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Article

Tissue-specific landscape of metabolic dysregulation during ageing

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Abstract: The dysregulation of cellular metabolism is a hallmark of ageing. To understand the metabolic changes that occur as a consequence of the ageing process and to find biomarkers for age-related diseases, we conducted a metabolomic analysis of brain, heart, kidney, liver, lung and spleen in young (9-10 weeks) and old (96-104 weeks) wild type (mixed genetic background of 129/J and C57BL/6) mice using NMR spectroscopy. We found differences in metabolic fingerprints of all tissues and identified several metabolites to be altered in most tissues, suggesting that they may be universal biomarkers of ageing. In addition, we found distinct tissue-clustered sets of metabolites throughout the organism. The associated metabolic changes may reveal novel therapeutic targets for the treatment of ageing and age-related diseases. Moreover, the identified metabolite biomarkers could provide a sensitive molecular read-out to age determine the age of biologic tissues and to validate the effectiveness and potential off-target effects of senolytic drug candidates on both a systemic and tissue-specific level.

Keywords: ageing; tissues-specific; metabolomics; biomarker.

1. Introduction

Ageing can be defined as the process during which structural and functional changes accumulate in an organism as a result of the passage of time. Overall, ageing is characterized by a reduction in the ability to maintain metabolic and functional homeostasis in multiple tissues (1). This can occur at vastly different compartments within the cell, and implies that ageing proceeds as a consequence of the interplay between a multitude of pathways, rather than from a single cause. Altogether, the following nine hallmarks are most frequently proposed to be epiphenomena of ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, stem cell exhaustion, altered intercellular communication and cellular senescence (1). In particular, senescence has been proposed to contribute to the course of ageing and

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age-related diseases (2) by imbalanced cellular function, leading to increased DNA damage, generation of reactive metabolites, oxidative stress and inflammation (3-5). These changes can lead to pathophysiological manifestations like tissue atrophy and loss of nerves, both of which are common in ageing tissues. In addition, they are associated with age-related pathologies, such as geriatric syndromes, Parkinson's and Alzheimer's disease, diabetes mellitus type 2 and atherosclerosis (6-13). Whereas those conditions differ greatly in their clinical manifestations, they share a common trait of dysregulated metabolism (13-18). As an example, blood concentrations of branched chain amino acids (BCAAs), lipids with low carbon numbers or sugar metabolites are increased in diabetes mellitus type 2 (16, 17), whereas methionine, histidine, lysine and phosphatidylethanolamine can be detected at increased levels in patients suffering from Alzheimer's disease (18). Moreover, there is increasing evidence that metabolic changes do not only occur as a consequence of ageing processes, but, vice versa, might be drivers thereof (14).

In each organism, tissues are combined in structural and functional units to form organs. Different organs are integrated and connected together through blood and lymph vessels to form an entire organism. The tissue conditions may affect basic vital functions and the health status of the entire organism and vice versa. In respect to alterations of the metabolome on the tissue level associated with ageing, only a few investigations have been performed so far in mice, and even less in humans (19-22). Mice are a key tool for ageing research due to their relatively short life span, allowing monitoring of the ageing process within a reasonable time frame, and due to the feasibility of performing genetic manipulations. Ageing research has so far mostly focused on genetically modified mice mimicking progeroid syndromes and not on animals that age by themselves. Therefore, normal aged mice are a highly valuable model to investigate the alterations in metabolites and metabolic pathways as a consequence of the spontaneous deterioration of the homeostatic balance over time. Metabolomics enables the capturing of the entire metabolic state of an organism, allows its temporal resolution at distinct time points during the ageing process, and helps to identify altered pathways and biomarkers during ageing and in disease (23, 24). Today´s biomarkers of ageing mainly include phenotypical read-outs such as frailty or grip-strength (25) and a small set of to-be-further-evaluated molecular markers, which provide a more general assessment of the physiological age (26). These biomarkers are a valuable tool to describe the physiological changes that occur with age, the process of ageing and the occurrence of age-related diseases.

Here, we aimed to provide a comprehensive set of ageing-related metabolic biomarkers in mouse tissues for the identification of tissue-specific and systemic metabolic changes in an ageing organism. To this end, we employed untargeted Nuclear Magnetic Resonance (NMR) spectroscopy and determined changes in polar metabolites in multiple tissues throughout different time points in the course of the lifespan of wild type mice. Specifically, we studied metabolic changes of brain, heart, kidney, liver, lung and spleen in young (9-10 weeks) and aged (96-104 weeks) wild type mice (mixed genetic background of 129/J and C57BL/6J). We found alterations in the metabolic phenotypes of all tissues and identified sets of both, tissue-specific and systemic metabolite biomarkers of ageing. We identified the following organ-specific biomarkers: i) BCAAs, uracil and glutamine in the brain, ii) leucine, isoleucine, valine and 4-aminobutyrate (GABA) in the heart, iii) succinate and choline in the kidney, iv) nicotinamide, glycerol and inosine in the liver, v) lysine, nicotinamide, aspartate and fumarate in the lung, and vi) taurine and uridine in the spleen. Uridine changed systemically in most tissues, indicating conserved mechanisms of ageing. Our comprehensive metabolic profiling of the key mouse tissues at different age provides a robust set of metabolic biomarker candidates to study the mechanisms of metabolic reprograming associated with ageing. A deeper understanding of the underlying processes might not only shed light onto the causes of age-related pathologies, but also help to discover novel targets for pharmacological interventions. These biomarker candidates could serve as a read-out of the biological age of tissues and may be utilized to validate the effectiveness of proposed senolytic therapies. Taken together, comprehensive analyses and utilization of metabolomics provide a useful tool to monitor changes of metabolites during ageing and

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degenerative process, respectively, and may eventually help to increase health span and, thus the life quality of the aged population.

2. Materials and Methods

2.1. Animals and diets

For all experiments, young (9-10 weeks) and old (96-104 weeks) female wild type mice (mixed genetic background of 129/J and C57BL/6J) were used (n=5). Mice were maintained in a clean, temperature-controlled (22 ± 1°C) environment with a regular light–dark cycle (12 h/12 h) and unlimited access to chow (Altromin 1324, Altromin Spezialfutter GmbH, Lage, Germany) and water. All experiments were performed in accordance with the European Directive 2010/63/EU and approved by the Austrian Federal Ministry of Education, Science and Research.

2.2. NMR sample preparation, data acquisition and analysis

Tissue samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. For NMR metabolomics analysis, 30-50 mg of each tissue were resected. To extract metabolites, 400 µl of ice-cold methanol and 200 µl MilliQ H_2O were added to the tissue and transferred to a tube containing Precellys beads (1.4 mm zirconium oxide beads, Bertin Technologies, Villeurbanne, France) for homogenization by Precellys24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). After centrifugation at 13,000 rpm for 45 min (4°C), the supernatant was transferred to a fresh tube, and the samples were lyophilized. For NMR experiments, samples were re-dissolved in 500 µl of NMR buffer [0.08 M Na_2HPO_4, 5 mM TSP (3-(trimethylsilyl) propionic acid-2,2,3,3-d_4 sodium salt), 0.04 (w/v) % NaN_3 in D_2O, pH adjusted to 7.4 with 8 M HCl and 5 M NaOH].

Tissue metabolic profiling analysis was conducted at 310 K using a 600 MHz Bruker Avance Neo NMR spectrometer equipped with a TXI 600S3 probe head. The Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence was used to acquire ^1H 1D NMR spectra with a pre-saturation for water suppression (cpmgpr1d, 512 scans, 73728 points in F1, 12019.230 Hz spectral width, 1024 transients, recycle delay 4 s). NMR spectral data were processed as previously described (27). Shortly, data were processed in Bruker Topspin version 4.0.2 using one-dimensional exponential window multiplication of the FID, Fourier transformation, and phase correction. NMR data were then imported to Matlab2014b, TSP was used as internal standard for chemical shift referencing (set to 0 ppm), regions around the water, TSP and methanol signals were excluded, NMR spectra were aligned, and a probabilistic quotient normalization was performed. Principal component analysis (PCA), orthogonal partial least squares discriminant analysis (O-PLS-DA), and partial least squares-discriminant analysis (PLS-DA) were performed in Matlab2014b and MetaboAnalyst 4.0 (28) as well as all associated data consistency checks and cross-validation. In order to validate the statistical significance of the determined differences, the quality assessment statistic Q^2 is reported. This measure provides information about cross-validation and is a qualitative measure of consistency between the predicted and original data, with a maximum value of 1. Metabolite identification was carried out using Chenomx NMR Suite 8.4 (Chenomx Inc., Edmonton, AB, Canada) and reference compounds. Quantification of metabolites was carried out by signal integration of normalized spectra. Univariate statistical analysis was carried out using Graph Pad Prism 5.01 (GraphPad Software, La Jolla, CA). Data are represented as mean ± standard deviation (SD). P-values were calculated using a two-tailed Student's t-test for pairwise comparison of variables, and are only stated for metabolite that had a p-value < 0.05.

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3. Results

We sought to establish both tissue-specific profiles and systemic metabolic signatures of ageing, which could serve as a basis for understanding the overall ageing process. ^{1}H-NMR spectroscopy is a powerful technique capable of simultaneous identification and quantification of multiple metabolites in complex biological matrices (29). To better understand the systemic and tissue-specific ageing process and to identify metabolites influenced by age, we carried out metabolic profiling of brain, heart, kidney, liver, lung and spleen from young and aged mice using NMR spectroscopy. The identified respective biomarker candidates will provide a valuable resource for a variety of applications in ageing research and drug discovery.

Neurocognitive ageing is characterized by a reduction in the information processing time, an impaired long-term memory (30), both of which are related to an imbalance in energy metabolism and redox homeostasis (31). Using untargeted NMR spectroscopy of brain samples, we first determined the metabolic fingerprints in young (9-10 weeks) and aged mice (96-104 weeks). The discriminant clustering between young and old tissues shown in the Orthogonal-Partial Least Squares - Discriminant Analysis (O-PLS-DA) plot in Figure 1A indicates the underlying differences in the metabolome, supported by the correlation coefficients R^2Y up to 0. 997 ($p=0.02$) and a positive Q^2 of 0.727 ($p=0.03$), validating the significance of these results. Reduced NMR spectra revealed altered metabolites in mouse brains of different ages (Figure 1B) with decreased concentrations of lactate, methionine, N-acetylaspartate, uridine and inosine. In contrast, concentrations of leucine, isoleucine, valine, glutamine, allantoin, uracil, tyrosine and phenylalanine were increased in the aged mice (Figure 1B, C).

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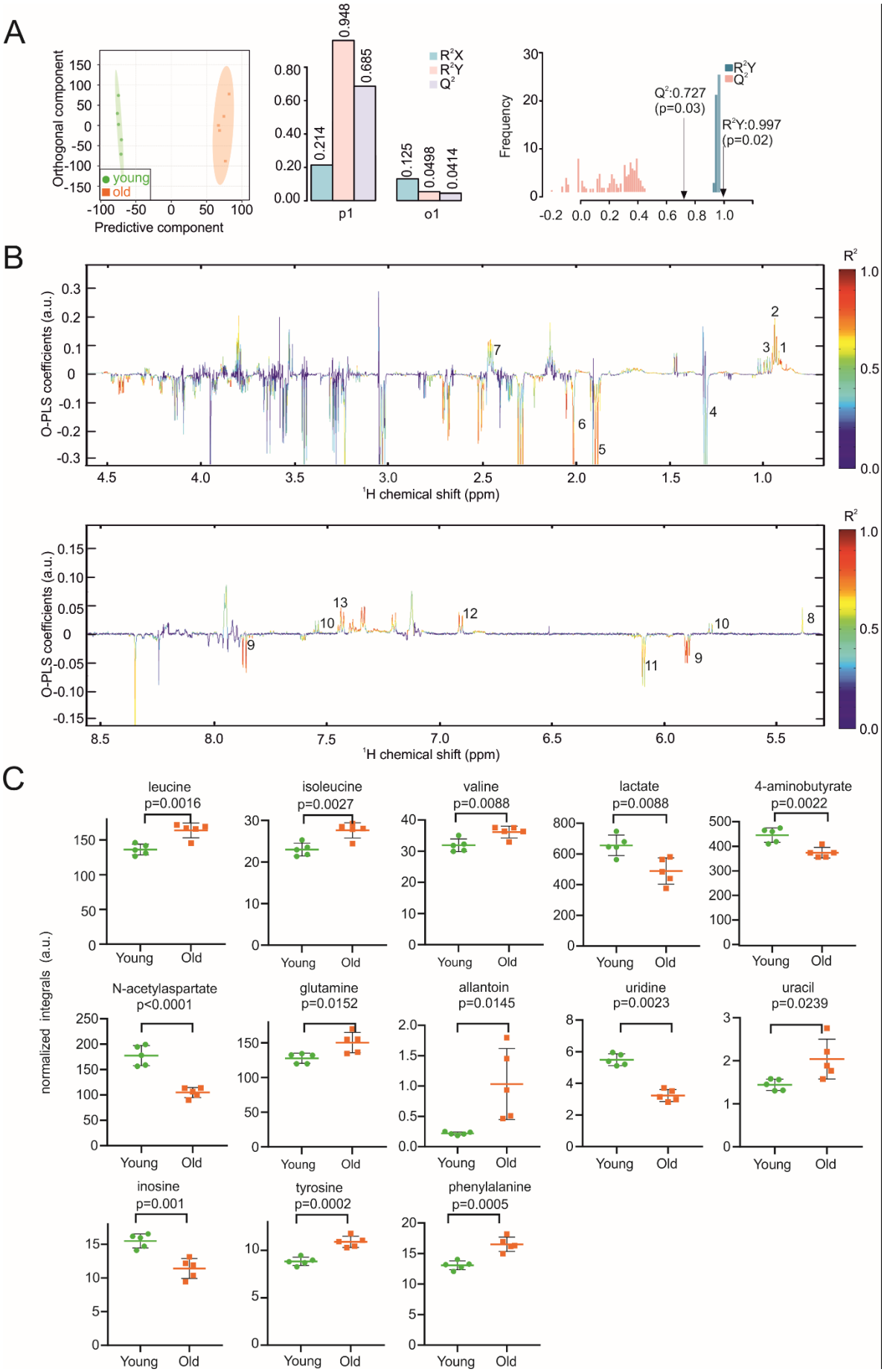

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Figure 1. NMR metabolomics analysis of mouse brain samples. (A) O-PLS-DA plot of brain samples, including cross validation. (B) The reduced NMR spectrum reveals altered components in normalized brain samples. Positive covariance corresponds to components present at increased concentrations, whereas negative covariance corresponds to decreased component concentration. Predictivity of the model is represented by R^2. 1. Leucine, 2. isoleucine, 3. valine, 4. lactate, 5. methionine, 6. N-acetylaspartate, 7. glutamine, 8. allantoin, 9. uridine, 10. uracil, 11. inosine, 12. tyrosine, 13. phenylalanine. (C) Statistical analysis using Student's t-test of individual metabolites in brain samples. $p < 0.05$ was considered statistically significant.

Impaired metabolic flexibility is a hallmark of the ageing heart, with decreased capacity to oxidize fatty acids and increased glucose metabolism (32). In order to assess metabolic differences between young and old murine heart samples, we recorded NMR spectra of heart lysates followed by metabolic profiling. When comparing the metabolic fingerprints between heart samples isolated from young and aged mice, O-PLS-DA revealed distinct clustering of respective heart samples with correlation coefficients R^2Y up to 0.999 ($p=0.19$) and a Q^2 of 0.786 ($p=0.02$) (Figure 2A). Reduced NMR spectra demonstrated altered abundance of metabolites in normalized heart samples (Figure 2B) and indicated decreased uridine concentrations in the hearts of aged mice, whereas the levels of leucine, isoleucine, valine, acetate, GABA, creatine, uracil, tyrosine and phenylalanine were increased (Figures 2B, C).

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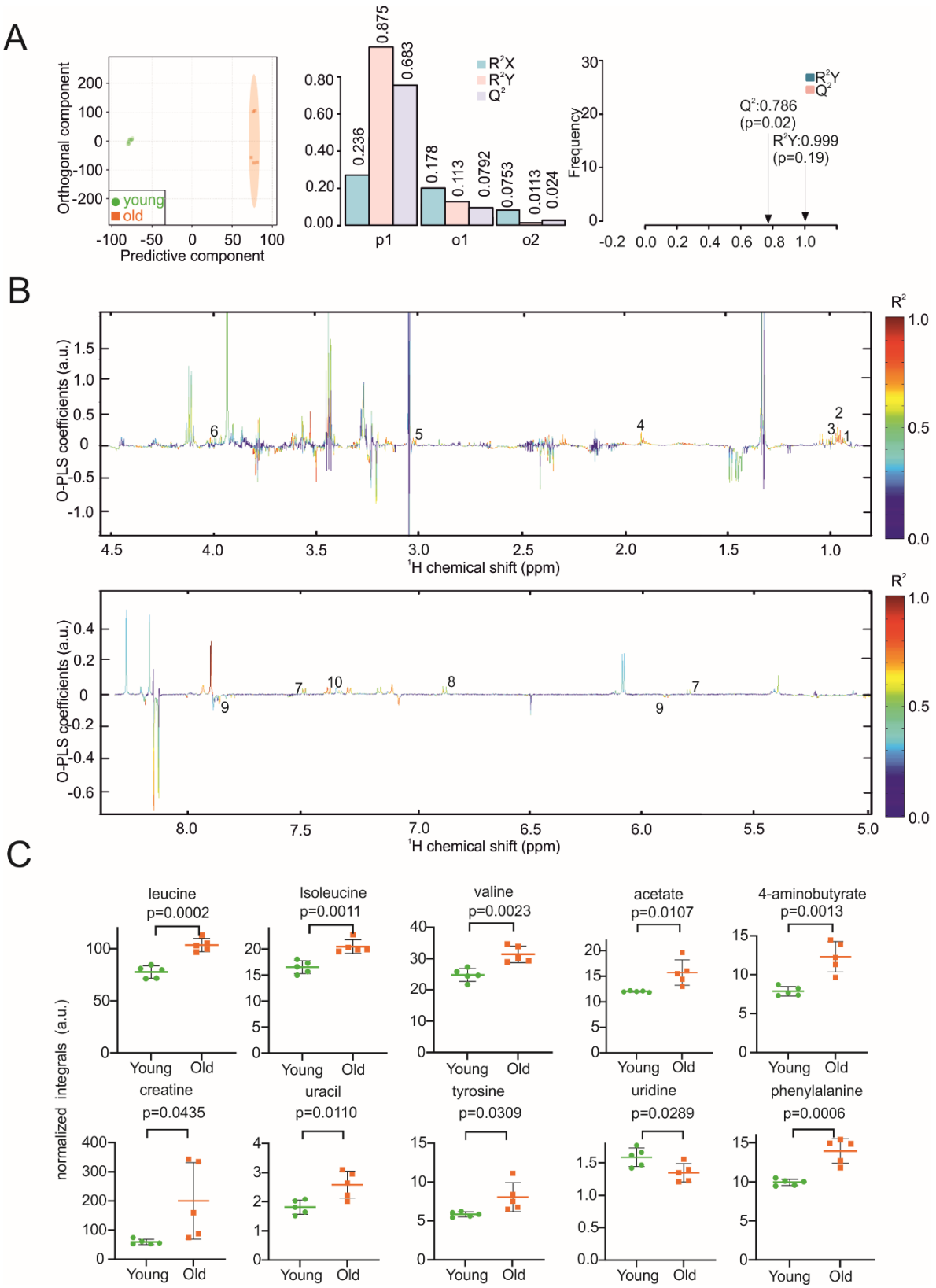

Figure 2. NMR metabolomics analysis of mouse heart samples. (A) O-PLS-DA plot of heart samples, including cross validation. (B) The reduced NMR spectrum reveals altered components in normalized heart samples. Positive covariance corresponds to component present at increased concentrations, whereas negative covariance corresponds to decreased component concentration. Predictivity of the model is represented by R2. 1. Leucine, 2. isoleucine, 3. valine, 4. acetate, 5. 4-aminobutyrate, 6.

 doi:10.20944/preprints202012.0174.v1

creatine, 7. uracil, 8. tyrosine, 9. uridine, 10. phenylalanine. (C) Statistical analysis using Student's t-test of individual metabolites in heart samples. $p < 0.05$ was considered statistically significant.

A plethora of abnormalities in kidney structure and function are positively correlated with advancing age (33). In the kidney, local immune responses induce cellular metabolic reprogramming that changes with ageing (34). To evaluate the age-specific alterations of metabolites in kidney of mice, O-PLS-DA and reduced NMR spectra analysis were performed. The distinct clustering of kidney samples from young and old mice is shown in the score and validation plots of the O-PLS-DA in Figure 3A. The two clusters with correlation coefficients R^2Y up to 0.997 ($p=0.03$) and a Q^2 of 0.898 ($p = 0.01$) are represented in Figure 3A. In reduced NMR spectra, we found differences in the abundance of 23 age-dependent metabolites (Figure 3B). Leucine, isoleucine, valine, alanine, methionine, glutamate, succinate, aspartate, asparagine, lysine, ethanolamine, choline, glycerol, creatine, serine, uridine, inosine, tyrosine and nicotinamide were decreased in the cohort representing the older mice, whereas the levels of allantoin and uracil were increased (Figures 3B, C).

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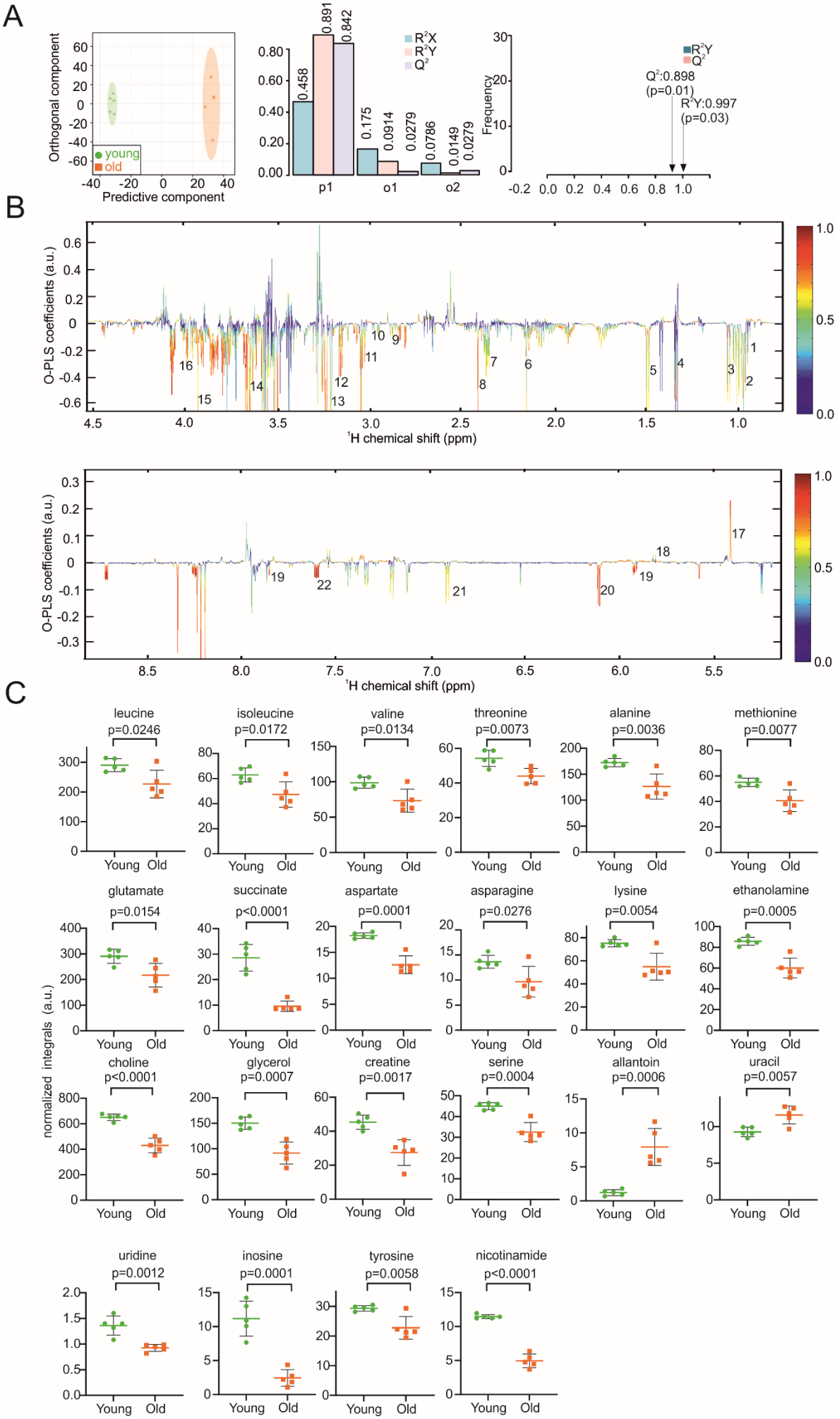

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Figure 3. NMR metabolomics analysis of mouse kidney samples. (A) O-PLS-DA plot of kidney samples, including cross validation. (B) The reduced NMR spectrum reveals altered components in normalized kidney samples. Positive covariance corresponds to component present at increased concentrations, whereas negative covariance corresponds to decreased component concentration. Predictivity of the model is represented by R2. 1. Leucine, 2. isoleucine, 3. valine, 4. threonine 5. alanine, 6. methionine, 7. glutamate, 8. succinate, 9. aspartate, 10. asparagine, 11. lysine, 12. ethanolamine, 13. choline, 14. glycerol, 15. creatine, 16. serine, 17. allantoin, 18. uracil, 19. uridine, 20. inosine, 21. tyrosine, 22. nicotinamide. (C) Statistical analysis using Student's t-test of individual metabolites in kidney samples. $p < 0.05$ was considered statistically significant.

Impaired fatty acid oxidation and increased *de novo* lipogenesis in the liver contribute to the risk for age-associated chronic liver disease (35). Comparable to the other tissues described above, we also identified two distinct metabolic clusters in livers of old and young mouse with correlation coefficients R^2Y up to 0.997 ($p<0.01$) and a positive Q^2 of 0.842 ($p<0.01$) (Figure 4A). Reduced NMR spectra revealed nine metabolites with varying concentrations (Figure 4B). In old mice, the concentrations of lactate, alanine, glycerol, glucose, uridine, inosine, fumarate and nicotinamide were decreased, whereas aspartate was increased (Figure 4B, C).

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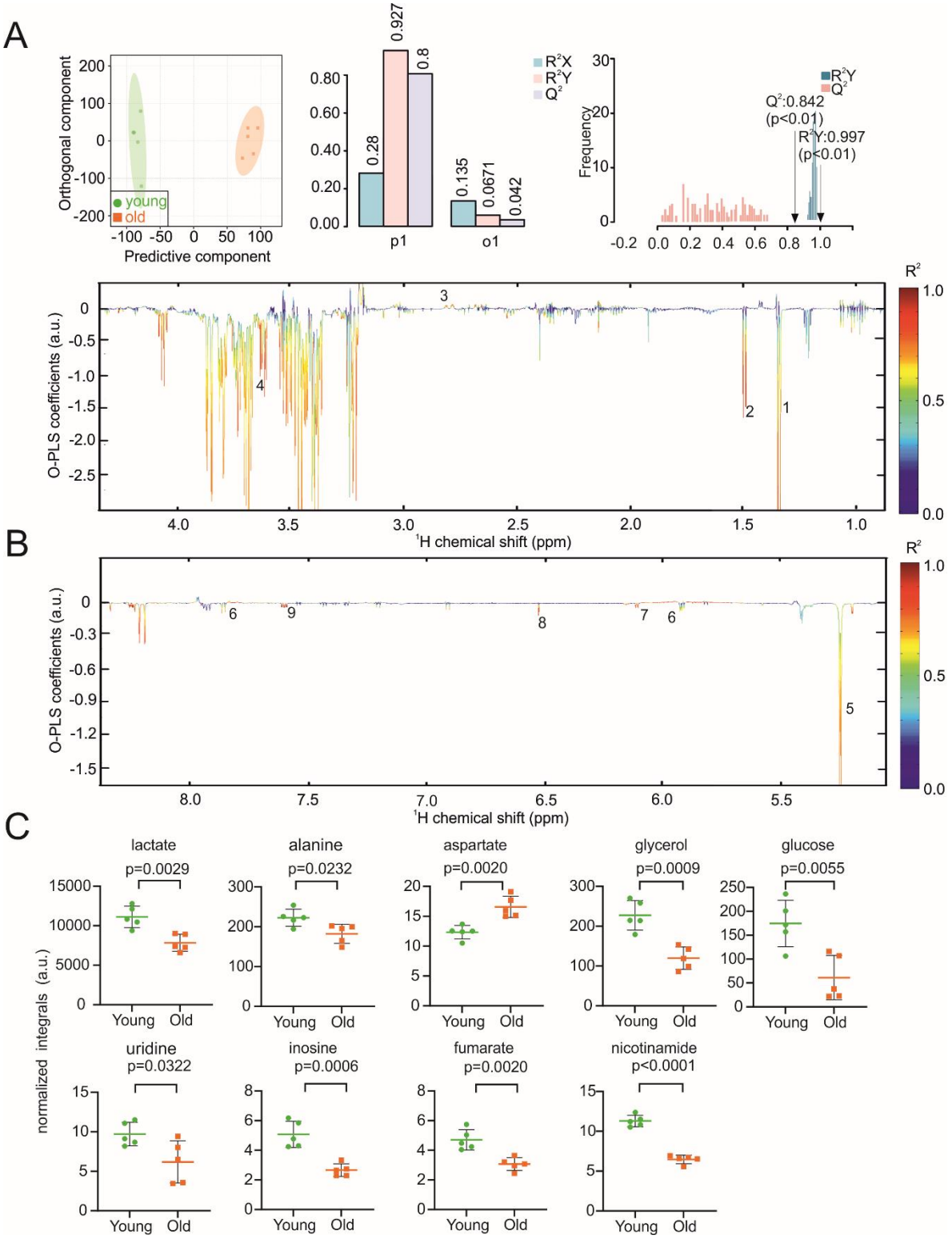

Figure 4. NMR metabolomics analysis of mouse liver samples. (A) O-PLS-DA plot of liver samples, including cross validation. (B) The reduced NMR spectrum reveals altered components in normalized liver samples. Positive covariance corresponds to component present at increased concentrations, whereas negative covariance corresponds to decreased component concentration. Predictivity of the model is represented by R^2. 1. Lactate, 2. alanine, 3. aspartate, 4. glycerol, 5. glucose, 6. uridine, 7. inosine, 8. fumarate, 9. nicotinamide. (C) Statistical analysis using Student's t-test of individual metabolites in liver samples. $p < 0.05$ was considered statistically significant.

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Lung ageing is related to structural remodeling, decreased respiratory function, and chronic lung diseases, which are closely linked to the ageing of the immune system (36). The hierarchical O-PLS-DA scores plots (Figure 5A) allowed a clear discrimination between lung samples from young and old mice with correlation coefficients R^2Y of up to 0.986 ($p=0.26$) and a positive Q^2 of 0.755 ($p=0.02$)). Malonate, fumarate, and nicotinamide were decreased in the old mice, whereas leucine, isoleucine, valine, threonine, methionine, aspartate, lysine, allantoin, tyrosine, and phenylalanine concentrations were increased (Figures 5B, C).

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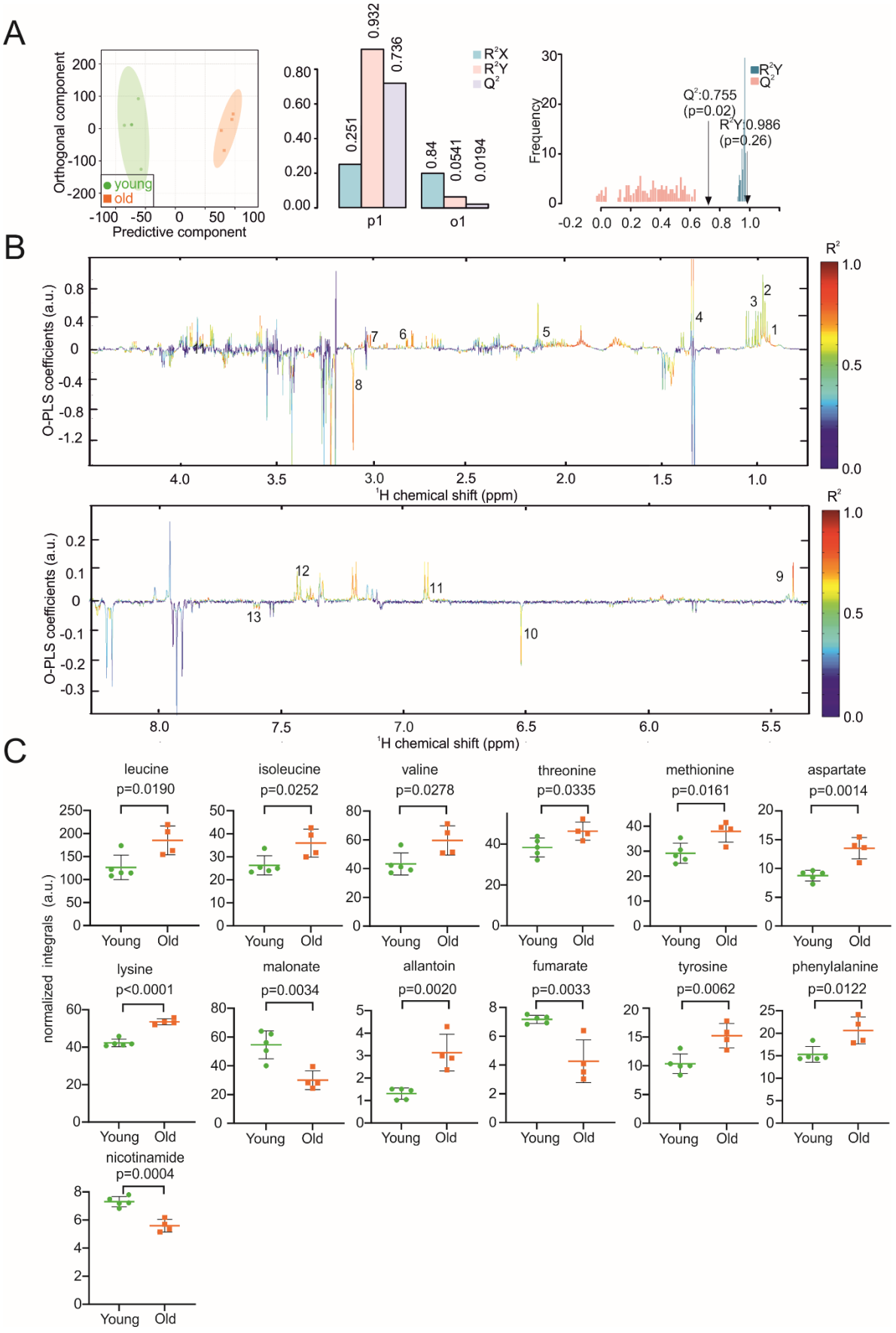

Figure 5. NMR metabolomics analysis of mouse lung samples. (A) O-PLS-DA plot of lung samples, including and cross validation. (B) The reduced NMR spectrum reveals altered components in normalized lung samples. Positive covariance corresponds to component present at increased

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concentrations, whereas negative covariance corresponds to decreased component concentration. Predictivity of the model is represented by R^2. 1. Leucine, 2. isoleucine, 3. valine, 4. threonine, 5. methionine, 6. aspartate, 7. lysine, 8. malonate, 9. allantoin, 10. fumarate, 11. tyrosine, 12. phenylalanine, 13. nicotinamide. (C) Statistical analysis using Student's t-test of individual metabolites in lung samples. $p<0.05$ was considered statistically significant.

The spleen plays an important role in the immune system. In aged mice, structural changes in the spleen result in a less effective or decreased immune response (37). O-PLS-DA models clearly discriminated NMR spectra of spleen samples from young and aged mice (Figure 6A). Reduced NMR spectra revealed decreased concentrations of alanine, methionine, glutamate, aspartate, asparagine, lysine, o-phosphocholine, taurine, glycine, uridine, fumarate, tyrosine, and phenylalanine in the aged mice, whereas lactate, glucose, and allantoin concentrations were increased (Figures 6B, C).

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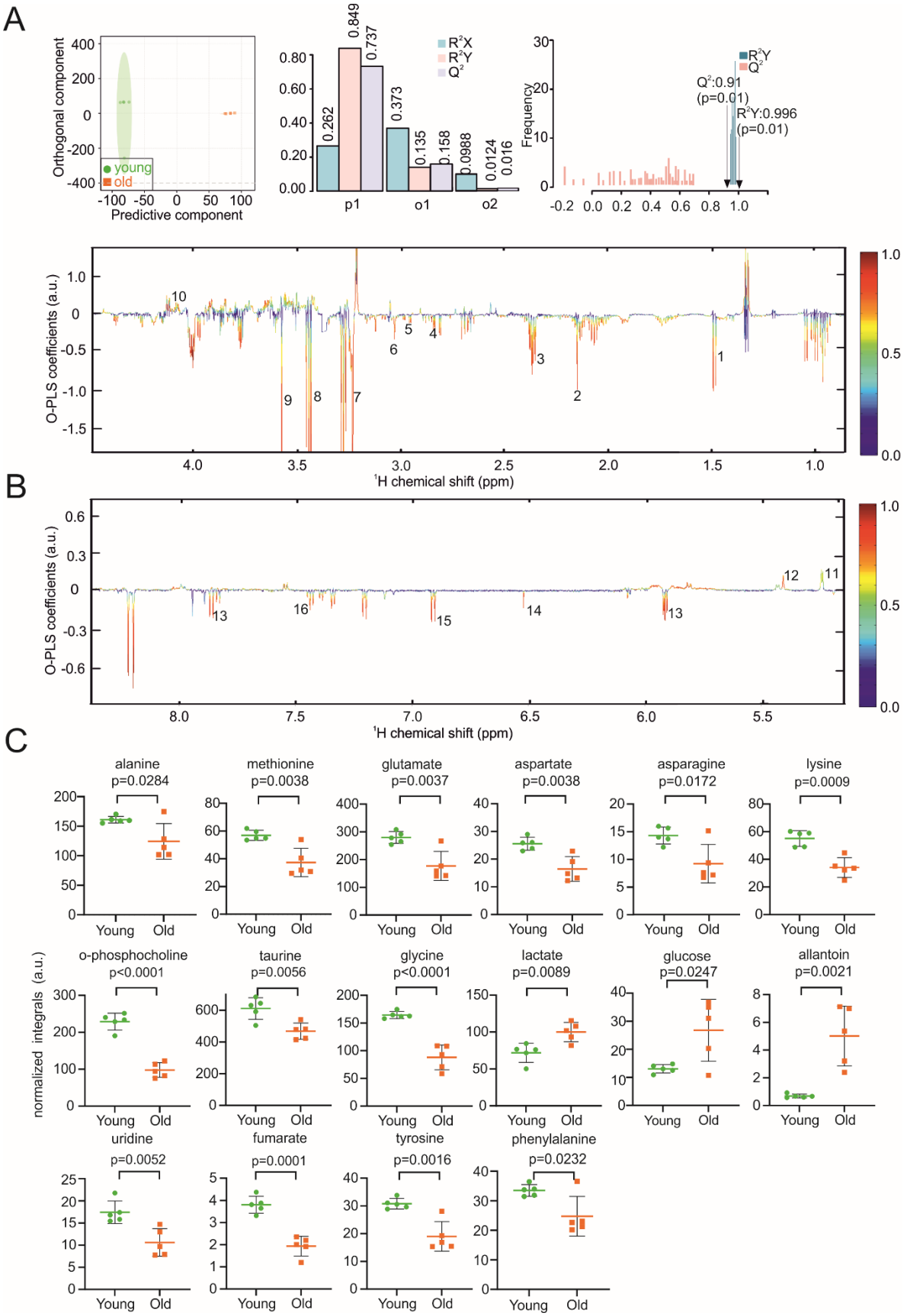

Figure 6. NMR metabolomics analysis of mouse spleen samples. (A) O-PLS-DA plot of spleen samples, including cross validation. (B) The reduced NMR spectrum reveals altered components in normalized spleen samples. Positive covariance corresponds to component present at increased concentrations, whereas negative covariance corresponds to decreased component concentration.

 doi:10.20944/preprints202012.0174.v1

Predictivity of the model is represented by R2. 1. Alanine, 2. methionine, 3. glutamate, 4. aspartate, 5. asparagine, 6. lysine, 7. o-phosphocholine, 8. taurine, 9. glycine, 10. lactate, 11. glucose, 12. allantoin, 13. uridine, 14. fumarate, 15. tyrosine, 16. phenylalanine. (C) Statistical analysis using Student's t-test of individual metabolites in spleen samples. A P-value of <0.05 was considered statistically significant.

4. Discussion

Ageing is a process that gradually increases the organism's vulnerability and affects multiple biological pathways, including metabolism. The health state of tissues plays a key role in ageing or *Vice versa*, as age-associated organ failure, for example, can lead to the death of an organism. Therefore, revealing the consequences of ageing on specific metabolites in distinct tissues is essential to provide information of the underlying mechanisms as they explain the metabolic activity in various tissues and provide functional evidence for biochemical activity. By studying metabolic reprogramming in ageing mice using untargeted NMR-based metabolomics, we identified a set of robust biomarkers for ageing in several murine tissues. NMR spectroscopy is a powerful tool with this regard, as it is characterized by its high reproducibility and simple analysis.

For all six tissues studied, we identified sets of tissue-specific biomarkers of ageing. In the brain, we identified 13 metabolites comprising amino acids and their derivatives, such as tyrosine, phenylalanine and N-acetylaspartate. In addition to amino acid metabolism, the ageing metabolome of mouse brain is characterized by alterations in the purine and pyrimidine metabolism, with a significant increase in uracil (22). In line, we also observed increased uracil in aged mice. Larsson *et. al.* reported that the elevated plasma levels of BCAAs (isoleucine, leucine and valine) are associated with Alzheimer's disease (38). In line with this observation, we also found increased levels of BCAAs in brain lysates of old mice. This phenomenon may be related to the production of the neurotransmitter glutamate known to be altered in the nervous system during ageing (39). Additionally, our results on changes in glutamine concentrations, in line with a study that investigated metabolites of the motor cortex of the brain in humans of different age (24 to 68 years) by ^{1}H-NMR, indicate that these metabolites represent stable ageing markers in the brain of both mice and humans (40). Thus, changes in BCAAs, uracil and glutamine are in accordance with recent studies of brain metabolites (38, 41).

With ageing, the heart exhibits alterations in amino acid and purine metabolism. Here we found a set of ten metabolites significantly changed in the ageing mouse heart. Downregulation of BCAA catabolism in cardiomyocytes has been previously reported to disrupt autophagy, which in turn may be associated with ageing (42, 43). Elevated BCAA levels can therefore be seen as detrimental, in line with large-scale human cohort studies, investigating heart failure (44) and risks of cardiovascular disease (45). Thus, increased BCAA levels suggest an increased risk of cardiovascular disease in course of aging. Similarly, the neurotransmitter GABA has been proposed to interfere with cardiac function and was increased by ageing (46, 47). A direct association with cardiac function has previously been demonstrated (44, 46, 47), rendering leucine, isoleucine, valine and GABA particularly promising as aging-heart biomarkers.

In the kidney, we identified profound changes in metabolic profiles, with more than 20 metabolites differing between old and young mice. Most metabolic changes were associated with amino acid, purine/pyrimidine metabolism, and the tricarboxylic acid (TCA) cycle. Among additional metabolites, changes in the choline status might indicate kidney damage (48). Choline deficiency has been reported to cause kidney damage in rats due to a decrease in the formation of phospholipids, which in turn causes degeneration of the kidney structure (49). Thus, the decreased choline status in aged mice point to similar mechanisms (48). Succinate activates the longevity regulator DAF-16 C in *C. elegans*, which increases stress resistance and may extend lifespan (50). Decreased succinate levels in old mice suggest a more important role of this metabolite in age-related

 doi:10.20944/preprints202012.0174.v1

metabolic adaptations than previously assumed. Choline and succinate might be used in the future as promising biomarkers to determine the health- and age-related status of the kidneys.

Metabolic profiles of mouse livers differ substantially in old compared to young mice with marked changes in the TCA cycle, as shown by the altered levels of alanine, aspartate and fumarate. Ageing reduces glycerol-3-phosphate acyltransferase activity (51) and glycerol (52), respectively, in rats, which is consistent with decreased glycerol concentrations in old mice. Nicotinamide, a poly ADP-ribose synthetase (PARS) inhibitor, attenuated ischemia-induced liver injury with potent anti-inflammatory effects (53). Inosine also has an anti-inflammatory potential, and has been shown to be decreased in 24 month- compared to 1.5 month-old rats (54). We also observed decreased nicotinamide and inosine levels in old mice, which may be related to chronic inflammation during ageing (55). Due to the low variability within each group and the clear distinction between the groups, we propose nicotinamide, glycerol and inosine as potential biomarkers for differentiating between young and old liver tissues.

In the lung, a panel of 13 metabolites was identified and linked mostly to amino acid metabolism and TCA cycle (alanine, aspartate and glutamine). The concentration of.two aromatic amino acids tyrosine and phenylalanine, all three BCAAs and methionine were increased. To date, few metabolome-wide analyses have been performed on lung tissues, and no data at all are available for heathy lung tissue in context of ageing. Thus, our results could set the base for further investigations, focusing on the general protein biosynthesis activity and its alterations as potential cause or consequence of the ageing process. Compared to other organs, lung lysates showed smaller differences in their metabolic profiles between the two respective mouse groups. This may suggest that the change in the metabolic phenotype of the lung is a consequence of metabolic derailing in other organs which in turn affects the lung metabolome. Nevertheless, the levels of lysine, nicotinamide, aspartate and fumarate differ between the two groups, implying these metabolites as biomarkers for the assessment of ageing in lung tissue. In addition to amino acids and metabolites of purine/pyrimidine metabolism, glucose and lactate levels were changed, indicating derailing of the glucose metabolism with age. Altered levels of nicotinamide and inosine in lung and liver might be linked to the ability of both molecules in triggering inflammatory responses (53).

Sixteen metabolites have been found as biomarker candidates for the ageing spleen. Among them, metabolites emerge as components of metabolic pathways linked to amino acid, glucose and lipid metabolism. A decreased concentration of taurine, a sulfur-containing amino acid that augments the proliferative responses of T-cells, decreased in old mice, indicating defective proliferative response of T-cells in old mice (56). Decreased levels of taurine point to a reduced potential for detoxification of reactive oxygen species found in splenic cells (57). In line with previous results (58), we observed decreased uridine levels in the ageing spleen, suggesting uridine and taurine as robust spleen-specific biomarker candidates for the ageing process. Accordingly, reduced levels of uridine might be associated with increased cellular senescence in all tissues, as uridine has been shown to affect senescence of human mammary epithelial cells (59). Thus, decreased uridine concentrations in brain, heart, kidney, liver and spleen of aged mice indicate that uridine could be a common biomarker for ageing.

5. Conclusions

Research desperately seeks for molecular markers of ageing (60). Our straightforward workflow of NMR-based untargeted metabolomics together with the identified metabolite biomarker panels is well-suited to study the impact of senolytic drug candidates, such as dasatinib, quercetin (61), FOXO4-DRI peptide (62), Bcl-2 family inhibitors (63) and Hsp90 inhibitors (64) , to help increasing the overall health span and facilitating healthy ageing. Although experiments with mouse models have already been performed to test the efficacy of certain senolytics in the past, these investigations mostly focused on measuring motor-activities, frailty or physical characteristics (26). The ageing-

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associated, tissue-specific metabolite biomarkers discovered in the current study should provide a novel molecular read-out for ageing-associated changes of an organism. Our results represent a powerful tool for future drug discovery projects to build a bridge between *in vitro* and *in vivo* studies, and to validate the molecular efficacy of investigated therapeutics.

Importantly, besides the power of metabolomics for biomarker discovery and validation, identification of metabolites altered in different states of health and disease can help tracing back the pathway(s) causing metabolic derailing during ageing. Following this approach, and assuming that ageing affects the state of health, identifying metabolites that change significantly with ageing are crucial in classifying pathways that are closely connected to key ageing processes such as cellular senescence (65). Senescent cells are "hypermetabolic", a condition which may potentially be therapeutically targetable in terms of therapy. As previously demonstrated, interventions such as rapamycin treatment and methionine restriction impact key aspects of metabolism and delay cellular senescence to extend cellular lifespan (66, 67). How metabolically targeted drugs can achieve sufficient specificity for senescent over non-senescent cells *in viv*o to allow successful translation remains an open question. Our study provides a protocol enabling evaluation of these metabolism-targeting drugs *in vivo* based on both universal and tissue-specific metabolite alterations that accompany the ageing process. In addition, our approach also depicts a metabolite panel for future *in vivo* NMR studies in living animals.

In this study, NMR-based untargeted metabolomics was applied to investigate the metabolic profile of key tissues in young and old mice. We revealed that ageing is associated with considerable metabolic alterations specifically in amino acids, neurotransmitters and other small molecules. Our study not only generated a high-quality untargeted analysis of ageing metabolism, but also provided a set of metabolic markers that may be used in further translational studies, such as the development of senolytic compounds. Taken together, our approach brought up a metabolite panel for future *in vivo* magnetic resonance studies.

6. Patents

Not applicable.

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