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ARTICLE

Evaluation of Individual Aging Degree by Standard-Free, Label-Free LC-MS/MS Quantification of Formaldehyde-modified Peptides

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In this study, a standard-free, label-free LC-MS/MS method is proposed to evaluate aging based on the cross-linkage theory. First, aging-biomarker screening model was set up *in vitro* by formaldehyde and the highest abundant protein in plasma, human serum albumin (HSA), based on Maillard Reaction. The modification level of peptides cleaved from HSA was investigated using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method with ^{18}O-labeling technique. One formaldehyde-insensitive peptide and six formaldehyde-sensitive peptides that would be verified for being putative peptide-biomarkers had been screened via *in vitro* aging model. These six putative biomarkers were then preliminarily verified by plasma samples with the aldehyde-insensitive peptide served as the internal standard. The verification results indicating that peptides LDELRDEGK and VFDEFKPLVEEPQNLIK showed significantly quantitative difference among young/mid-aged/elderly groups of people.

Introduction

Aging in humans physically refers to a multidimensional process that all the changes were accumulated in a person over time.[1-3] These aging changes are responsible for the progressive increases in the chance of disease and death associated with them.[4-6] Aging changes can be attributed to many factors, such as human development,[7] genetic defects,[8, 9] environment,[10, 11] disease[12-14] and the inborn aging process.[15] Several theories have been developed to explore and understand the aging process.[3] For example, the aging process has been attributed to molecular cross-linking,[16, 17] changes in immunologic function,[18, 19] damage by free-radical reactions,[20] senescence genes in the DNA,[21] and telomere shortening.[22, 23]

Among these aging theories, cross-linking is a well-known and thoroughly studied theory: it is led by Maillard Reaction, which is a complex series of reactions between reducing sugars and amino groups on proteins.[24, 25] In the early stage of Maillard Reaction, proteins are modified to form Schiff Base, then to give Amadori Products (early glycation products) after arrangement. During this stage, Maillard Reaction is reversible. Next, after a complex series of processes, such as condensation, dimerization and cross-linking, advanced glycation end products (AGEs) are finally formed, and the reaction is no longer reversible in the late stage. AGEs accumulate in long-lived proteins, and may contribute to the development of age-related diseases.[26-28] The Maillard Reaction can also happen between formaldehyde and amine groups of proteins. Because the formyl group of formaldehyde can be attacked by the lone pair electrons in the amine group as well as the reduced sugars'. Then proteins can be modified to form AGEs, and lose their functions with manifestations of aging through macro view.

Under physiological conditions, aldehydes are products of various exogenous and endogenous amines catalyzed by semicarbazide-sensitive amine oxidase (SSAO; E.C.1.4.2.6.), a family of heterogeneous enzyme. SSAO can catalyze various amines to form hydrogen peroxide and the corresponding aldehydes, such as formaldehyde.[29] It was reported that SSAO shows a significant higher catalytic activity, producing more aldehydes in age-related diseases and other diseases such as diabetes.[30] Methylamine is a physiological substrate of SSAO and it can be catalyzed by SSAO to give formaldehyde, which can finally cause protein cross-linkage.[29] As a result, formaldehyde level in elderly people would be higher than that of young and mid-aged people. Formaldehyde can modify the side chains of lysine and arginine on proteins via Maillard Reaction, which would cause protein cross-linkage and lose their functions. However, SSAO is not a perfect candidate to be monitored for evaluating aging, because it is widely distributed in tissues (particularly in vascular smooth muscle and adipose tissue), which are not readily accessible sample-sources for clinical applications. Formaldehyde is also unsuitable for being a biomarker in vivo, since other enzymatic catalysis reactions (such as monoamine oxidase, MAO) would also produce formaldehyde.

In this work, a method is proposed to characterize and evaluate individual aging by monitoring the level of protein modifications by formaldehyde. For this purpose, formaldehyde induced human serum albumin (HSA, the most abundant protein in plasma) modification was quantified to represent the average modification levels on plasma proteins. As shown in

Fig. 1, a label-free, standard-free quantitative LC-MS/MS method was used to quantify HSA modification level through measuring formaldehyde-sensitive peptides versus formaldehyde-insensitive peptides in clinial trails. In our experiments, an *in vitro* model was first set up to select formaldehyde-insensitive and formaldehyde-sensitive peptides. Next, one formaldehyde-insensitive peptide was verified then used as the internal standard to measure those formaldehyde-sensitive peptides in plasma samples. Fianlly, two formaldehyde-sensitive peptides were screened for putative biomarkers to evaluate aging.

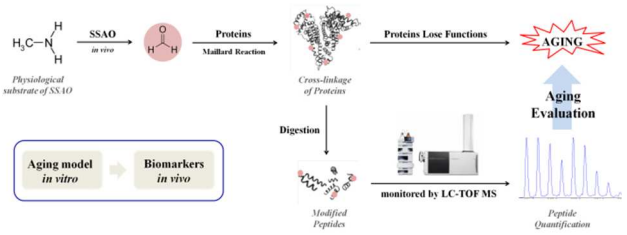

Fig. 1 Research idea and schematic workflow of the experiments.

Experiments

Materials

Sequencing grade modified trypsin was purchased from Promega (WI, USA); $H_2{}^{18}O$ (97%) was purchased from the China Isotope Company (Beijing, China); HPLC-grade acetonitrile and formic acid (FA) were purchased from Fisher Scientific (NJ, USA and Edmonton, Canada); human serum albumin (HSA), dithiothreitol (DTT), iodoacetamide (IAA), urea, 2,4,6-three nitrobenzene sulfonic acid (TNBS), *o*-phthalaldehyde (OPA), *L*-leucine and NH_4HCO_3 were purchased from Sigma (MO, USA); Formaldehyde solution (30% in H_2O), Borax ($Na_2B_4O_7{\cdot}10H_2O$), β-mercaptoethanol, toluene, KH_2PO_4 and K_2HPO_4 were purchased from Beijing Chemical Company (Beijing, China). Water was prepared by a Milli-Q system (Millipore, MA, USA). A reverse-phase LC column (C18, 2.1 × 150 mm, 300 Å) was purchased from Grace Vydac (IL, USA).

HPLC-ESI-TOF MS

An Agilent 1100 series HPLC system was coupled with an Agilent ESI-TOF MS (6210) with a Grace C18 column (300 Å, 2.1 × 150 mm) at a flow rate of 0.2 mL/min for quantitative analysis. The sample injection amount was 10 μg. The mobile phase consisted of buffer A (0.1% FA in H_2O) and buffer B (0.1% FA in acetonitrile). The gradient elution was as follows: 3% buffer B from 0 to 5 min, 40% buffer B from 5 to 35 min, 40%–95% buffer B from 35 to 40 min, 95% buffer B from 40 to 43 min, and 95%–3% buffer B from 43 to 45 min, with a final flow from 45 to 55 min. Nitrogen was used as both the drying and nebulizing gas (flow rate, 10 L/min; pressure, 35 psi; temperature, 350 °C). The MS ionization mode was positive; the capillary voltage was set at -3.5 kV; the mass window of m/z 300–1800 was used.

HPLC-ESI-Ion Trap MS/MS

The same Agilent 1100 series HPLC system was coupled with an Agilent MSD trap for qualitative analysis. The same chromatographic and ESI conditions were used. MS and MS/MS analyses were performed on favorable fragmentations to doubly and triply charged ions. Ion trap MS also had a mass window of m/z 300–1800. Mascot (version 2.4.1.0, Matrix Science, UK) was used to identify peptide sequences associated with each MS/MS spectrum, and the human protein database SwissProt was used for protein identification.

Sample Preparation

The *in vitro* aging model was set up by standard HSA and different concentrations of formaldehyde solution. Brifely, unmodified HSA (40 mg/mL in 50 mM KH_2PO_4-K_2HPO_4 buffer, pH 7.4, containing 0.1 mM toluene as a bacteriostat) was incubated with formaldehyde at 4 different molar ratios (HSA: formaldehyde = 1:10, 1:41.5, 1:83, and 1:415) at 37 °C for 10, 20, or 30 days and then lyophilized. These four concentrations (6.0, 24.9, 49.8 and 249.0 mM) were calculated by the molar ratio of HSA: formaldehyde to give a few of site-occupancy, theoretical 50% site-occupancy, theoretical 100% site-occupancy, and 5-fold formaldehyde excess, respectively. HSA incubated under the same conditions without formaldehyde was used as a control.

Protein Digestion and Peptide ^{18}O-labeling

Two hundred micrograms of HSA (both modified and control HSA) was denatured and reduced by a 20-μL solution containing 8 M urea and 10 mM DTT in 50 mM NH_4HCO_3 buffer (pH 8.3) at 37°C for 4 h. Alkylation was then performed in a 50 mM IAA solution at room temperature for 1 h in the dark. After alkylation, the sample was diluted using a 50 mM NH_4HCO_3 buffer (pH 8.3) to give a final urea concentration of 1 M. Tryptic digestion was then performed at a concentration ratio of 50:1 (protein:trypsin, w/w) for 28 h at 37 °C. All digested peptide samples were lyophilized to complete dryness and then used for the labeling reaction.

Peptide ^{18}O-labeling was carried out under optimized conditions (see supplementary material). Peptide samples were dissolved in 50 mM KH_2PO_4-K_2HPO_4 buffer (pH ~4). This step was followed by a second lyophilization to completely dry the sample. Finally, the control HSA peptides and the modified HSA peptides were labeled in $H_2{}^{18}O$ and $H_2{}^{16}O$, respectively, with 1.6 μg trypsin. After labeling at 37°C for 16 h, residual trypsin activity was quenched by boiling the sample for 10 min and subsequently adding 5% FA (v/v). All samples were centrifuged at 17,000 × g for 15 min before HPLC-MS analysis.

Handling of Human Plasma Samples

The protein concentration of a human plasma sample was determined by the Bradford assay (depends on different individual, generally 60–80 μg/μL). Two μL of each case was lyophilized and then denatured and reduced by adding a solution (0.1 μL solution per μg protein) containing 8 M urea and 10 mM DTT in 50 mM NH_4HCO_3 buffer (pH 8.3) at 37 °C for 4 h. Alkylation was performed in 50 mM IAA (IAA : DTT = 5 : 1, n/n) at room temperature for 1 h in the dark. After alkylation, the sample was diluted using a 50 mM NH_4HCO_3 buffer to give a final urea concentration of 1 M. Tryptic digestion was then performed at a trypsin-to-protein ratio of 1 : 50 (w/w) for 28 h at 37 °C. The trypsin remaining in the sample was deactivated in a boiling water bath for 10 min and the

addition of 1% (v/v) FA. Samples were then centrifuged at 17,000 × g for 15 min before HPLC/ESI-TOF MS analysis. All conditions and parameters of LC and MS were as same as the detection of standard samples. More details about plasma samples were given in supporting information.

Results and Discussion

Aging process is characterized by a decreased physical activity, and it is probably because of proteins losing their functions.[3] Maillard Reaction might play a key role in protein modifications by formaldehyde, because it does not mediated by enzymatic catalysis. Under the physiological condition, aldehydes, including formaldehyde, were produced by oxidative deamination of primary aromatic and aliphatic amines via SSAO. SSAO is mainly expressed in blood vessels in the brain, but it is also present in plasma.[31] It was reported that the activity of SSAO in normal people's blood is very low; however, it shows a significant higher catalytic activity in typical age-related diseases, such as cardiovascular disease (CVD), hypertension, Alzheimer's Disease (AD) and so on.[30, 31]

TNBS assay was carried out first to confirm the Maillard Reaction happened.[32] TNBS reacts with free amine groups of lysines on the surface of proteins, and the characteristic absorption peak of products (three nitrobenzene derivatives) could be detected at ~ 420 nm. Standard HSA was used for making a standard curve. HSA was incubated with increasing concentration of formaldehyde solution (6.0, 24.9, 49.8 and 249.0 mM, respectively) for 10 days at 37°C was used as an example to simulate accumulation effects. Lysine availability of HSA decreases significantly with increasing concentration of formaldehyde. As a reult, the *in vitro* model was confirmed by Fig. 2 that the reaction between lysine in HSA with formaldehyde was happened. OPA assay was also performed as a comparing experiment to confirmed the TNBS assay. The results of OPA assay were shown in the Section 3 of supplementary materials.

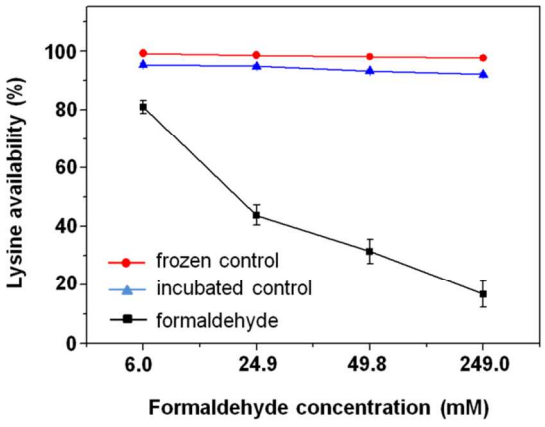

Fig. 2 Confirmations for HSA cross-linking experiments *in vitro*: the measurement of free amine groups by TNBS assay in HSA, which was incubated in formaldehyde solution (6.0, 24.9, 49.8 and 249.0 mM), respectively.

The ^{18}O-labeling technique was used for searching formaldehyde-sensitive and formaldehyde-insensitive peptides based on the *in vitro* aging model. High quality digestion and labeling were the presupposition for the accurate and reliable quantitation. As a result, it was necessary to optimize the conditions of tryptic digestion and ^{18}O-labeling, and the processes of optimization were summarized in supplementary material (Fig. S1-S6 and Table S1-S2). As shown in Fig. 3A, after being incubated with formaldehyde solution, the ^{16}O-to-^{18}O ratios of peptide AAFTECCQAADKAACLLPK (*m/z* = 977.4 with retention time of 28.7 min) was similar to that of control, indicating that AAFTECCQAADKAACLLPK is insensitive to formaldehyde. Furthermore, the signal intensity of peptide AAFTECCQAADKAACLLPK in TOF MS was strong enough (10^4 in plasma samples) to ensure analysis accuracy. As a result, peptide AAFTECCQAADKAACLLPK was finally selected as the internal standard to quantify formaldehyde-sensitive peptides.

On the other hand, the ^{16}O-to-^{18}O ratios of formaldehyde-sensitive peptides would generally decrease with longer incubation time or higher formaldehyde concentration. As shown in Fig. 3B and 3C, the ^{16}O-to-^{18}O ratios of peptide LDELRDEGK (*m/z* = 537.7 with retention time of 22.8 min) and VFDEFKPLVEEPQNLIK (*m/z* = 682.4 with retention time of 30.6 min) decrease as incubation time and formaldehyde concentration increase. Detailed decreasing ratios among peak area ratios (^{16}O-to-^{18}O) of formaldehyde-sensitive peptides with different incubation durations or different formaldehyde concentration were described in supplementary materials (Table S3-S6). Fianlly, six peptide ions (Table 1) were provisionally recognized as formaldehyde-sensitive peptides for HSA based on aging model *in vitro*. These peptides were able to be stably detected in each MS run and gave strong enough intensity for quantification, and their ^{16}O-to-^{18}O ratios showed gradual decreasing with increasing incubation duration and/or formaldehyde concentration. The comparing quantitative results of formaldehyde-sensitive peptides with/without internal standard and labeling have been performed to demonstrate the standard-free and label-free method. The accuracy, precision and repeatability of methodology have been investaged. All detailed information was supplied in Section 2 and Table S7 of supplementary materials. It can be seen from the comparing experimets that the developed standard-free and lable-free method can give a simiar quantitative results with using ^{18}O-labeling technique.

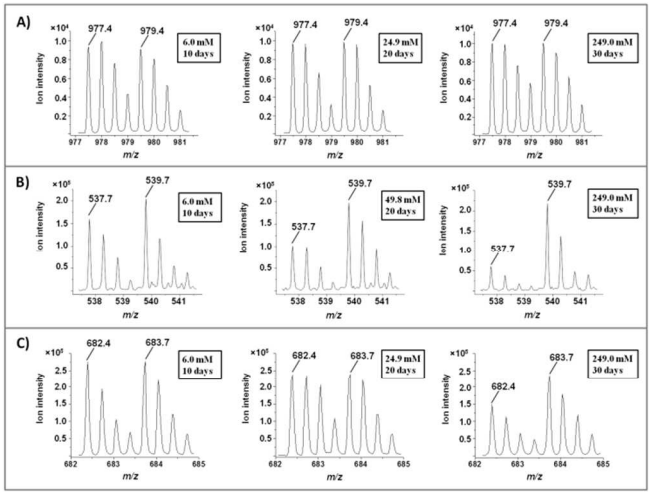

Fig. 3 The ^{16}O/^{18}O peak area ratios of HSA-peptides after being incubated in different concentration of foramldehyde solution for a certain period of time: A) peptide AAFTECCQAADKAACLLPK shows stable ^{16}O/^{18}O peak area ratios; B) and C) the ^{16}O/^{18}O peak area ratio of peptide LDELRDEGK and VFDEFKPLVEEPQNLIK decreased as modification increased.

It was found that formaldehyde-induced modification on HSA has concentration-dependency and cumulative effect in the situation of peptide biomarker screening based on the *in vitro* model. It is reasonable to investigate the modification of HSA, because HSA is a high abundant protein with a relatively long degradation half-life (21 days) *in vivo*. According to the scheme of the Maillard Reaction, modification of proteins may give various of products, including early modification products (Amadori products) and advanced glycation end products (AGEs). Measuring the modified peptides directly will be more difficult than simply targeting the unmodified peptide. The disappearance of unmodified HSA-peptides are basically caused by formaldehyde-induced modification, because glucose was not used in aging model *in vitro* and all diabetes cases were excluded for plasma samples.

Table 1. Formaldehyde-sensitive peptide screened by the *in vitro* aging model

Peptide Location	*m/z*	Rt[a] (min)	z[b]	Peptide Sequence
206–214	537.7	22.8	2	LDELRDEGK
287–298	694.3	24.3	2	YICENQDSISSK
301–310	596.8	32.7	2	ECCEKPLLEK
376–383	492.3	29.9	2	TYETTLEK
570–581	672.8	20.3	2	AVMDDFAAFVEK
397–413	682.4	30.6	3	VFDEFKPLVEEPQNLIK

[a] Retention Time;
[b] Charge

The six formaldehyde-sensitive peptide ions were further validated in plasma samples. Participants who provided blood samples were classified into three groups: young (23 to 40), mid-aged (41 to 60) and elderly people (over age of 61). Table S8 in supplementary materials summarized the basic information and biochemical examination results of individuals who participated in this study. Because Maillard Reaction could also be induced by reduced sugar (such as glucose), cases with glucose level ≥ 6.1 mmol/L were all excluded to eliminate the influence caused by high blood sugar.

Formaldehyde-insensitive and formaldehyde-sensitive peptide were quantified in plasma samples of 253 healthy people. Two peptide ions (*m/z* = 537.7 and 682.4) out of the six potential peptide biomarkers showed significant differences among the three groups (young, middle-aged and elderly people). Fig. 4 showed the box plot of peptide LDELRDEGK and VFDEFKPLVEEPQNLIK. These two peptides showed significant difference among the young, middle-aged and elderly groups. T test was used for processing the MS data between different groups. Compared to the young group, peptide LDELRDEGK showed significant decreases ($p < 0.05$) in its concentrations in the mid-aged group, and peptide VFDEFKPLVEEPQNLIK showed highly significant decreases ($p < 0.01$) in the young group and mid-aged group. Furthermore, there were highly significant differences ($p < 0.01$) between the mid-aged and elderly groups for both LDELRDEGK and VFDEFKPLVEEPQNLIK. These results confirms the hypothesis mentioned in introduction section that formaldehyde level in elderly people would be higher than that of young and mid-aged people. Thus, the amount of residue unmodified peptide LDELRDEGK and VFDEFKPLVEEPQNLIK were fewer than that of young and mid-aged groups. The two formaldehyde-sensitive peptides were located on the surface of the HSA protein, and their locations may contribute to their formaldehyde-sensitivities, because it would be easy for small molecules (such as formaldehyde) to attack these modification sites. Wherase, the modification site of the internal standard peptide AAFTECCQAADKAACLLPK might be hided in the center of HSA, making it difficult to be modified by other molecules. Figure S8 shows the location of peptides AAFTECCQAADKAACLLPK, LDELRDEGK and VFDEFKPLVEEPQNLIK on HSA in supplementary matierals.

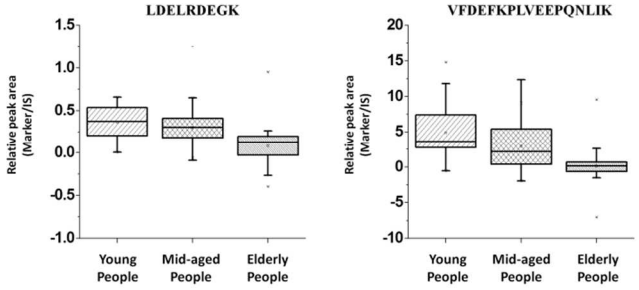

Fig. 4 Behavior of the two potential peptide biomarkers (LDELRDEGK and VFDEFKPLVEEPQNLIK) in 253 cases of human plasma samples among young people, mid-aged people and elderly people groups, with the selected glucose-insensitive peptide (AAFTECCQAADKAACLLPK) used as the internal standard.

Conclusions

This paper describes a new strategy for screening putative peptide-biomarkers for aging evaluation. The method was developed that can quantify peptides in plasma samples without labeling and any internal standard. Basic screening model was first set up *in vitro* by the highest abundant protein (standard HSA) and formaldehyde. Both of formaldehyde-sensitive and formaldehyde-insensitive peptides were monitored using ^{18}O-labeling techniques. The selected peptides were then verified by human plasma samples, peptides LDELRDEGK exhibited significant differences between young and mid-aged group, and peptide VFDEFKPLVEEPQNLIK showed highly significant differences between the two groups. Moreover, both of the two peptides showed significant differences between both young/mid-aged and mid-aged/elderly groups. These results indicated that peptides LDELRDEGK and VFDEFKPLVEEPQNLIK could be putative biomarkers for characterizing human aging.

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Notes and references

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1 M. R. Bonjar and L. S. Bonjar, *Drug Design Development and Therapy*, 2015, **9**, 663.
2 M. I. Miyamoto, K. Djabali and L. B. Gordon, *Glob Heart.* , 2014, **9**, 211.
3 D. C. Park and S. G. Yeo, *Korean J Audiol.* , 2013, **17**, 39.
4 K. Jin, J. W. Simpkins, X. Ji, M. Leis and I. Stambler, *Aging Dis.* , 2014, **6**, 1.
5 E. Buckley, T. Sullivan, G. Farshid, J. Hiller and D. Roder, *Breast.* , 2015, pii: S0960-9776(15)00007-7. doi: 10.1016/j.breast.2015.01.006. [Epub ahead of print].
6 P. Stattin, A. J. Vickers, D. D. Sjoberg, R. Johansson, T. Granfors, M. Johansson, K. Pettersson, P. T. Scardino, G. Hallmans and H. Lilja, *Eur Urol.*, 2015, pii: S0302-2838(15)00024-X. doi: 10.1016/j.eururo.2015.01.009. [Epub ahead of print].
7 F. Beleggia, Y. Li, J. Fan, N. H. Elcioğlu, E. Toker, T. Wieland, I. H. Maumenee, N. A. Akarsu, T. Meitinger, T. M. Strom, R. Lang and B. Wollnik, *Hum Mol Genet.* , 2015, pii: ddu744.
8 J. Li, S. Han, W. Cousin and I. M. Conboy, *Stem Cells.*, 2014, doi: 10.1002/stem.1908. [Epub ahead of print].
9 N. A. Damaschke, B. Yang, S. Bhusari, J. P. Svaren and D. F. Jarrard, *Prostate.*, 2013, **73**, 1721-1730.
10 L. Xu and C. M. Schooling, *J Thromb Haemost.*, 2015, doi: 10.1111/jth.12862. [Epub ahead of print].
11 A. J. Lehning, R. J. Smith and R. E. Dunkle, *Res Aging.* , 2014, 36, 72.
12 S. Greco, M. Gorospe and F. Martelli, *J Mol Cell Cardiol.*, 2015, pii: S0022-2828(15)00023-1. doi: 10.1016/j.yjmcc.2015.01.011. [Epub ahead of print].
13 C. Rizzo, C. Caruso and S. Vasto, *Immun Ageing.*, 2014, 11:16. doi: 10.1186/1742-4933-11-16. eCollection 2014.
14 S. Ikehara and M. Li, *Front Cell Dev Biol.* , 2014, 2:16. doi: 10.3389/fcell.2014.00016. eCollection 2014.
15 S. J. Park, Y. H. Chung, J. H. Lee, D. K. Dang, Y. Nam, J. H. Jeong, Y. S. Kim, T. Nabeshima, E. J. Shin and H. C. Kim, *Endocrinol Metab (Seoul).* 2014, **29**, 336.
16 E. Dumont, T. Držata, C. F. Guerra and F. Lankaš, *Biochemistry.*, 2015, **54**, 1259.
17 M. Kyriazis, *Rejuvenation Res.*, 2014, **17**, 390.
18 M. D. l. Fuente, J. Cruces, O. Hernandez and E. Ortega, *Curr Pharm Des.* , 2011, **17**, 3966.
19 W. A. Howard, K. L. Gibson and D. K. Dunn-Walters, *Rejuvenation Res.*, 2006, **9**, 117.
20 B. F. liveira, J. A. Nogueira-Machado and M. M. Chaves, *ScientificWorldJournal.* , 2010, **10**, 1121.
21 P. Wu, H. L. Li, Z. J. Liu, Q. Q. Tao, M. Xu, Q. H. Guo, Z. Hong and Y. M. Sun, *Clin Interv Aging.* , 2015, **10**, 371.
22 P. A. Kruk, N. J. Rampino and V. A. Bohr, *Proc Natl Acad Sci U S A.*, 1995, **92**, 258.
23 M. E. Brault, S. M. Ohayon, R. Kwan, H. Bergman, M. J. Eisenberg, J. F. Boivin, J. F. Morin, Y. Langlois, C. Autexier and J. Afilalo, *J Am Geriatr Soc.*, 2014, **62**, 2205.
24 J. E. Hodge, *J. Agric. Food Chem.* , 1953, **1**, 928.
25 F. Ledl and E. Schleicher, *Angew. Chem. Int. Ed. Engl.*, 1990, **29**, 565.
26 R. D. Semba, M. F. Cotch, V. Gudnason, G. Eiríksdottir, T. B. Harris, K. Sun, R. Klein, F. Jonasson, L. Ferrucci and D. A. Schaumberg, *JAMA Ophthalmol.*, 2014, **132**, 464.
27 W. Cai, J. Uribarri, L. Zhu, X. Chen, S. Swamy, Z. Zhao, F. Grosjean, C. Simonaro, G. A. Kuchel, M. Schnaider-Beeri, M. Woodward, G. E. Striker and H. Vlassara, *Proc Natl Acad Sci U S A.*, 2014, **111**, 4940.
28 F. Roca, N. Grossin, P. Chassagne, F. Puisieux and E. Boulanger, *Ageing Res Rev.*, 2014, **15**, 146.
29 P. H. Yu and Y. L. Deng, *Atherosclerosis.* , 1998, **140**, 357.
30 M. Kazachkov, K. Chen, S. Babiy and P. H. Yu, *J Pharmacol Exp Ther.*, 2007, **322**, 1201.
31 M. d. M. Hernandez, M. Esteban, P. Szabo, M. Boada and M. Unzeta, *Neurosci Lett.*, 2005, **384**, 183.
32 R. Fields, *Biochem J.*, 1971, **124**, 581.