Innovative (electro-driven) sample preparation tools for metabolomics study of muscle aging

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Chapter 1

General introduction and scope

1. Aging and sarcopenia

One of the most impressive achievements of the past century has been the remarkable linear rise of average life expectancy: in Europe this was ~45 years in 1850, whereas children born in 2016 are predicted to have a more than 50% chance of becoming 100 or older [1]. Hopefully, many of these extra years will be spent in good health. However, it is also evident that age-associated diseases will become much more prevalent in the near future, which imposes extra pressure on healthcare resources and will have enormous socio-economic consequences. For these reasons it is imperative to find ways to promote someone's health span, *i.e.*, the period of one's life during which a person is generally healthy and able to contribute to society. Only this will tilt the balance in favor of the active versus inactive fraction of the population, bend the steep curve of increasing healthcare expenses in a more favorable direction, and at the same time improve wellbeing.

Sarcopenia is characterized by declining muscle mass and function and is one of the main drivers of loss of independence in the elderly [2]. Muscle mass usually starts to decline after 25–30 years of age, such that on average 40% of muscle mass is lost by 80 years [3]. In the world, 11–50% of those aged 80 or above suffer from sarcopenia [4], and this number keeps increasing. The loss of muscle mass and strength is caused by multi-factorial endocrine and cellular processes weakening the contractile function of skeletal muscle [5, 6]. So far, there is still not an approved medication for sarcopenia [7]. The molecular mechanisms underlying sarcopenia still need to be well understood.

1.1 Metabolomics study of sarcopenia

The past decades have seen major advancements in understanding the building blocks of life on a molecular level. Especially sequencing the human genome was a scientific breakthrough [8]. However, it also became clear that information about gene-expression (transcriptomics) and protein profiles (proteomics) alone is not sufficient to fully understand the processes that cause certain disease phenotypes. The metabolite profile (metabolome) can be considered an important link between the genotype and phenotype. Therefore, the analysis of the metabolome is of great importance.

Recent reports about the influence of aging and aging-related diseases on metabolite levels suggest that oxidative stress and molecular inflammation play important roles in sarcopenia [9, 10] and that they are associated with the loss of muscle mass [11] and poor grip strength,

i.e., an indicator of muscle strength [12]. Also, the monounsaturated fatty acids oleate and palmitoleate increase in aged rats, suggesting altered lipid metabolism [13]. Energy production related metabolites were reported to be highly associated with weak muscle contractile function, and other age-associated diseases [14-16]. For example, reduced glycolytic metabolism, including accumulation of glycolytic, glycogenolytic, and pentose phosphate pathway intermediates, was observed in aged rat skeletal muscle [17]. Furthermore, 3-methylhistidine and free amino acids, including branched amino acids, were reported to possibly indicate muscle atrophy, and associate with sarcopenia and other aging related diseases [15, 18, 19]. A study in humans also reported altered amino acid levels, as well as phosphatidylcholine and lysophosphatidylcholine levels in the elderly [20].

Dietary restriction (DR) is currently recognized as an effective intervention to extend the lifespan and alleviate the aging related diseases in various species, including some mammals [21-25] and humans [26-28]. Colman *et al.* found that dietary restriction with a decrease of caloric intake of 30% can attenuate sarcopenia in rhesus monkeys [25]. Almundarij *et al.* reported that the work efficiency of rat muscle was significantly increased after 50% of reduction of caloric intake [29]. However, the fundamental metabolomics mechanisms of how dietary restriction attenuates sarcopenia remain unclear. More investigations need to be systematically conducted to identify potential biomarkers for diagnosis, prevention, and treatment of sarcopenia.

1.2 Mouse model for sarcopenia study

Aging and aging-related diseases could be influenced by many factors [30]. One of the main causal hallmarks of aging is time-dependent accumulation of DNA damage [31-33]. The cloning of the first human DNA repair gene by researchers of the Erasmus Medical Center (Rotterdam), followed by many more was instrumental for elucidation of the nucleotide excision repair (NER) pathway [32, 34, 35]. This also revealed the very strong evolutionary conservation of DNA repair and resolved the basis of cockayne syndrome, xeroderma pigmentosum, trichothiodystrophy, Fanconi anemia and XFE1 syndrome [32, 36, 37]. Subsequently, an extensive series of mouse repair mutants was generated to cover the range from molecule to patient [33, 38]. These mouse mutants turned out to not only mimic the corresponding human syndromes to an exceptional degree but also enabled detailed insight into the complex aetiology of human repair diseases. Overall, these mouse mutants showed

a very strong connection between accumulating DNA damage and accelerated but truly *bona fide* aging.

DNA repair deficiency causes accumulation of endogenous DNA lesions, interfering with DNA functions, triggering premature cellular senescence, cell death and consequent dramatically accelerated, but otherwise normal aging. *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice (Figure 1), which are deficient in several repair processes, *i.e*., transcription-coupled repair (TCR), global genome (GG-)NER and interstrand cross-link (ICL) repair (and likely sub-pathways of double strand break (DSB) repair), exhibit in a progressive manner premature and even in some cases excessive aging features in almost all organs and tissues including multi-morbidity and overall frailty [32, 33]. These mice has a lifespan of ~25 weeks and exhibit a remarkably wide range of pathological, physiological, and behavioral features related to accelerated aging, such as sarcopenia; osteoporosis; progressive neurodegeneration (dementia, ataxia, hearing and vision loss); liver, kidney, vascular and haematological aging; *etc*. similar to those of natural aging [24, 36, 39, 40]. Progeroid *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice were also previously successfully used for the studies of aging and aging-related diseases [41-44]. Therefore, *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice will be utilized for the sarcopenia studies in this thesis.

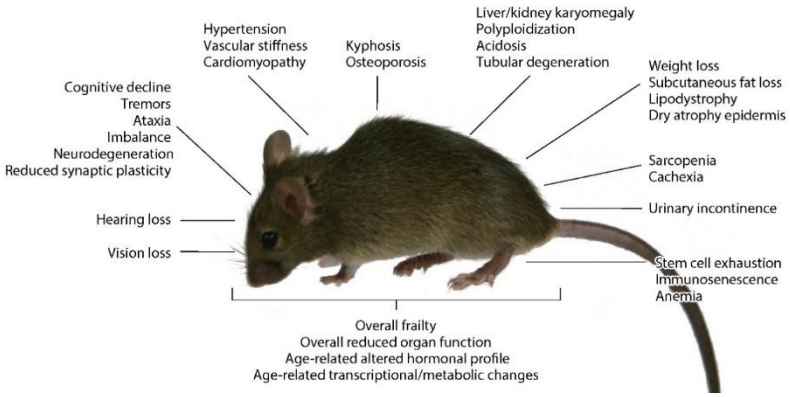

Figure 1. Aging features and age-related pathology in *Ercc1*$^{\Delta/-}$ mice, accelerated muscle aging (sarcopenia) was observed [33].

2. Sample-preparation methods for sarcopenia

Metabolomics is a powerful approach for obtaining molecular insights into complex diseases and for discovery of disease biomarkers, including the above-described metabolites related to oxidative stress, inflammation, energy production, and nutrition for sarcopenia

[45]. These metabolites greatly differ in physico-chemical properties: some are very non-polar (lipids), some are polar (amino acids), and some are cationic (*e.g*. acylcarnitines) or anionic metabolites. Currently, no single metabolomics platform can be used to measure all these metabolites within one experiment. Multiple metabolomics platforms, *i.e*., reversed-phase ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS), and capillary electrophoresis-mass spectrometry (CE-MS), should be considered for the analysis of these metabolites. Typically, samples have to be split into aliquots in order to analyze parts of the sample on different metabolomics platforms. However, this approach requires a sufficient starting amount of the sample to meet the analytical requirements of the different platforms. As the progeroid *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice exhibit early cessation of growth, only small amount of (skeletal) muscle, *i.e*., often down to ±5 mg, can be isolated. Therefore, it is important to develop sensitive and reproducible sample-preparation methods that are capable of subjecting the 5-mg mouse muscle tissue sample to at least three different metabolomics platforms, thus covering the largest part of the metabolome, *i.e*., non-polar, polar, cationic, and/or anionic metabolites. This will enable us to better understand the biochemistry mechanism underlying sarcopenia, and identify potential biomarkers for diagnosis, prevention, and treatment of sarcopenia.

2.1 Electro-extraction (EE) method for charged analytes

2.1.1 Manual EE setup

Electro-driven extraction is an emerging and promising sample-preparation method with advantages for extraction of charged analytes from small amounts of samples, especially the ionic analytes with low concentrations. This extraction method is based on the active migration of ionic molecules in an applied electric field, which drives the ionic molecules from the sample to the acceptor phase. There are two main advantages of electro-driven extraction: firstly, high enrichment and low loss of selected analytes during extraction. 100% extraction recovery can be achieved in principle [46-48], and peptides and charged metabolites can be enriched up to 1000-fold from the sample into the small acceptor droplet within a few minutes, resulting in limits of detection (LOD) values as low as 10 nM for peptides in plasma [49] and 15 nM for acylcarnitines in urine [47] analyzed by LC- and CE-MS. The second important advantage of this technique is the efficient clean-up of sample.

It can selectively enrich the analytes of interest from complex, protein- and salt-rich samples, and remove interference to reduce the matrix effect in mass spectrometry [46-48, 50, 51].

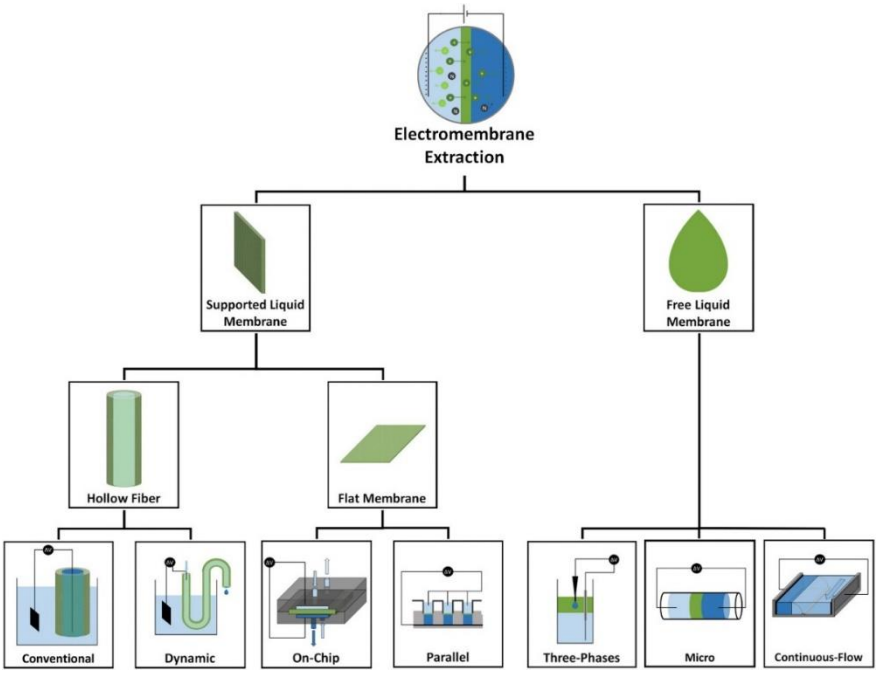

Figure 2. Classification of electro-driven extraction methods and setups overviewed in [52].

Supported liquid membrane electroextraction (SLM-EE) and free liquid membrane electroextraction (FLM-EE) are the two main variants of electro-driven extraction (Figure 2). The difference between them is the application of a stable solid membrane between the sample and acceptor phase in SLM-EE to hold the organic solution [52]. SLM-EE was first reported in 2006 by Pedersen-Bjergaard *et al.*, and uses a membrane with organic solvent held in its pores, between the aqueous sample and an acceptor solution [53]. FLM-EE was first developed for analytical purposes in 1994 by Van der Vlis *et al.* [54], but was not used for bioanalysis until 2010 by Lindenburg *et al.* [55]. Due to the omission of a membrane, FLM-EE is more straightforward in operation and necessary equipment [56]. Single droplet EE (including three-phase and two-phase EE), micro-EE and continuous flow on-chip EE are recently reported FLM-EE methods [52]. For simple extraction setup and operation, single droplet EE is the most straightforward of the FLM-EE methods to be applied to bio-analysis. Depending on the number of phases, single droplet EE can be categorized as two-

phase or three-phase electroextraction (EE). In two-phase EE, the phases consist of an organic phase and an aqueous acceptor phase, and analytes have to be dissolved in the organic phase before extraction, which may limit the analytes coverage and application of the method for the analytes that cannot be easily dissolved in the organic phase [55]. Raterink *et al.* first reported three-phase EE in 2013, with an aqueous acceptor droplet formed in the organic phase which is separated from the aqueous sample (Figure 3) [51]. However, only a few acylcarnitines were utilized by Raterink *et al.* [51] for the three-phase EE optimization and performance evaluation, further systematically investigation needs to be conducted before the application of the three-phase EE method.

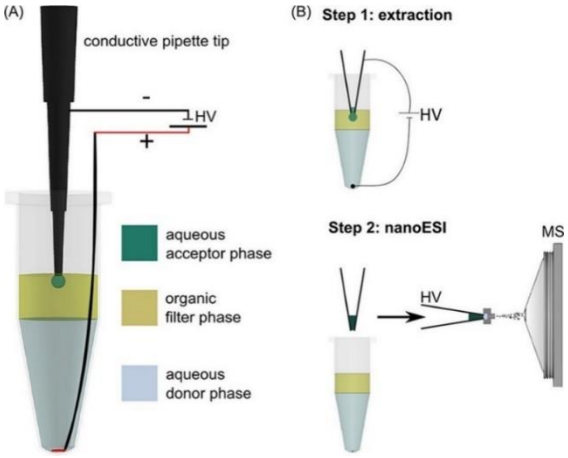

Figure 3. The schematic diagram of three-phase EE [51], where an aqueous acceptor droplet is formed in the organic phase and separated from aqueous sample.

2.1.2 Automated and high-throughput EE setup

Sample preparation is often the major bottleneck in bio-analytical workflows [57-60], and can be very laborious and time consuming when dealing with large numbers of samples; especially for those with limited amounts of starting material [61-64], more strategies have to be considered to enrich the molecules of interest, for example, the sample from progeroid *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice (with muscle tissue amount down to ±5 mg, and number over 400) for our sarcopenia study. To avoid these bottlenecks, an ideal solution is to automate and improve the throughput of the sample-preparation method. An automated and high-throughput sample-preparation method should be easy to coordinate with the analysis

instrumentation, *e.g.*, liquid chromatography–mass spectrometry (LC-MS), should be able to clean up and pre-concentrate analytes, should be easily applied to amount-limited samples, and should be environmentally friendly [57, 61, 65-68].

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the most commonly used sample-preparation methods [69-71]. However, both are laborious and time intensive. Three-phase EE is a promising alternative sample-preparation method to be automated offering a simple extraction process, high enrichment, and clean-up for low concentration analytes from biomass-limited samples. Raterink *et al.* applied three-phase EE on an automated nanoESI robot (Triversa NanoMate) [51]. The pipette tip used for the automated three-phase EE on the nanoESI robot allowed direct infusion of extracted sample [51], however, hyphenation of the setup to separation instruments, such as liquid chromatography (LC) or capillary electrophoresis (CE), was limited. Lack of separation may cause ion suppression of extracted analytes during MS analysis. The CTC PAL robotic autosampler (Figure 4) (www.palsystem.com) is an established auto-sampler platform that provides flexibility for integration of in-house developed setups, hyphenation to LC, and the precise positioning of the robot arms. Therefore, it is an ideal platform to achieve both automation and high-throughput for three-phase EE method.

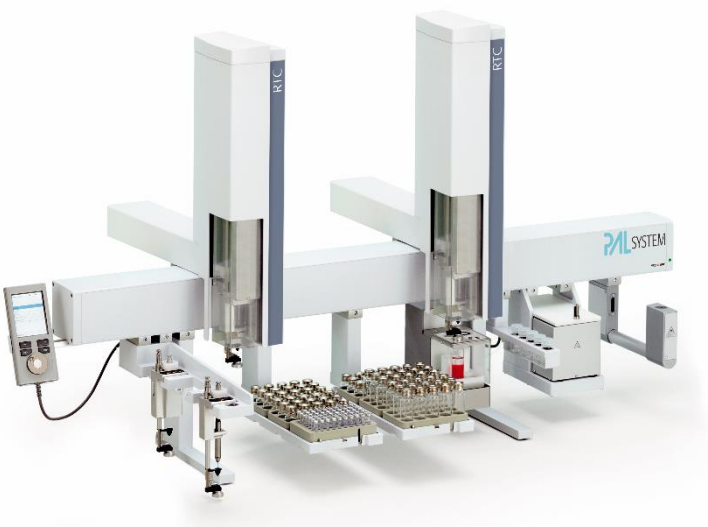

Figure 4. A CTC PAL3 RSI/RTC dual-headed robotic autosampler with syringe, syringe holder tool, sample plate.

A single small aqueous droplet was utilized as the acceptor phase in three-phase EE because of its high surface-area-to-volume ratio, which may contribute to high extraction efficiency of the three-phase EE method [51]. So far, the droplet has only been visualized by a digital camera to monitor its status. Droplet stability during electroextraction and whether its stability effects three-phase EE performance still remain unclear. Machine vision is a good approach to calculate the acceptor droplet size and estimate its stability in real time. Therefore, firstly, an automated three-phase EE setup with machine vision (Figure 5) on a CTC PAL robot is necessary to evaluate the performance of the automated three-phase EE method before its application.

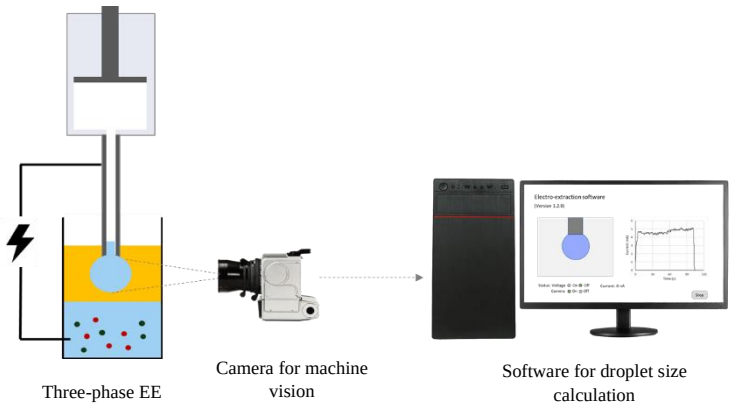

Figure 5. The schematic diagram of three-phase EE with machine vision. The camera is connected with an in-house developed software with algorithm for droplet size and is utilized for monitoring the acceptor droplet of the three-phase EE.

Acylcarnitines play important roles in transport fatty acids from cytoplasm into mitochondria, which improves the β-oxidation and energy production, and is highly related to muscle strength and function. Raterink *et al.* optimized the extraction of some acylcarnitines by using a manual three-phase EE setup and evaluated its performance in human plasma samples [51]. However, the performance of automated three-phase EE setup still needs to be investigated. To apply the setup to large numbers of samples, improving throughput of the evaluated automated three-phase EE method is necessary. Therefore, a high-throughput and automated three-phase EE setup needs to be developed and integrated with a CTC PAL robot, and the parameters of the setup needs to be optimized for acylcarnitines extraction to analyze large numbers of minute sarcopenia samples.

2.2 Liquid-liquid extraction (LLE) for uncharged analytes

Electroextraction is a good sample extraction method with advantages for classes of ionic molecules, however, for other classes of analytes, *i.e*., uncharged metabolites, other sample preparation-methods need to be considered. Liquid-liquid extraction (LLE) is well established and one of the most commonly used sample-preparation methods for (uncharged) non-polar and/or polar molecules. For the small amount of sarcopenia samples (muscle tissue down to ±5 mg) can be isolated from progeroid *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice, it is challenging to split the samples for separate extraction of non-polar and polar metabolites for the multiple metabolomics platforms analysis. A single-step LLE method allowing simultaneous extraction of the non-polar and polar metabolites from one samples is necessary for the sarcopenia mechanism study.

The Bligh and Dyer method is a traditionally used LLE method, which is able to simultaneously and non-selectively extract a wide range of non-polar and polar metabolites [72, 73]. 1-Butanol(BuOH)-methyl tert-butyl ether (MTBE)-Citrate is a sensitive sample-preparation method for amount-limited sample applications, Di Zazzo *et al.* applied for analysis of oxylipins, oxidative stress markers, endocannabinoids, and bile acids for ocular surface cicatrizing conjunctivitis. However, the performance of BuOH-MTBE-Citrate method on small amount of muscle tissues (±5 mg) from progeroid mouse for sarcopenia study still remains unknown, and because of the utilization of a non-volatile (citric acid/phosphate) buffer, the extracted polar metabolites in the aqueous phase was not compatible with mass spectrometric measurement [74].

Therefore, a LLE method specific for minute mouse muscle tissue needs to be developed for the concomitant extraction of non-polar and polar metabolites related to sarcopenia in a single step (Figure 6). This is necessary for the investigation of dietary restriction effects on sarcopenia, and identification of potential biomarkers for diagnosis, prevention, and treatment of sarcopenia.

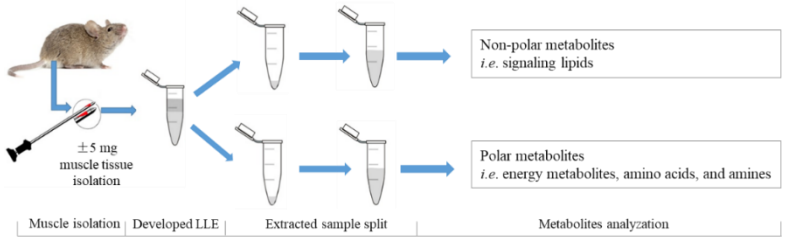

Figure 6. The developed LLE method for small amount of sarcopenia samples

3. Scope of the thesis

This thesis focusses on the development of sample-preparation methods for small amounts of samples and applying the developed methods to muscle tissues to investigate the mechanisms involved in sarcopenia. The idea underlying this thesis was that (automated) enrichment of charged metabolites using electroextraction will allow analysis of charged metabolites from small samples for further MS-based analysis. In addition, the hypothesis was that metabolomics can facilitate the understanding of metabolic processes underlying sarcopenia and can help to identify treatment options.

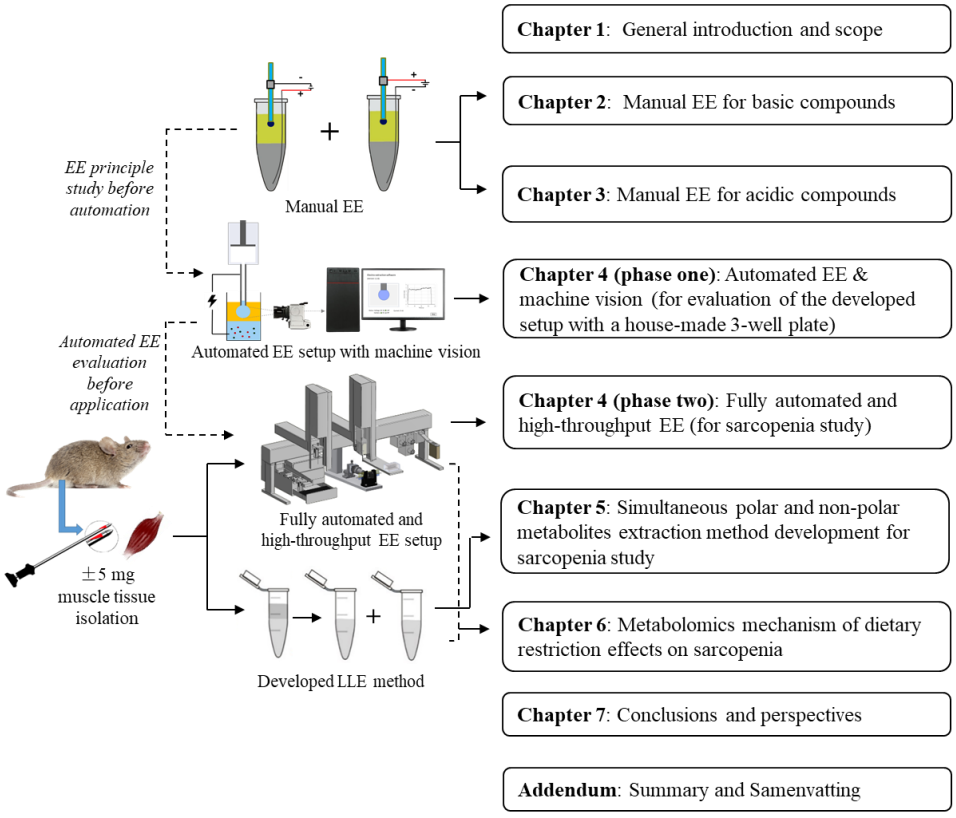

Figure 7. Overview structure of the thesis and the aim of each chapter.

The challenge is to extract charged and uncharged metabolites related to aging or aging associated diseases from small amounts of mouse muscle tissues for analysis using multiple metabolomics platforms analysis. Therefore in the first part of this thesis, the aim was the

development and application of innovative and miniaturized preparation methods/setups, for example, the fully-automated and high-throughput three-phase EE setup. In the second part of this thesis, the developed methods were applied to study the metabolic effects underlying mechanism of dietary restriction effects on sarcopenia and identify biomarkers for clinical therapy of sarcopenia. The structure of the thesis is shown in Figure 7.

The aims of **Chapter 2 and 3** were to develop a manual three-phase EE setup and systematically investigate the fundamental properties of three-phase EE methods by exploring the effects of different experimental parameters on some model basic and acidic compounds. Different types and composition of organic phases, the pH of sample and acceptor phase, composition of acceptor phase, extraction voltage, and extraction time were investigated and optimized for the positive (for basic compounds) and negative (for acidic compounds) three-phase EE methods. The results showed that the above parameters are crucial for both positive and negative three-phase EE method for achieving a high enrichment or extraction recovery of analytes from small amounts of sample. However, the labor- and time-consumption of the manual three-phase EE setup limits its application in routine analysis.

The aims of **Chapter 4 (phase one)** were to automate the three-phase EE method and evaluate the automated setup by a machine-vision system. A robotic sample preparation and handling platform (PAL CTC robotic autosampler) was used for the automation of the three-phase EE method. The aqueous acceptor droplet in three-phase EE was monitored by a high-resolution digital camera controlled by in-house developed software with machine vision to calculate the droplet size and evaluate its stability. At the same time, the current during three-phase EE was also monitored and recorded by the software. The results revealed a stable and repeatable automated three-phase EE setup for samples with low concentrations of basic model compounds. The first part of Chapter 4 utilized an in-house developed 3-well plate, digital camera visualization and machine-vision calculation for the acceptor droplet, while a design more suitable for high throughput was presented in **the phase two of Chapter 4** where a fully automated and high-throughput three-phase EE was developed by using a house-made 96-well plate with a bottom electrode on an PAL CTC robotic autosampler. Acylcarnitines were selected as model analytes for optimization of the automated and high-throughput three-phase EE, because of their important role in fatty acid transport into mitochondria, which is highly associated with muscle strength and function.

The optimum setup obtained a high enrichment factor and extraction recovery for acylcarnitines and was successfully applied to *Ercc1*$^{\Delta/-}$ mouse muscle tissues to study the effect of the muscle tissue isolation speed on acylcarnitine stability.

Chapter 5 focused on the development of LLE methods for concomitant extraction of polar and non-polar metabolites in a single step from small amounts of muscle tissues. Two previously reported and two newly developed sample-preparation methods were compared to find an optimal method with the highest extraction recovery and acceptable matrix effect for metabolite classes associated with sarcopenia, including signaling lipids, energy metabolites, amino acids and amines. The optimal method was applied to *Ercc1*$^{\Delta/-}$ mice muscle tissues to investigate whether fast muscle tissue isolation is necessary for sarcopenia study. We demonstrated that fast muscle isolation is crucial for metabolite stability for mechanistic studies of sarcopenia.

The aim of **Chapter 6** was to apply the developed miniaturized sample preparation methods on mouse muscle samples to investigate the dietary restriction effects on sarcopenia. Behavioral studies, *i.e.*, limb grip strength, motor coordination and learning, and mouse body weight, were conducted first to determine the dietary restriction effects on wild type and progeroid (*Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$) mice. Then the metabolites related to oxidative stress, pro-inflammation, anti-inflammation, energy production, and nutrition were analyzed by multiple optimized liquid chromatography-mass spectrometry platforms to try to reveal the sarcopenia metabolomics mechanism underlying the behavioral study results. The results demonstrated that dietary restriction improves the muscle quality for sarcopenia, and potential biomarkers for diagnosis, prevention, and treatment of sarcopenia were identified.

Finally, **Chapter 7** provides general conclusions and perspectives of this thesis. The overall performance of the developed sample-preparation setups, for example, the fully automated and high-throughput three-phase EE setup, are discussed. Future perspectives to upgrade these setups and to improve sarcopenia mechanistic studies and biomarker identification are also included in this chapter.

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