrial proteome | Chang et al. [90]             |
| Subproteomic analysis of mitochondria-enriched fraction from aged rat gastrocnemius muscle       | Increased levels of mitochondrial creatine kinase, NADH dehydrogenase, ATP synthase, succinate dehydrogenase, and ubiquinol cytochrome c reductase during muscle aging                      | O'Connell and Ohlendieck [97] |
| Analysis of total extracts and mitochondria-enriched fraction from aged rat gastrocnemius muscle | Differential effect on mitochondrial enzymes, such as pyruvate dehydrogenase, cytochrome c oxidase, isocitrate dehydrogenase, and ATP synthase during muscle aging                          | Lombardi et al. [96]          |
| Subproteomic analysis of mitochondria-enriched fraction from aged mouse hind limb muscles        | Differential effects on the abundance and carbonylation of various mitochondrial enzymes, including NADH dehydrogenase, cytochrome c oxidase, and ATP synthase during muscle aging          | Alves et al. [98]             |
| Analysis of detergent phase-extracted protein complement from aged rat gastrocnemius muscle      | Increase in mitochondrial marker enzymes, such as ATP synthase and isocitrate dehydrogenase during muscle aging   | Donoghue et al. [99]          |
| Proteomic analysis of nitration in aged rat skeletal muscle                                      | Increased nitration levels in succinate dehydrogenase   | Kanski et al. [84]            |
| Phosphoproteomic analysis of total extracts from aged rat gastrocnemius muscle                   | Decreased phosphorylation levels in cytochrome c oxidase and aconitase during muscle aging  | Gannon et al. [92]            |
| Proteomic analysis of carbonylation in aged rat skeletal muscle mitochondria                     | Altered carbonylation levels in numerous mitochondrial proteins, including ATP synthase, NADH dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase during muscle aging       | Feng et al. [91]              |

as an ideal analytical tool for the study of skeletal muscle aging. The biochemical establishment of a robust protein marker signature for sarcopenia of old age will be extremely useful for (i) formulating a coherent cellular theory of muscle aging, (ii) the development of proper diagnostic criteria that can differentiate between different degrees of age-related muscle weakness, (iii) the identification of novel therapeutic targets to counteract cellular stress and fibre degeneration during aging, and (iv) the evaluation of improved treatment regimes to slow down the aging process.

Recent proteomic studies of mitochondria-enriched fractions and total skeletal muscle extracts have demonstrated altered levels of key mitochondrial enzymes in senescent muscle tissues. Aged neuromuscular systems appear to contain a higher degree of certain mitochondrial enzymes. Thus, although mitochondrial dysfunction and oxidative stress are associated with sarcopenia, muscle aging is also clearly linked to metabolic alterations. This suggests that abundant mitochondrial enzymes may be useful for general muscle profiling and are excellent biomarker candidates for

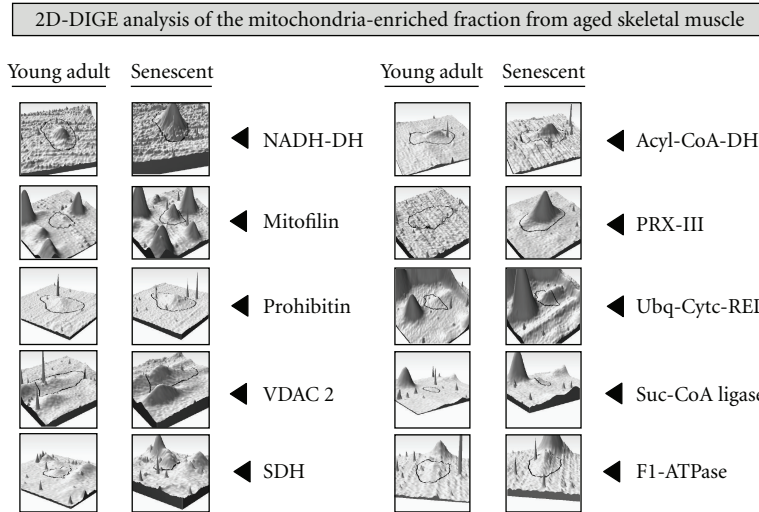


FIGURE 3: Proteomic profiling of the mitochondria-enriched fraction from aged skeletal muscle tissue. Shown is the comparative graphic representation of distinct two-dimensional protein spots with a changed expression in aged muscle as judged by fluorescence difference in-gel electrophoretic analysis [97]. Individual panels document alterations in the abundance of NADH dehydrogenase (NADH-DH), the inner mitochondrial membrane protein mitofilin, prohibitin, the porin isoform VDAC 2, succinate dehydrogenase (SDH), acyl-coenzyme A dehydrogenase (Acyl-CoA-DH), peroxiredoxin isoform PRX-III, ubiquinol-cytochrome c reductase core I protein (Ubq-Cytc-RED), succinate-coenzyme A (Suc-CoA) ligase, and mitochondrial F1-ATPase.

the biochemical classification of cellular changes during the natural aging process.

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