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Chapter 3

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Abstract

Background: Rheumatoid arthritis (RA) is a complex, chronic autoimmune disease characterized by various inflammatory symptoms, including joint swelling, joint pain, and both structural and functional joint damage. The most commonly used animal model for studying RA is mice with collagen-induced arthritis (CIA); the wide use of this model is due primarily to many similarities with RA in human patients. Metabolomics is used increasingly in biological studies for diagnosing disease and for predicting and evaluating drug interventions, as a large number of disease-associated metabolites can be analyzed and interpreted from a biological perspective.

Aim: To profile free amino acids and their biogenic metabolites in CIA mice plasma.

Method: Ultra-high-performance liquid chromatography/tandem mass spectrometry (UPLC-ESI-MS) coupled with multiple reaction monitoring (MRM) was used for metabolomics study.

Results: Profile of 45 amine metabolites, including free amino acids and their biogenic metabolites, in plasma was obtained from CIA mice. We found that the plasma levels of 20 amine metabolites were significantly decreased in the CIA group.

Conclusion: The results suggest that a disordered amine response is linked to RA-associated muscle wasting and energy expenditure.

Key words: Collagen-induced arthritis, mouse model, amine metabolites, systems biology.

1. Introduction

Rheumatoid arthritis (RA) is a highly prevalent chronic disease, currently affecting approximately 1% of the world's population [1]–[3]. Patients with RA typically have destruction of joint cartilage and bone accompanied by joint stiffness, hyperplasia, microvascular injury, swelling, and pain. The pathogenesis of RA is mainly associated with the secretion of cytokines such as interleukins (e.g., IL-1 and IL-6), tumor necrosis factor (TNFα), interferon gamma (IFNγ), and various pro-inflammatory mediators [4], [5]. Increased activity of the nuclear factor (NF)-κB pathway, which inhibits apoptosis in immune cells, also plays a role in RA [6]–[10]. A variety of cellular immune responses are also activated and/or dysregulated by increased cytokine levels in RA [11]–[14]. Interestingly, nearly two-thirds of patients with RA develop cachexia and sarcopenia, with a loss of skeletal muscle mass, degradation of proteins, and energy expenditure [15]–[18]. This perturbation in catabolic processes drives the body into a state of negative energy balance, leading to skeletal muscle atrophy, loss of muscle strength, and reduced physical activity [18], [19].

Considering the complex nature of RA, animal models have been useful for studying the underlying pathology and disease mechanisms. The most widely used animal model for studying chronic RA is the collagen-induced arthritis (CIA) mouse model; in addition to high reproducibility and easy induction, the physiological processes and pathogenic features of CIA mice are strikingly similar to the clinical features associated with patients with RA [20]–[23]. For example, increased levels of IL-6 , IL-1, and TNFα play a role in the development of CIA [24]. In addition, high correlation between muscle wasting and the severity of clinical arthritis has also been observed in animal models, including both monkeys and mice with CIA [25], [26].

Applying a systems biology approach using metabolomics can provide a comprehensive functional readout of the organism's physiological status [27]. Recently, van Wietmarschen and van der Greef summarized the putative

inflammatory mediators identified in RA patients using metabolomics [28]. Although some pro-inflammatory mediators have been observed in CIA mice [29], the complexity of the disease warrants a search for additional compound classes and a study of their relationship with the biochemical processes underlying RA. Free amino acids and their derivative biogenic amines play essential roles in both energy production and protein synthesis/degradation; thus, changes in the levels of these amine metabolites may reflect changes in the body's state and catabolism of proteins in RA disease. Therefore, we used a liquid chromatography mass spectrometry (LC/MS)-based amine platform to measure the levels of amine metabolites in the plasma of CIA and control mice. We observed reduced levels of amine metabolites in the plasma of CIA mice, possibly reflecting systemic changes in this model of RA. Based on these results, we speculate that decreased amine metabolite levels likely reflects muscle mass loss and protein degradation and may associates with inflammatory activity.

2. Materials and Methods

2.1 Induction of Arthritis by Co-Administration of Collagen Type II and Lipopolysaccharide

A total of 20 male DBA/1J mice (age 6–7 weeks) were obtained from Charles River Laboratories (Yokohama, Japan). The animals were randomly divided into two groups, with ten mice in the experimental (CIA) group and ten mice in the control (Ctrl) group. The protocol for inducing arthritis is well established and has been described in detail [29]–[31]. In brief, the mice were given intraperitoneal (i.p.) injections containing collagen type II (extracted from bovine nasal cartilage and dissolved in acetic acid) and lipopolysaccharide (extracted from *Escherichia. coli* 011:B4 and dissolved in phosphate-buffered saline) in order to induce chronic polyarthritis by stimulating an autoimmune response; control mice received i.p. injections of vehicle (acetic acid and phosphate-buffered saline) only. All animals were housed in a temperature- and light-controlled environment with free access to standard rodent chow and water throughout the experiments. After repeated injections (administered on days 0, 14, 28, 42, and 56), blood samples were collected from each animal on day 70 and stored in pre-cooled Vacutainer tubes (BD Vacutainer, Plymouth, UK) containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. After centrifugation, the EDTA-plasma fractions were collected and aliquots—including individual study samples and pooled quality control (QC) samples—were stored at -80ºC until further analysis. During sampling, one mouse in the CIA group died; thus, the final analysis is based on 9 CIA mice and 10 control mice.

2.2 Extraction of Amine Metabolites and Analysis using UPLC-MS/MS

The methods for extracting and analyzing amine metabolites were adapted for mouse plasma samples based on a previously described protocol [32]. For each sample, a 5-µl aliquot of plasma was used for the analysis. A mixture of internal

standards containing $^{13}C^{15}N$-labeled amine metabolites was added to each 5-µl plasma sample. After the proteins were precipitated using MeOH, the supernatant was transferred to a fresh Eppendorf tube and dried under N_2. The residue was then dissolved in borate buffer (pH 9), and 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) derivatization reagent (Waters, Etten-Leur, The Netherlands) was added. The reaction mixture was then neutralized by the addition of formic acid (20%), and the solution was transferred to injection vials for ultra-high-performance LC tandem MS (UPLC-MS/MS) analysis (injection volume: 1.0 µl) using an ACQUITY UPLC system (Waters) equipped with an AccQ-Tag Ultra column (2.1 mm × 100 mm, 1.7 µm particles, Waters) coupled to a Xevo mass spectrometer with electrospray ionization source (Waters). Multiple reaction monitoring was performed in the positive ion mode in order to monitor the analytes. A gradient elution starting with Eluent A (water containing 2% formic acid) and ramping to Eluent B (aqueous acetonitrile containing 2% formic acid) was used as the mobile phase in the UPLC system. The samples were analyzed in random order.

2.3 Data Processing and Statistical Analysis

The integrated peak areas of the target analytes were calculated using Quanlynx software (Waters) and corrected using the appropriate internal standards. The response ratio (calculated at the ratio between the target analyte and the respective internal standard) was used for further statistical analysis. The reproducibility and reliability of each metabolite measurement was determined using repeated measurements of the QC pool performed after every ten samples. By defining the acceptable relative standard deviation as <15%, 45 amine metabolites (from a starting list of 74) were considered high quality and were included in the final list for further analysis. The data were log-transformed to correct for distribution skewness and auto-scaled to achieve uniform units.

To visualize clustering of individual samples, unsupervised principal component analysis (PCA) was performed using MetaboAnalyst version 3.0 (http://www.metaboanalyst.ca) [33]. To measure the significance of differences in each individual amine metabolite between the CIA group and the Ctrl group, a two-sided unpaired Student's *t*-test was performed, assuming unequal variance; differences with a *p*-value <0.05 were considered significant (H_0: group means are equal). Fold change (FC) was then calculated in order to determine the direction ($\log_2$ of FC) and magnitude (FC ratio reflecting the CIA/Ctrl ratio) of differences between two group mean values. A positive value for the $\log_2$ of FC indicates higher levels of metabolites in the CIA group, whereas a negative value indicates lower levels of metabolites in the CIA group. In the FC analysis, a minimum threshold of 1.5 was used, meaning that the ratio of metabolites between the CIA and Ctrl groups exceeded 1.5.

3. Results

A 2D plot of the PCA scores was generated using an unsupervised pattern recognition method and was used to provide a visual overview of the natural distribution of amines detected in the plasma samples of the nine CIA and ten Ctrl mice (Fig. 1). PC1 and PC2 accounted for 56.6% and 13.9% of the variation, respectively; thus, these two principal components (i.e., PC1 and PC2) explained a total of 70.5% of the variance. From the 2D plot of the PCA scores, the CIA group (depicted with triangles symbol) and the Ctrl group (depicted with the "+" symbol) were generally distributed in distinct regions with respect to PC1, with the CIA samples clustering largely on negative side of the plot and the Ctrl samples clustering largely on the positive side of the plot, thereby reflecting group differences with respect to the composition of free amine metabolites in the plasma samples.

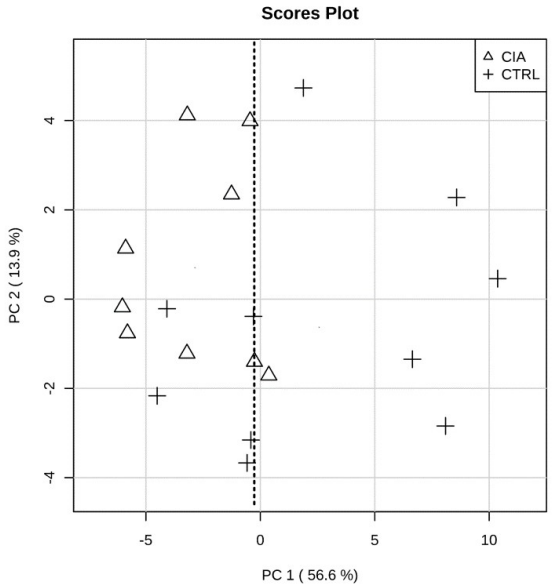

Fig. 1 2D plot of the PCA scores for the amine metabolites measured in the plasma samples from CIA (△) and control (+) mice. The plot of the PCA scores shows that the two groups form distinct clusters along the *x*-axis (corresponding to PC1), indicated by the vertical dashed line.

To measure whether the differences between the two groups were significant, we performed a Student's *t*-test for each amine metabolite. In total, 20 of the 45 detected amine metabolites differed significantly differences between the two groups ($p<0.05$); these 20 amine metabolites are shown in Fig. 2, and all 45 detected amine metabolites are summarized in Table 1. Metabolite changes were reported in the table 1 only when the *p*-values from the Student's test were lower than 0.1. Table 1 also lists the false discovery rate-adjusted *p*-values. Fold change (FC) analysis was performed to indicate the direction of change and the magnitude of change for the detected amine metabolites (FC of the CIA/Ctrl ratio). The analysis revealed that 11 amine metabolites decreased by more than one-third in the CIA group ($FC_{CIA/Ctrl}$ <0.67). The $\log_2$ value of FC indicates that 43 of the 45 amine metabolites detected (95.6%) were lower in the CIA group (i.e., a negative $\log_2$ value of FC), whereas the remaining two metabolites (methylcysteine and O-phosphoethanolamine) were higher in the CIA group.

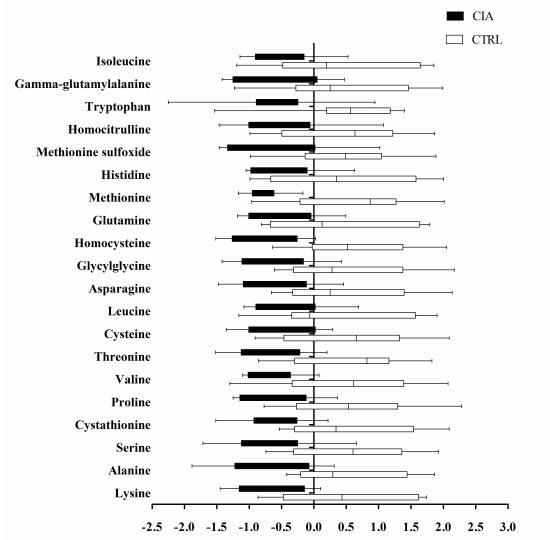

Fig. 2 Summary of the 20 amine metabolites that differed significantly between the collagen-induced arthritis (CIA) and control groups ($p<0.05$). The values are presented as the response ratio of the peak area (determined as the ratio of the target amine metabolite to its corresponding internal standard) after logarithmic transformation and auto-scaling.

Table 1. Summary of the 45 amine metabolites detected in CIA and control mice

Amine Metabolite	HMDB	*p*-value	FDR	$FC_{CIA/Ctrl}$	Direction of Change
Methionine	HMDB00696	**0.001**	0.017	0.533	↓
Homocysteine	HMDB00742	**0.001**	0.017	0.640	↓
Threonine	HMDB00167	**0.001**	0.017	0.628	↓
Proline	HMDB00162	**0.003**	0.031	0.521	↓
Alanine	HMDB00161	**0.003**	0.031	0.573	↓
Cystathionine	HMDB00099	**0.005**	0.032	0.818	↓
Valine	HMDB00883	**0.005**	0.032	0.650	↓
Glycylglycine	HMDB11733	**0.006**	0.032	0.660	↓
Lysine	HMDB00182	**0.007**	0.032	0.686	↓
Serine	HMDB00187	**0.007**	0.032	0.815	↓
Asparagine	HMDB00168	**0.009**	0.036	0.667	↓
Cysteine	HMDB00574	**0.012**	0.044	0.802	↓
Tryptophan	HMDB00929	**0.015**	0.051	0.789	↓
Homocitrulline	HMDB00679	**0.017**	0.051	0.737	↓
Methionine sulfoxide	HMDB02005	**0.017**	0.051	0.582	↓
Isoleucine	HMDB00172	**0.020**	0.056	0.682	↓
Gamma-glutamylalanine	HMDB06248	**0.021**	0.056	0.616	↓
Histidine	HMDB00177	**0.041**	0.103	0.799	↓
Glutamine	HMDB00641	**0.047**	0.107	0.775	↓
Leucine	HMDB00687	**0.048**	0.107	0.741	↓
Citrulline	HMDB00904	0.052	0.112	0.818	↓
Saccharopine	HMDB00279	0.086	0.168	0.698	↓
Ornithine	HMDB00214	0.087	0.168	0.727	↓
2-Aminoadipic acid	HMDB00510	0.093	0.168	0.732	↓
Phenylalanine	HMDB00159	0.094	0.168	0.755	↓
Homoserine	HMDB00719	0.103	0.178	0.852	-
Methylcysteine	HMDB02108	0.114	0.187	1.330	-
Sarcosine	HMDB00271	0.119	0.187	0.860	-
Arginine	HMDB00517	0.120	0.187	0.866	-
Tyrosine	HMDB00158	0.154	0.231	0.723	-
Alpha-aminobutyric acid	HMDB00452	0.165	0.239	0.765	-
Kynurenine	HMDB00684	0.270	0.379	0.861	-
Glycine	HMDB00123	0.335	0.439	0.902	-
Beta-alanine	HMDB00056	0.338	0.439	0.809	-
Putrescine	HMDB01414	0.341	0.439	0.777	-
Norepinephrine	HMDB00216	0.371	0.463	0.660	-
Glutamic acid	HMDB00148	0.399	0.485	0.850	-
5-Hydroxylysine	HMDB00450	0.417	0.494	0.630	-
Glutathione	HMDB00125	0.497	0.573	0.841	-
4-Hydroxyproline	HMDB06055	0.565	0.635	0.934	-
Aspartic acid	HMDB00191	0.713	0.781	0.960	-
Serotonin	HMDB00259	0.729	0.781	0.699	-
Spermidine	HMDB01257	0.785	0.812	0.933	-
O-Phosphoethanolamine	HMDB00224	0.794	0.812	1.015	-
Ethanolamine	HMDB00149	0.963	0.963	0.858	-

CIA, collagen-induced arthritis; Ctrl, control; HMDB, Human Metabolome Database; FC, fold change; FDR, false discovery rate

4. Discussion

RA is a chronic disease in which the immune response is dysregulated and the levels of several cytokines and factors are elevated, including TNFα, IL-1β, IL-6, IFNγ, and ROS [34], [35]; in addition, NF-κB activation is increased [36]. Changes in metabolic factors such as arachidonic acid–derived inflammatory mediators have also been reported in RA [37], suggesting that a metabolomics approach may provide insight into the biochemical processes underlying this disease.

In addition to the well-characterized inflammatory dysregulation in RA, muscle wasting and energy expenditure are also common features and are linked to the production of cytokines during the immune response [38]–[40]; muscle wasting and energy expenditure can then dysregulate the protein degradation pathway, leading to perturbed metabolic processes [15], [16], [19], [41]–[43]. Given the close relation between amine metabolites and proteins, it is therefore reasonable to speculate that changes in amine metabolites may reflect protein dysregulation which owing to muscle wasting and energy expenditure. However, few studies have focused on measuring muscle wasting in RA by measuring the plasma levels of amine metabolites.

Studies of the biochemical processes associated with RA revealed that activated NF-κB is linked to skeletal muscle loss [44], and this activation has been observed in animal models of RA [45], [46]. Moreover, injecting TNF and IL-1 into healthy rats causes muscle wasting [47]. Previously, we reported increased levels of inflammatory mediators and ROS-generated oxylipins in the plasma of CIA mice, and this was associated with the production of cytokines and increased NF-κB activation [29]. Increased ROS levels, which affect muscle signaling pathways, have also been measured in CIA mice [48]; similar results have been reported in tumor-bearing rats [49]. Given that increased cytokines, ROS, and NF-κB activation robustly affect muscle metabolism, we expected to identify a metabolic “signature” in the plasma of CIA mice.

Building on the previous report of increased inflammatory mediators and increased ROS-generated oxylipins in CIA mice [29], we used a targeted amine platform to evaluate the changes in plasma amine metabolites in age- and gender-matched CIA mice compared with control mice. We found that the plasma amine metabolomes were clearly distinguishable between CIA mice and control mice. Specifically, 20 amine metabolites were significantly lower in the plasma of CIA mice.

Given that certain free amino acids such as branched-chained amino acids are closely associated with protein degradation, amino acids—and their biogenic amines—might be used as a biomarker of muscle wasting [50]. In support of this notion, decreased plasma levels of some amine metabolites have been reported in other diseases (e.g., chronic obstructive pulmonary disease) and have been linked to resting energy expenditure and muscle wasting [51]. Increased excretion of nitrogen into the urine due to muscle wasting has been reported in RA patients [17], [52], and increased levels of acyl-carnitines in the urine of RA patients reflect muscle breakdown [53]. Together, these lines of evidence suggest that muscle wasting is a highly relevant phenomenon related to RA. However, to date relatively few clinical studies examined muscle wasting in RA by measuring amine metabolite levels. The large decrease in plasma amine metabolite levels (e.g., histidine, valine, leucine, phenylalanine, and tryptophan metabolites) is consistent with a previous study of CIA rats by Zhang et al. [54]. The earliest studies of amino acids regulation in RA patients date back to the mid-20th century [55], when researchers found decreased levels of several amino acids but were relatively limited with respect to the biological interpretation. Kobayashi et al. measured a similar decrease in some amine metabolites in the plasma of Japanese patients with RA [56]; although the authors used these results to demonstrate a relationship between ornithine metabolism and inflammation, they did not discuss the possible biological interpretation of non-significantly changed amine metabolites, including alanine, isoleucine, leucine, lysine, serine, and valine [56]. In addition, other clinical studies have reported inconsistent changes in the levels of amine

metabolites, and did not attribute these changes to muscle wasting [57]–[61]. The difference between our CIA mouse model and RA patients with respect to changes in amine metabolites may be due to differences in catabolic processes between mice and humans. Alternatively, the relative complexity of clinical data in patients may mask certain changes in amine metabolites, as various confounding variables are not always taken into consideration in clinical studies, including factors such as age, gender, illness stage, treatment protocol, and diet. Our findings indicate that CIA mice are a valuable tool for studying the pathological processes that underlie RA; specifically, this model is easy to induce, and researchers can easily control/exclude confounding factors that may affect the study results, including age, gender, genetic background, and drug exposure.

In summary, combining our previous oxylipin results and our current amine metabolomics results allows us to speculate upon the biological relationship between muscle wasting and the inflammatory response in RA (Fig. 3). In addition, our results indicate that muscle wasting conditions such as cachexia can be measured using a metabolomics approach (for example, by measuring amine metabolites). Lastly, our results indicate that changes in branched-chain amino acids as well as other amine metabolites may reflect muscle wasting status in RA.

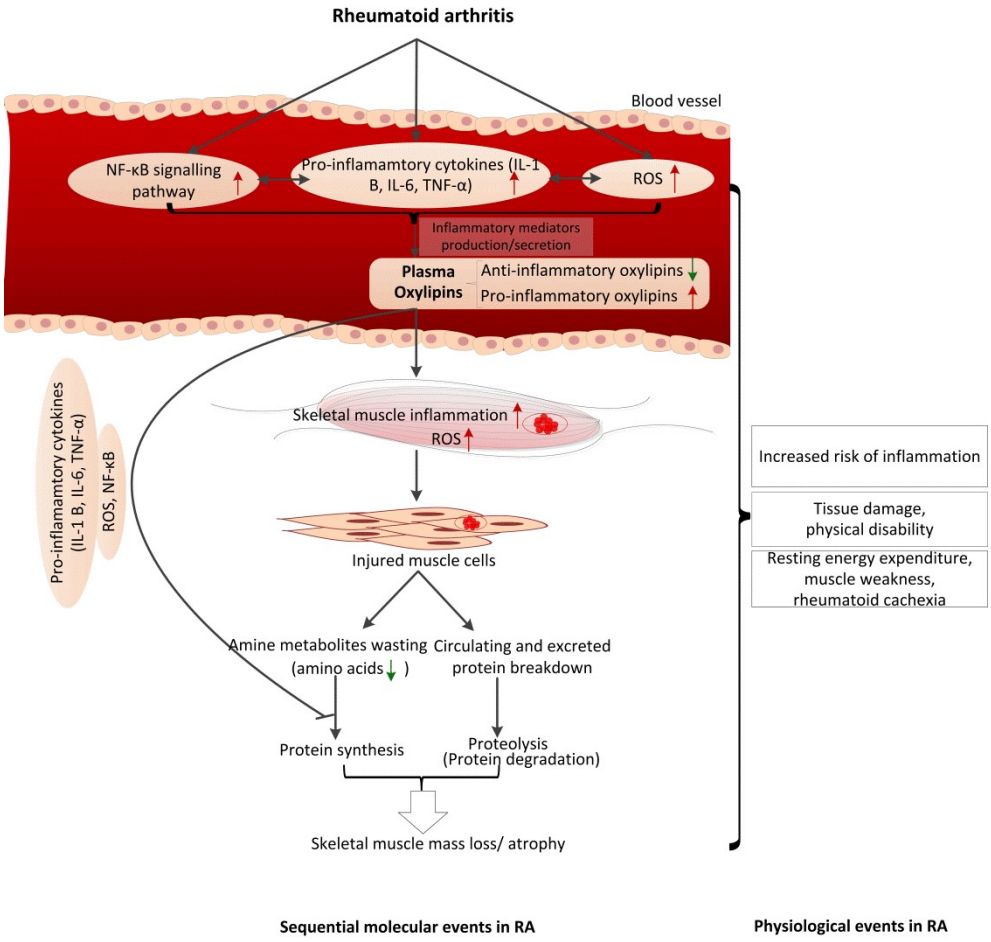

Fig. 3. Proposed biological interpretation of muscle wasting in RA. In RA, increased levels of inflammatory cytokines, ROS, and NF-κB activation play a role in the production of inflammatory oxylipins, which then trigger an inflammatory response in muscle cells. The inflammatory response then increases resting energy expenditure and thermogenesis, leading to amino acid wasting and accelerating protein breakdown. Thereafter, the accelerated protein catabolism and the subsequent reduction in amines—accompanied by the excretion of nitrogen in the urine, causes the muscle mass loss/atrophy that manifests clinically as muscle weakness / cachexia in RA patients.

5. Conclusion

In summary, using metabolomics, we found that the levels of amine metabolites are systematically decreased in the plasma of CIA mice, which is consistent with similarities between our CIA mouse model and RA patients at the metabolomics level. This result indicates that the muscle wasting and energy expenditure issues (e.g., cachexia) associated with RA—and models of RA—are highly complex.

The cachexia and sarcopenia associated with muscle atrophy, protein breakdown, and energy expenditure are not unique to RA. For example, several other chronic inflammatory diseases have been associated with catabolic wasting, including cancer [62], HIV/AIDS) [63], type 2 diabetes [64], renal failure, uremia [65], and heart failure [66]. We therefore hypothesize that systemic decreases in the levels of amine metabolites may reflect muscle mass loss and protein degradation due to inflammation.

Considering the complexity and consequences of muscle wasting in a wide variety of chronic diseases, using a metabolomics-based approach may provide a clearer understanding of the biological processes involved in these diseases.

6. Acknowledgement

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7. Compliance with Ethical Requirements

Conflict of interest: We have no conflict of interest to declare.

Ethical approval: The study was performed in accordance with the guidelines established in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All experiments were approved by the Tohoku Institute of Technology Research Ethics Committee, Sendai, Japan (approval date: January 18, 2009).

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