Association of age-related changes in circulating intermediary lipid metabolites, inflammatory and oxidative stress markers, and arterial stiffness in middle-aged men

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Bo Yeon Ra

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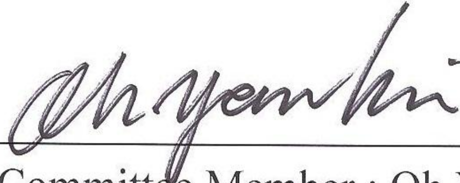

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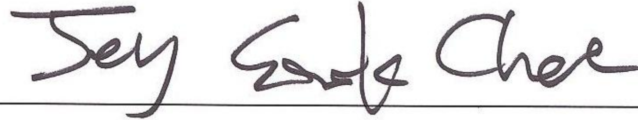

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감사의 글

많은 기대와 설렘으로 시작했던 대학원 생활을 마무리하며, 길고도 짧았던 2년의 과정동안 사랑해주시고 제 곁에서 함께 해주신 분들에게 짧게나마 감사하는 마음을 전하려 합니다.

가장 먼저, 많이 부족한 저에게 임상영양학을 배울 수 있는 기회를 주신 이종호 교수님께 진심으로 감사드리며, 존경을 표합니다. 자상하게 저희를 챙겨주시고 이끌어주셨던 지숙 언니, 세심하게 논문을 지도해주신 환한 미소가 매력적인 오연 언니, 바쁘신 중에도 저희에게 언제나 따뜻한 말을 아끼지 않으신 지영 언니, 스터디와 실험에 대해 많은 가르침을 주신 정현 언니, 항상 친절하게 대해주셔서 감사했던 진경언니, 밝은 미소로 반겨주셨던 정임언니, 꼼꼼하게 많은 것을 알려주셨던 령우 언니, 5층으로 내려가서 아쉬웠던 민쥬킴에게도 감사를 전합니다.

2년 동안 잊지 못할 시간들을 함께 했던 나의 동기들. 언제나 뒤에서 우리를 보듬어주고, 힘든 일, 어려운 일 모두 들어줬던 내 첫 첨단짝꿍 맏언니 용인언니^^, 인생의 가장 중요한 일에 좋은 결실 맺기를 기도할게. 약한 몸으로 궂은 일 다 맡아서 해줬던 걱정대장 하지만 내 징징대는 얘기들 다 들어준 고마운 주영언니, 당신도 빨리 가세요^^, 홀로 남겨져 걱정되기는 하지만 믿음직스러운 작은 손 비타민 현영언니^^, 결혼 준비하느라

얼굴 살이 쪽 빠진 예쁜 수다쟁이 림언니^^, 소년에서 여자가 된 귀여운 은희언니^^, 먼저 사회로 떠나간 내 두 번째 첨단짝꿍 자이언트 베이비 지원이^^, 개성 있는 책들을 좋아했던 어른스러운 우리 동기막내 포스짱 은경이^^. 당신들이 있어서 너무 즐겁고 힘이 됐소~!! 사랑해♥ 나의 부족한 부분들도 잘 채워주었던 PA라인 나연이와 가영, 영주언니, 그리고 바플라인 재현오빠를 비롯한 많은 후배님들께 감사한 마음을 전합니다. 앞으로 대학원 생활이 후배님들의 삶에 꼭 필요한 시간이 될 수 있기를 바랍니다.

오랜 시간 제 옆에서 큰 기쁨과 힘이 되어주었던 사랑하는 사람들에게도 감사와 사랑하는 마음을 전하고 싶습니다. 사랑하는 나의 베프들 보경이, 단이, 예원아 앞으로 우리가 어떤 삶을 살게 되든 언제나처럼 서로에게 힘이 되주자. 평생 함께 갈 나의 영혼의 동반자들, 최유, 쩡유, 최자, 지공이, 보마걸. 너희들과의 만남이 어느 순간 내 삶에 활력소가 되었어. 우리의 만남을 정말 감사한다^^. 또 짱이 가족도 사랑해요. 부모님 다음으로 나에 대해 가장 많이 알고 있는 계희언니, 나의 영원한 리더 성경언니, 언니들에게서 정말 많은 것을 배웠어요. 고맙고 사랑합니다^^. 주일마다 저에게 사랑을 가득 실어준 청년부 언니, 오빠, 동생들. 특히 모퉁이돌 식구들 너무 너무 사랑합니다.

마지막으로 제 인생의 가장 큰 버팀목인 가족들에게 감사의 말을 전합니다. 불안하고 힘들 때에 언제나 제가 듣고 싶은 위로의 말을 해주셨던 아

빠, 짜증내고 퉁명스럽게 굴 때에도 왜 기분이 안 좋은지 조용히 물어봐 주셨던 엄마, 슬프고 마음이 답답할 때 아무말없이 옆에서 함께 해주었던 바라핸, 애교는 없지만 능글능글 누나들에게 큰 웃음을 주는 막둥이 준이, 언제나 귀여운 눈으로 나를 바라봐주는 세상에서 제일 귀여운 꼬맹이. 모두 정말 사랑합니다. 앞으로 우리 가족의 한 멤버로서 더 사랑하고 헌신하는 라보가 될게요.

대학원을 마치고 앞으로 제가 어떤 길을 가게 되더라도 저를 사랑해주신 많은 분들의 격려를 힘입어 두렵지만 헤쳐 나갈 용기를 얻게 되었습니다. 지난 2년의 시간이 제가 앞으로 살아가는 데 있어서 소중한 밑거름이 될 것이라 생각합니다. 앞으로 저의 인생을 감사함으로 채워가고, 최선을 다해 많은 분들에게 부끄럽지 않은 제가 될 수 있도록 노력하겠습니다.

마지막으로 26년 동안 언제나 저와 함께 해주신 하나님께 감사드립니다.

2012년 7월

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ABSTRACT

Association of age-related changes in circulating intermediary lipid metabolites, inflammatory and oxidative stress markers, and arterial stiffness in middle-aged men

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The relationships between age-related changes in circulating endogenous metabolites, inflammatory and oxidative stress markers, and arterial stiffness in 57 middle-aged (34–55 years), nonobese men were studied over the course of 3 years. Arterial stiffness was measured using brachial-ankle pulse wave velocities (ba-PWV). Plasma metabolomic profiling was performed using

ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS). After 3 years, decreased HDL-cholesterol and increased malondialdehyde (MDA) and ox-LDL levels were observed. Among 15 identified lipids, lysoPCs (C16:0, C18:0, C18:2, C20:4, and C20:5) and linoleyl carnitine were the major plasma metabolites that contributed to the age-related differences. LysoPC16:0 (variable importance in the projection [VIP] value: 6.2029) was the most important plasma metabolite for evaluating these changes. LysoPC16:0 levels were positively correlated with changes in MDA (r=0.413), high-sensitivity C-reactive protein(hs-CRP) (r=0.509), IL-6 (r=0.497), and ba-PWV (r=0.283) levels. ba-PWV levels were positively correlated with changes in inflammatory and oxidative stress markers. In a subgroup analysis of subjects with decreased ba-PWVs vs. increased ba-PWVs, changes in waist-to-hip ratios(WHR) and levels of lysoPC16:0, ba-PWV, IL-6, MDA, and P-selectin were significantly different. Our results suggest that age-related increases in lysoPC16:0 can contribute to lipid peroxidation, the activation of proinflammatory phenotypes, and arterial stiffness.

Key Words: age-related changes, intermediate metabolites, inflammation,
oxidative stress, arterial stiffnes

1. INTRODUCTION

Aging and increased levels of circulating proinflammatory markers and oxidized LDLs (ox-LDLs) are associated with arterial stiffness.[1-4] In particular, arterial stiffness increases with age, even in healthy individuals without clinical cardiovascular disease (CVD).[5] Because aging is a complex physiological process involving numerous endogenous metabolites,[6,7] understanding age-related changes in these metabolites will allow better understanding of the pathological or physiological processes underlying arterial stiffness, a condition closely associated with aging.

In the present study, 57 nonobese men between the ages of 34 and 55 years, without disease histories, were followed for 3 years to study the relationship of age-related changes in levels of endogenous metabolites and inflammatory and oxidative stress markers with arterial stiffness. To compare age-related changes between the baseline and 3-year follow-up data, we used a metabolomics approach based on the combination of ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS) coupled with multivariate data analyses. Additionally, to analyze the oxidative and inflammatory status of the study subjects at baseline and after 3 years, we measured the levels of inflammatory and oxidative markers using specific immunoassays, and measured arterial stiffness using brachial-ankle

pulse wave velocity (ba-PWV).

2. BACKGROUND

2.1 Aging and inflammatory markers

2.1.1 Interleukin-6(IL-6)

Inflammation is very important in the pathogenesis of atherothrombosis.[8] Interleukin 1 (IL 1), IL 6, and tumor necrosis factor (TNF) are typical markers of multifunctional cytokines involved in the control of the immune response and inflammation. Their functions are widely overlapping but each shows its own characteristic properties. IL 6 was originally identified as a B cell differentiation factor, and thus one of the major functions of IL 6 is antibody induction.[9] IL-6, an inflammatory cytokine produced mainly by T cells, macrophages, and adipocytes, supports inflammatory responses via the membrane-bound or circulating soluble interleukin-6 receptor (IL6R) on monocytes, hepatocytes, and endothelial cells.[10] The functions of Interleukin-6 are summarized in Figure 1.

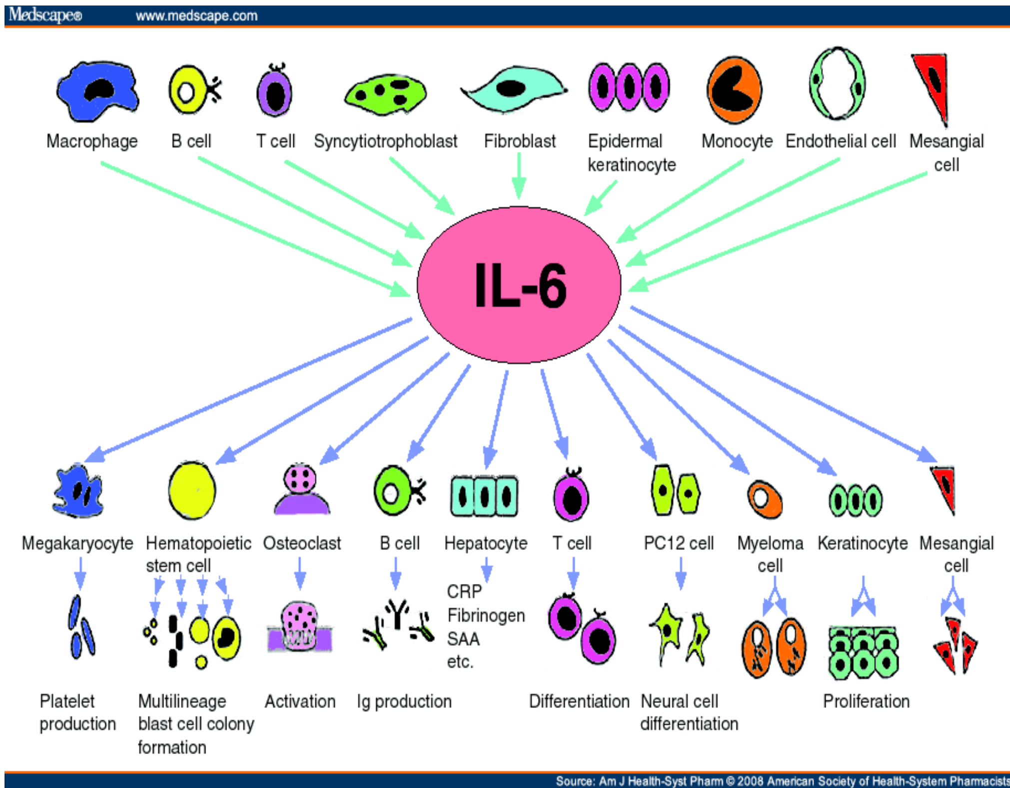

Figure 1. Cells producing interleukin-6 (IL-6) and the actions of IL-6 in the body. Ig = immunoglobulin, CRP = C-reactive protein, SAA = serum amyloid A protein. (adapted from reference 11)

2.1.2 HS-CRP

CRP is a critical component of the immune system, a complex set of proteins that our bodies make when faced with a major infection or trauma.[12]

High-sensitivity C-reactive protein (hs-CRP) has also been evaluated as potential tools for prediction of the risk of coronary events. Among them are markers of systemic inflammation produced in the liver.[12] hs-CRP is secreted from the liver in response to inflammation. Consequently, patients who have coronary artery disease (CAD) have elevated hs-CRP levels.[13] hs-CRP is well standardised and it has limits of detection as low as 0.02g/9-10dl. Various trials like Physician Health Study (PHS) and Women′'s Health Study (WHS) have shown that predictive values of hs-CRP are significantly higher than other traditional biochemical cardiovascular risk markers.[14]

2.2 Aging and oxidative stress

2.2.1 Malondialdehyde (MDA)

One of the major secondary oxidation products of peroxidized polyunsaturated fatty acids is malondialdehyde (MDA)[15] and, which has been inferred to have mutagenic and cytotoxic effects, and possibly to participate in the onset of atherosclerosis.[16] MDA readily splited up with several functional

groups on molecules including proteins, lipoproteins, and DNA. MDA-modified proteins may show altered physico-chemical behavior and antigenicity. Antibodies to MDA will help to visualize the MDA-adducts. Lipid peroxidation is a well-settled mechanism of cellular injury in plants or animals, and is used as an indicator of oxidative stress in several cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, which is the most plentiful malondialdehyde (MDA). Thus, measurement of MDA is generally used as an indicator of lipid peroxidation. If our body′s levels of lipid peroxidation products are increased, It will be associated with a variety of chronic diseases in both humans and model systems.

2.2.2 LDL oxidation

Free radical is a highly reactive chemical that contains oxygen and produced when molecules are split to give products that have unpaired electrons (a process called oxidation). These free radicals can make damage to

cellular molecules such as DNA or lipid. Meanwhile The oxidative stress has negative effect in our body. A role of oxidative stress has been maden several disease including atherosclerosis, heart failure, myocardial infarction, Alzheimer′s disease and the process of aging. One of the most toxic process is oxidized LDL cholesterol. It is a very important risk factor for CVD. Indeed, oxidized LDL (oxLDL) has multiple proatherogenic properties, which include induction of cholesterol accumulation in macrophages as well as potent proinflammatory, immunogenic, apoptotic and cytotoxic activities.[17]

The process of LDL oxidation is summarized in Figure 2. LDL particles break in to the arterial wall and accumulate in the intima. Modified LDL can create Both endothelial cell activation and expression of adhesion molecules. Additionally, intimal macrophages can internalize modified LDL particles through scavenger receptors and become foam cells—a key process in the development of atherosclerotic plaque.[18]

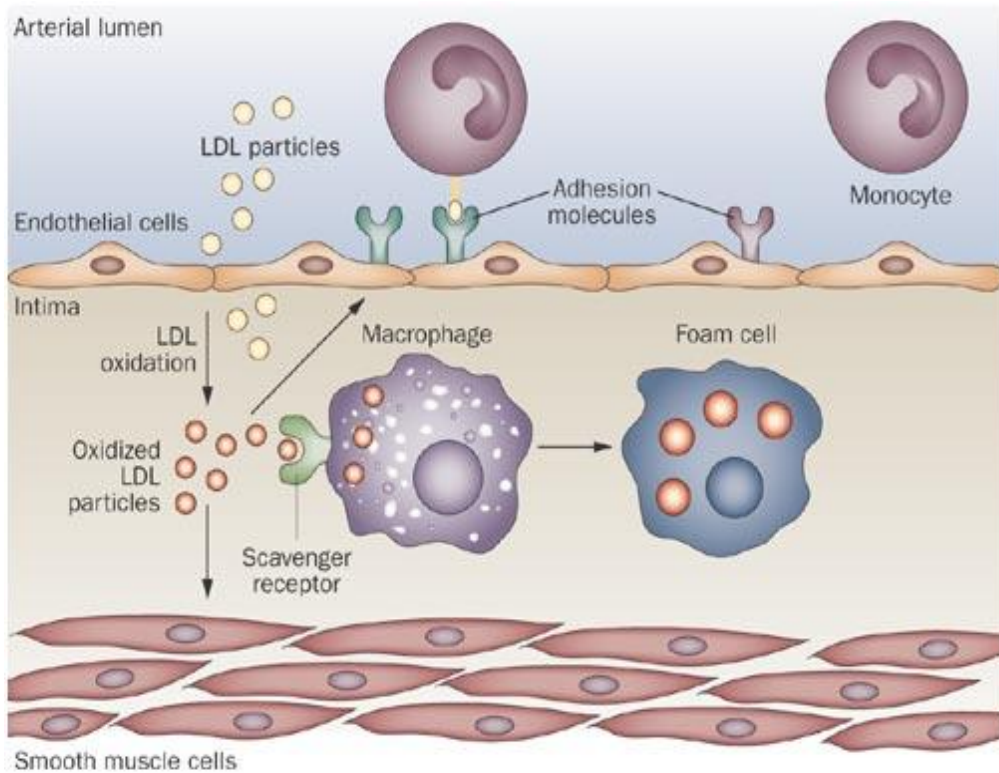

Fig.2 Effects of LDL particles on the vessel wall (adapted from reference 19)

2.3 Arterial stiffness

To prevent CVD, It is very important to control the risk of atherogenic diseases, such as hypertension, diabetes mellitus, and dyslipidemia.[20] Arterial stiffness can make consequence of age and arteriosclerosis. Hardening of the arteries is a common disorder. Fat, cholesterol, and other substances build up

in the walls of arteries over time and form plaques. Arterial stiffness can be performed by measurement of PWV or by pulse wave analysis(PWA).

2.4 Metabolite

Metabolomics is the science of investigating the unique chemical fingerprint of a cell, tissue, or organ by measuring the global variation of metabolites present (the metabolome). The metabolome may represent dynamic and physiologic changes that occur at the cellular level and that expose themselves as changes in metabolite concentrations before pathologic changes can be seen on a static histologic slide.[21]

There are several analytical strategies that can be used to analyse the metabolome[22], such as nuclear magnetic resonance (NMR), Fourier transformation infrared spectroscopy (FT-IR)[23], and mass spectrometry (MS) separated by high performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE). The combination of these different analytical techniques offers important advantages when analyzing the complete metabolome.[24]

There are two experimental approaches to metabolite analysis ; (1)global metabolic profiling and (2)targeted metabolic profiling. Global metabolic profiling is an experimental technique that attempts to measure all detectable metabolites in a sample without selective enrichment or concentration[25]. Global metabolic profiling has long been used in the assessment of pathophysiological processes that affect health status. In the traditional view, metabolic profiling data represent the end result of endogenous metabolism, which is more closely related to the individual phenotype than proteomics.[25] On the other hand, targeted metabolic profiling or targeted metabolomics attempts to measure certain classes of metabolites using selective enrichment or selective concentration via solid-phase extraction, liquid-liquid extraction, chemical derivatization, or chromatographic partitioning.[26]

3. EXPERIMENTAL

3.1 Subjects

The study protocol was approved by the Institutional Review Board of the National Health Insurance Corporation (NHIC)-sponsored Ilsan Hospital, Korea, and was conducted in accordance with the Helsinki Declaration.

Fifty-seven healthy, nonobese male subjects ($20 \leq \text{BMI} < 30$ kg/m^2) between the ages of 34–55 years visiting a health promotion center at the NHIC-sponsored Ilsan Hospital in Korea between August 2007 and October 2007 were enrolled in this study. The subjects led a sedentary lifestyle, and had not participated in weight reduction programs within the previous 3 years. The subjects also completed a personal health and medical history questionnaire that served as a screening tool for enrollment. Exclusion criteria included the presence of type 2 diabetes, CVD, or psychiatric problems, or the use of anti-hypertensive, lipid- lowering, anti-platelet, or anti-diabetic medications.

The duration of the study was 3 years. At baseline, the usual dietary intake of the study subjects was assessed using a semiquantitative food frequency questionnaire and a 24-h recall method. The subjects were encouraged to maintain their body weight within ±3 kg, and were given general oral and written information about healthy food choices and exercise at baseline and at the subsequent visit (week 4). The subjects were instructed by trained dietitians, and were also asked to keep 3-day food records (2 weekdays and 1 weekend) at each visit. Nutrient intake was determined and

calculated based on the 3-day food records using the Computer-Aided Nutritional Analysis Program (CAN-pro 2.0; Korean Nutrition Society, Seoul, Korea). Total energy expenditure (TEE) (kcal/day) was calculated based on the activity patterns of the study subjects, such as basal metabolic rate, 24-h physical activity, and specific dynamic actions of food.

3.2 Anthropometric parameters, blood pressure, and blood collection

Body weights and heights were measured in the morning while the study subjects were unclothed and without shoes. BMIs (kg/m^2) were calculated based on body weights and heights. Percent body fat was analyzed using a TBF-105 body-fat analyzer (Tanita Co., Tokyo, Japan). Waist circumference was measured at the umbilical level, with the subjects standing after normal expiration. Blood pressure (BP) was measured in the left arm of seated patients using an automatic blood pressure monitor (TM-2654, A&D, Tokyo, Japan) after a 20-min rest period. After a 12-h fast, venous blood specimens were collected in EDTA-treated or untreated tubes. Plasma or sera were separated and stored at -70 °C until further analysis.

3.3 Serum lipid profiles and fasting glucose levels, insulin concentrations, and homeostasis model assessment for insulin resistance

Fasting total cholesterol and triglyceride levels were measured using commercially-available kits and a Hitachi 7150 autoanalyzer (Hitachi Ltd., Tokyo, Japan). After precipitation of serum chylomicrons using dextran sulfate magnesium, HDL cholesterol concentrations in the supernatants were enzymatically measured. For subjects with serum triglyceride levels <400 mg/dL, LDL cholesterol levels were estimated directly using the Friedwald formula: LDL cholesterol = total cholesterol − (HDL cholesterol + [triglycerides/5]). For subjects with serum triglyceride levels ≥400 mg/dL, LDL cholesterol levels were measured indirectly. Fasting glucose levels were measured by the glucose oxidase method using a Beckman glucose analyzer (Beckman Instruments, Irvine, CA). Insulin levels were measured by a radioimmunoassay using a commercial kit (Immuno Nucleo Corporation, Stillwater, MN). Insulin resistance (IR) was calculated based on the homeostasis model assessment (HOMA) using the following equation: HOMA-IR = (fasting insulin [μIU/mL] × fasting glucose [mmol/L])/22.5.

3.4 Measurement of serum IL-6 levels, serum high-sensitivity C-reactive protein (hs-CRP) levels, and white blood cell (WBC) counts

Serum interleukin (IL)-6 concentrations were measured using Bio-Plex™ Reagent Kits and a Bio-Plexz™ system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The hs-CRP levels were measured on an Express Plus™ auto-analyzer (Chiron Diagnostics Co., Walpole, MA) using commercially-available high-sensitivity CRP-Latex (II) X2 kits (Seiken Laboratories Ltd., Tokyo, Japan). WBC counts were determined using a hematology analyzer from HORIBA ABX Diagnostic (HORIBA ABX SAS, Parc Euromedicine, France).

3.5 Plasma oxidized LDL, adiponectin, malondialdehyde, sVCAM-1, sICAM-1, and P-selectin levels

Plasma ox-LDL levels were measured using an enzyme immunoassay (Mercodia, Uppsala, Sweden). Plasma adiponectin concentrations were measured using an enzyme immunoassay (Human Adiponectin ELISA kit, B-Bridge International Inc., CA). The absorbencies of the resulting color

reactions (ox-LDLs and adiponectin) were measured at a wavelength of 450 nm using a Wallac Victor^2 multilabel counter (Perkin Elmer Life Sciences, Turku, Finland). The wavelength correction was set to 540 nm. Plasma MDA concentrations were measured based on the production of thiobarbituric acid-reactive substances (TBARS Assay Kit, Zepto-Metrix Co., Buffalo, NY). Plasma vascular cell adhesion molecule (VCAM)-1, inter-cellular adhesion molecule (ICAM)-1, and P-selectin levels were measured using Bio-Plex™Reagent Kits with a Bio-Plex™ system (Bio-Rad) according to the manufacturer's instructions.

3.6 Brachial-ankle pulse wave velocity (ba-PWV) measurement

ba-PWVs were measured using an automatic waveform analyzer (model VP-1000; Nippon Colin Ltd., Komaki, Japan) according to a previously described method.[27] The average ba-PWV from both left and right sides was used for analysis (correlation between the right and left ba-PWVs: r^2=0.925, P<0.001).

3.7 Plasma metabolic profiling

Plasma samples were prepared and injected into an ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometer (UPLC/Q-TOF MS; Waters, Milford, MA) according to previously described methods.[28] The Q-TOF MS was operated in a positive electrospray ionization (ESI) mode. The capillary and sampling cone voltages were set at 2.78 kV and 26 V, respectively. The desolvation flow was set to 700 L/h at 300 °C, and the source temperature was set to 110 °C. The TOF MS data was collected in the range of 50–1000 *m/z*, with a scan time of 0.2 s and interscan delay time of 0.02 s. All analyses were performed using lock spray to ensure accuracy and reproducibility; leucine-enkephalin (556.2771 Da in the positive ESI mode) was used as the lock mass at 200 pmole and a flow rate of 3 μL/min. The lock spray frequency was set at 10 s.

For quality control (QC), a mixture of five standard compounds (caffeine, sulfadimethoxine, terfenadine, 4-acetoaminophenol, and reserpine) was injected after every seven samples. The MS/MS spectra of the metabolites were obtained by a collision energy ramp from 10–30 eV. Accurate masses and

compositions of the precursor and fragment ions were calculated and sequenced using MassLynx 4.1 software (Waters) incorporated in the instrument. All MS data, including retention time, *m/z*, and ion intensity, were extracted using the MarkerLynx 4.1 software package (Waters) incorporated in the instrument, and the resulting MS data were assembled into a data matrix.

Peaks were collected using a peak width of 5% height of 1 s, a noise elimination of 6, and an intensity threshold of 120. Data were aligned with a mass tolerance of 0.04 Da and a retention time window of 0.15 min. All spectra were aligned and normalized to the total peak intensity. Assignment of metabolites contributing to the observed variance was performed using the elemental composition analysis software using calculated mass, mass tolerance (mDa and ppm), double-bond equivalent (DBE), and the i-Fit algorithm (the likelihood that the isotopic pattern of the elemental composition matches a cluster of peaks in the spectrum) implemented in the MassLynx software by the ChemSpider database (www.chemspider.com), and by the Human Metabolome Database (www.hmdb.ca). Authentic standards were used to confirm the assignments and to perform quantitative analyses.

3.8 Statistical Analysis

Statistical analyses were performed using the SPSS ver12.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL). The skewed variables were logarithmically-transformed for statistical analysis. For descriptive purposes, mean values were presented using untransformed values. Results are expressed as means ± standard error (SE). A two-tailed *P* value of <0.05 was considered statistically significant. Paired *t*-tests and Wilcoxon signed rank tests were used to evaluate differences between baseline and 3-year follow-up levels. Differences in the clinical variables between the decreased ba-PWV and increased ba-PWV groups were tested by independent *t*-tests and Mann-Whitney U-tests. A general linear model test was applied to adjust for baseline values. Pearson′s correlation coefficients were used to examine the relationships between variables.

Multivariate statistical analysis was performed using the SIMCA-P$^+$ software version 12.0 (Umetrics, Umeå, Sweden). Partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling discrimination between the baseline and 3-year follow-up data by visualizing score plots or S-plots using the first and second PLS components.

To validate the model, a 7-fold validation was applied to the PLS-DA model, and the reliabilities of the model were further rigorously validated by a permutation test (n=200). The goodness of the fit was quantified by R^2Y, while the predictive ability was indicated by Q^2Y. Generally, R^2Y, which describes how well the data in the training set are mathematically reproduced, varies between 0 and 1, with 1 indicating a model with a perfect fit.

4. RESULTS

4.1 Clinical characteristics, inflammatory markers, arterial stiffness, lipid peroxides, adhesion molecules, and nutrient intakes at baseline and the 3-year follow-up

After 3 years, subjects showed decreased levels of HDL-cholesterol (*P*<0.001) and increased levels of MDA (*P*<0.001) and ox-LDLs (*P*<0.001) (Table 1). There were no significant differences in the levels of inflammatory markers, arterial stiffness, and adhesion molecules between the baseline and 3-year follow-up data. The estimated total calorie intake at baseline was 2441±27 kcal/d and at 3-years was 2429±23 kcal/d. There was no significant difference in macronutrient intake, TEE, smoking, and drinking between the baseline and 3-year follow-up data (data not shown).

Table 1. Clinical characteristics, inflammatory markers, brachial-ankle pulse wave velocity (ba-PWV), lipid peroxides, and adhesion molecules at baseline and at the 3-year follow-up

	Baseline	**3-yr follow-up**	***P***
Age (year)	44.9±0.73	47.9±0.74	<0.001
Body mass index (kg/m^2)	24.6±0.32	24.6±0.29	0.953
Waist-to- hip ratio	0.90±0.01	0.91±0.01	0.267
Systolic BP (mmHg)	120.1±1.50	120.8±1.99	0.940
Diastolic BP (mmHg)	75.9±1.35	75.2±1.60	0.954
Triglyceride (mg/dL)	137.8±10.7	138.8±10.7	0.953
Total-cholesterol (mg/dL)	192.4±4.61	190.3±4.31	0.987
HDL-cholesterol (mg/dL)	51.5±1.87	45.3±1.50	<0.001
LDL-cholesterol (mg/dL)	114.9±4.73	118.8±3.95	0.119
Glucose (mg/dL)	93.9±1.55	96.0±1.72	0.117
Insulin (μU/mL)	8.34±0.43	8.04±0.48	0.581
[1]HOMA-IR	1.94±0.11	1.90±0.12	0.946
hs-CRP (mg/dL)	1.23±0.25	0.84±0.08	0.941

Serum IL-6 (pg/mL)	4.83±0.50	4.17±0.46	0.130
White blood cells (×10^9/L)	6.16±0.31	5.77±0.17	0.639
Adiponectin (µg/mL)	5.27±0.31	5.39±0.27	0.224
ba-PWV (cm/sec)	1340.3±27.5	1357.2±29.5	0.264
Malondialdehyde (nmol/mL)	9.92±0.31	12.4±0.47	<0.001
Oxidized LDL (U/L)	34.0±1.46	43.0±1.89	<0.001
sICAM-1 (pg/mL)	204.3±9.30	205.2±12.1	0.668
sVCAM-1 (pg/mL)	511.4±23.3	498.1±21.5	0.909
P-selectin (pg/mL)	30.0±1.81	31.2±1.89	0.346

Mean ± S.E.∮tested by logarithmic transformation; *P*-values derived from paired *t*-test with the Wilcoxon signed rank test. [1]HOMA-IR = {fasting insulin (μU/mL) x fasting glucose (mmol/L)}/22.5.

4.2 Multivariate statistical analysis and identification of plasma metabolites

The MS data of plasma metabolites obtained from healthy men at baseline and the 3-year follow-up were applied to a PLS-DA score plot (Fig. 3a). The first two-component PLS-DA score plots of the plasma metabolites showed distinct clustering for each group of healthy men at baseline and the 3-year follow-up. Both groups could be clearly differentiated from each other by the primary component t(1) or the secondary component t(2) based on the model with R^2X (cum) and R^2Y (cum) values of 0.391 and 0.990, respectively, indicating the goodness of fit of the data. The Q^2Y (cum) value of 0.818 estimated the predictive ability of the model. In addition, the PLS-DA models were validated using a permutation test, and indicated an R^2Y intercept value of 0.0971 and a Q^2Y intercept value of 0.0113. To identify the metabolites contributing to the discrimination between the baseline and 3-year follow-up data, *S*-plots of *p*(1) and *p*(corr)(1) were generated using centroid scaling (Fig. 3b). The *S*-plots revealed that the metabolites with higher or lower *p*(corr) values served as the more relevant ions for discriminating between the two groups.

Among the 813 metabolites in the plasma, the metabolites that play an important role in determining age-related changes after the 3-year follow-up

were selected according to their variable importance in the projection (VIP) scores. The normalized intensities of whole metabolites were statistically analyzed by a nonparametric *t*-test; the metabolites with significant differences between the baseline and 3-year follow-up data were included. Thus, 17 metabolites were selected based on their VIP values and independent *t*-tests. Finally, 15 metabolites were identified (two were unknown). The results of the UPLC-Q-TOF analysis are shown in Table 2.

Eight plasma metabolites, including l-valine, lysophosphatidylcholines (lysoPCs) containing C16:0, C18:2, C20:4, C20:5, C22:5, and C22:6, and lysophosphatidylethanolamines (lysoPEs) containing C18:0 and C22:6, showed significant increases at the 3-year follow-up, whereas three metabolites, including pyrrolinehydroxycarboxylic acid, linoleyl carnitine, and total lysoPCs, showed decreased levels. Linoleyl carnitine, and lysoPCs containing C16:0, C18:0, C18:2, C20:4, and C20:5 (with VIP values >1.0, indicating a high relevance to the difference between the sample groups) were the major plasma metabolites contributing to the discrimination between the baseline and 3-year follow-up data on the PLS-DA score plot (Table 2). In particular, lysoPC 16:0 with a VIP value of 6.2029 served as the most important plasma metabolite for evaluating the differences between the baseline and 3-year follow-up data.

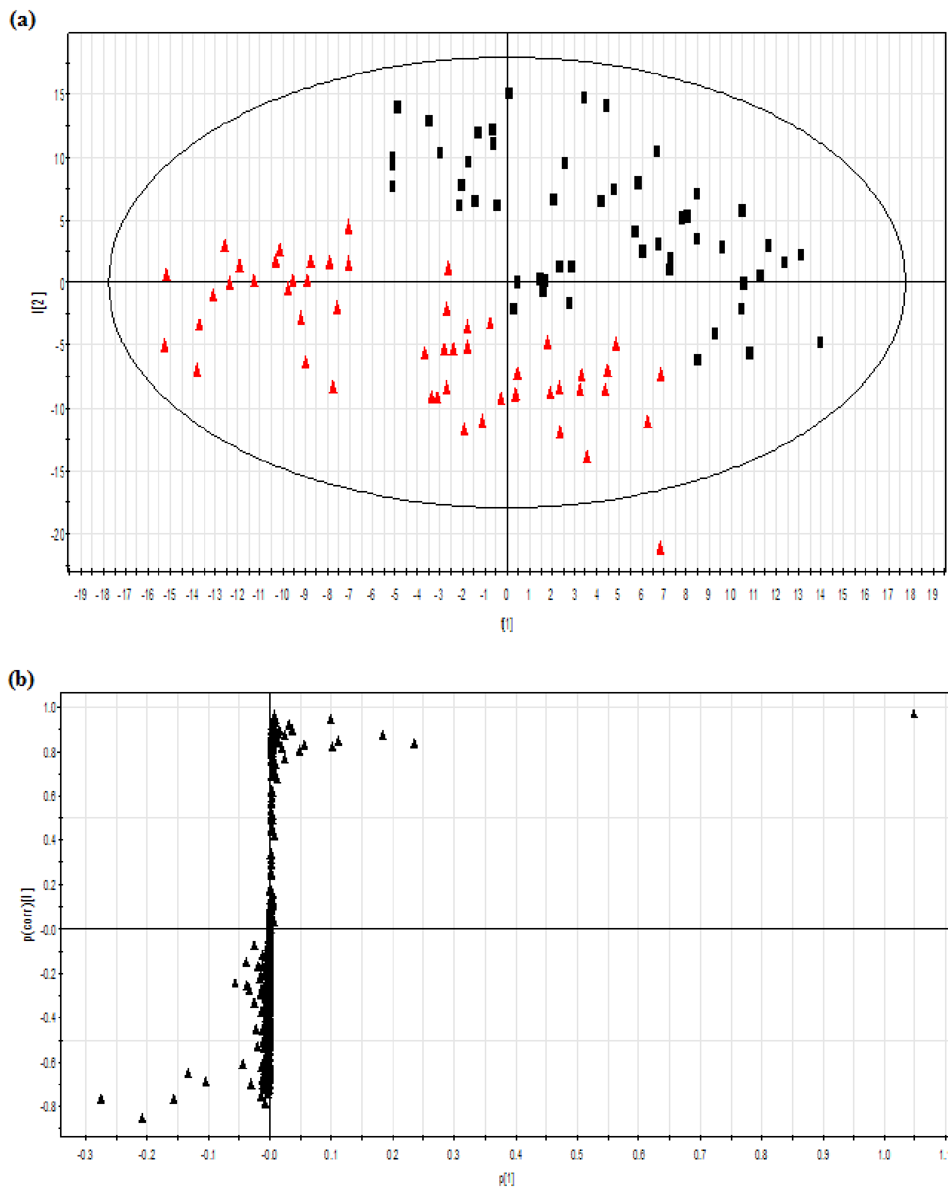

Fig.3 (a) Score plots from PLS-DA models classifying healthy men at baseline (■) and at the 3−year follow-up (▲). (b) Splot for covariance [p] and reliability correlation [p(corr)] from PLS-DA models

Table 2. Identification of plasma metabolites at baseline and at the 3-year follow-up

no.	Identity	Formula $[M+H]^+$	Exact mass (M + H)	Mass error (mDa)	Normalized peak intensities (mean ± SE)		Fold change[a] (vs. controls)	P^b	VIP
					Baseline	Follow-up			
1	L-Valine	$C_5H_{11}NO_2$	118.0789	-6.0	13.0±0.45	15.3±0.53	1.140	0.001	0.2058
2	Pyrroline hydroxy carboxylic acid	$C_5H_7NO_3$	130.0425	-6.4	3.30±0.10	2.54±0.10	0.737	<0.001	0.2809
3	L-Tryptophan	$C_{11}H_{12}N_2O_2$	205.0898	-7.6	32.1±1.56	31.4±1.64	0.944	0.704	0.8036
4	Linoleyl carnitine	$C_{25}H_{45}NO_4$	424.3348	-4.4	25.6±1.09	21.9±1.29	0.845	0.009	1.4369
5	LysoPC (16:0)	$C_{24}H_{50}NO_7P$	496.3324	-6.0	718.7±16.2	778.1±27.1	1.064	0.028	6.2029
6	LysoPC (18:0)	$C_{26}H_{54}NO_7P$	524.3637	-6.2	545.9±15.0	574.0±17.0	1.043	0.231	4.6709
7	LysoPC (18:2)	$C_{26}H_{50}NO_7P$	520.3325	-5.9	467.8±11.4	510.6±14.4	1.081	0.004	4.6890
8	LysoPC (18:3)	$C_{26}H_{48}NO_7P$	518.3168	-6.2	12.5±0.66	14.8±0.98	1.152	0.068	0.4923

Table 2. Identification of plasma metabolites at baseline and at the 3-year follow-up

9	LysoPC (20:4)	$C_{28}H_{50}NO_7P$	544.3325	2.5	54.7±3.56	66.1±6.38	1.175	0.021	1.4186
10	LysoPC (20:5)	$C_{28}H_{48}NO_7P$	542.3168	-0.7	39.1±1.52	45.4±1.32	1.134	0.002	1.1194
11	LysoPC (22:5)	$C_{30}H_{52}NO_7P$	570.3481	-8.3	1.88±0.18	2.56±0.22	1.272	0.002	0.0565
12	LysoPC (22:6)	$C_{30}H_{50}NO_7P$	568.3325	3.6	48.3±1.36	53.0±1.16	1.083	0.007	0.5201
13	LysoPE (18:0)	$C_{23}H_{48}NO_7P$	482.3168	-7.4	8.88±0.50	10.7±0.43	1.176	0.004	0.2748
14	LysoPE (22:6)	$C_{27}H_{44}NO_7P$	526.2855	-5.8	28.1±0.77	31.5±0.98	1.107	0.003	0.5602
15	Total LysoPC	-	-	-	3.14±0.08	3.69±0.12	0.713	0.001	-
16	Unknown1	-	417.3290	-7.3	-	-	0.100	-	0.5015
17	Unknown2	-	585.2634	-8.5	-	-	1.720	-	0.5597

Mean ± S.E. [a]Calucated by the mean of intensity of each metabolite from cases by the mean of intensity of each metabolite from controls. P^b-values derived from paired *t*-test with the Wilcoxon signed rank test.

4.3 Relationship between the changes in the major plasma metabolite levels

The changes in lysoPC 16:0 levels were positively correlated with the changes in lysoPC 18:0 (r=0.624, $P<0.001$), lysoPC 18:2 (r=0.620, $P<0.001$), and lysoPC 20:5 (r=0.414, $P=0.003$) levels. The changes in lysoPC 18:0 were positively correlated with the changes in lysoPC 18:2 (r=0.773, $P<0.001$) and lysoPC 20:5 (r=0.600, $P<0.001$). The changes in lysoPC 18:2 were positively correlated with the changes in lysoPC 20:4 (r=0.325, $P=0.023$) and lysoPC 20:5 (r=0.395, $P=0.005$). The changes in linoleyl carnitine were positively correlated with the changes in lysoPC 20:4 (r=0.423, $P=0.002$).

4.4 Relationship between the changes in levels of lysoPC 16:0, arterial stiffness, lipid peroxides, inflammatory markers, and adhesion molecules

The changes in lysoPC 16:0 between the baseline and 3-year follow-up data were positively correlated with the changes in MDA (r=0.413, *P*=0.004) (Fig. 4), hs-CRP (r=0.509, *P*<0.001), IL-6 (r=0.497, *P*=0.001) (Fig. 4), and ba-PWV (r=0.283, *P*=0.049). The changes in ba-PWV were positively correlated with the changes in ox-LDLs (r=0.312, *P*=0.023), MDA (r=0.302, *P*=0.024) (Fig. 4), hs-CRP (r=0.329, *P*=0.013), IL-6 (r=0.298, *P*=0.038) (Fig. 4), and P-selectin (r=0.345, *P*=0.034). The changes in hs-CRP were positively correlated with the changes in ox-LDLs (r=0.296, *P*=0.033) and IL-6 (r=0.635, *P*<0.001).

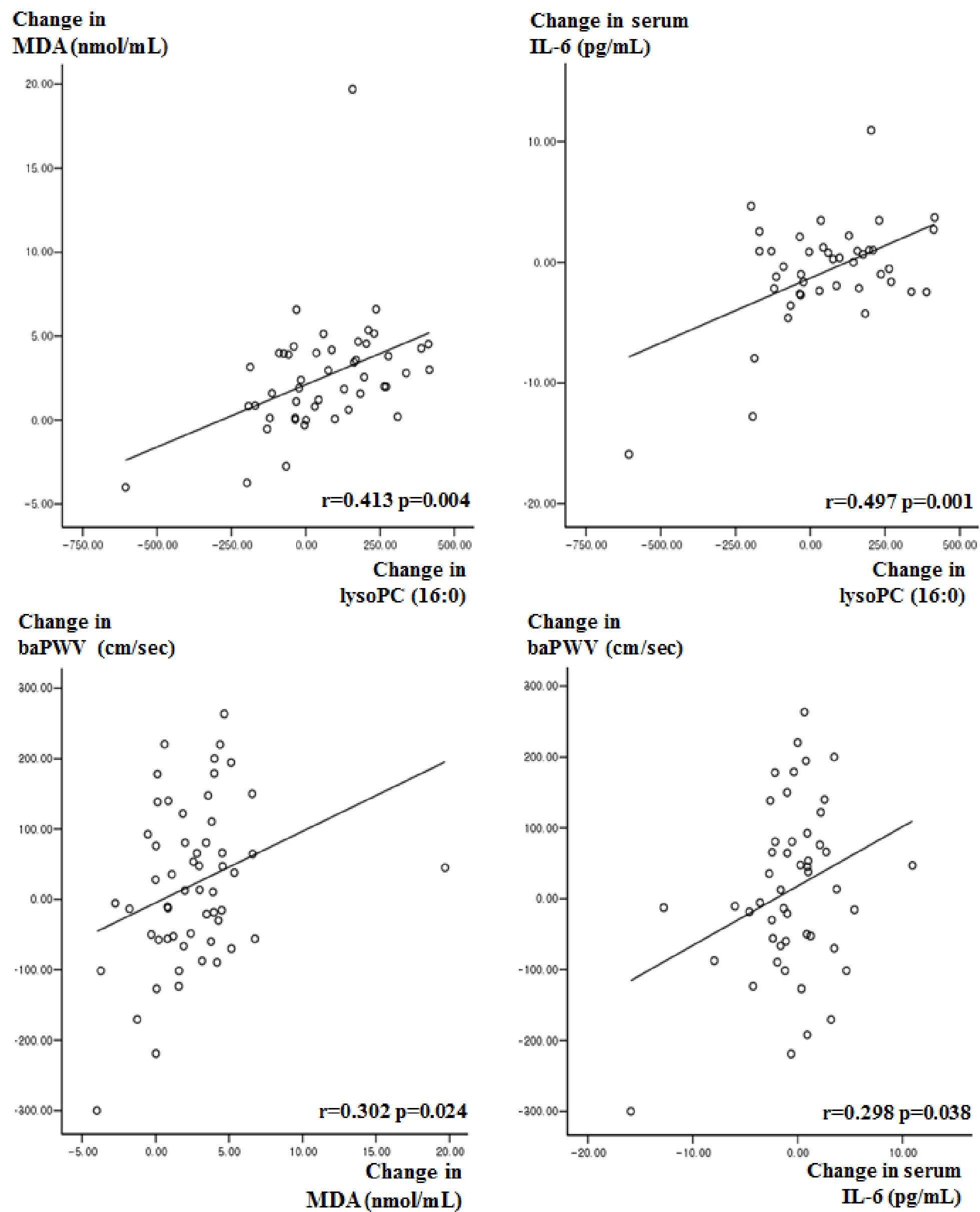

Fig.4 Relationship of the changes in lysoPC(16:0) levels and ba-PWVs with the changes in serum IL-6 and MDA levels in healthy men after 3 years.

§Tested by Pearson correlation analysis. r: correlation coefficient

4.5 Association of the levels of inflammatory markers, lipid peroxides, adhesion molecules, and metabolites with the changes in arterial stiffness

Because of the close relationship between the changes in levels of lysoPC 16:0, arterial stiffness, lipid peroxides, inflammatory markers, and adhesion molecules, we divided our subjects into two groups according to the changes in arterial stiffness from baseline to the 3-year follow-up (decreased ba-PWV vs. increased ba-PWV groups). At the baseline, men in the increased ba-PWV group (n=30) had higher BMIs (P=0.014) and lower hs-CRP levels (P=0.042) than did those in the decreased ba-PWV group. After 3 years, men in the decreased ba-PWV group (n=27) showed a significant reduction in ba-PWVs (P<0.001) and serum IL-6 (P=0.029) levels, and significant increases in the levels of MDA (P=0.019) and ox-LDLs (P=0.006) (Table 3). Men in the increased ba-PWV group showed significant increases in waist-to-hip ratio (WHR) (P=0.022) and levels of ba-PWV (P<0.001), MDA (P<0.001), ox-LDLs (P=0.001), and P-selectin (P=0.005) after 3 years. At the 3-year follow-up, men in the increased ba-PWV group had higher ba-PWVs (P=0.008) than those with decreased ba-PWVs. The changes in WHR (P=0.047), ba-PWV (P<0.001), IL-6 (P=0.031), MDA (P=0.037), and P-selectin (P=0.024) levels were significantly different between the two ba-PWV groups after adjustment

for baseline values (Table 3). Additionally, changes in lysoPC 16:0 levels were significantly different between the decreased and increased ba-PWV groups (-14.7±50.7 vs. 106.4±29.5; *P*=0.049) after adjustment for baseline values. There were no significant changes in other plasma metabolite levels between the ba-PWV groups before or after adjustment for baseline values (data not shown).

Table 3. Inflammatory markers, lipid peroxides, and adhesion molecules according to changes in arterial stiffness at baseline and the 3-year follow-up

	Controls (PWV decreased) (n=27)	P^a	Cases (PWV increased) (n=30)	P^a	P^b	P^c
Body mass index (kg/m^2)						
Baseline	23.9 ±0.47	0.313	25.3±0.41	0.220	0.014	
Follow-up	24.1 ±0.35		25.1±0.44		0.112	
Change	0.16 ±0.27		-0.16±0.13		0.155	0.776
Waist-to-hip ratio						
Baseline	0.91 ±0.01	0.400	0.89±0.01	0.022	0.183	
Follow-up	0.90 ±0.01		0.91±0.01		0.216	
Change	-0.01 ±0.01		0.02±0.01		0.030	0.047
[1]ba-PWV (cm/sec)						
Baseline∮	1385.9±49.7	<0.001	1299.3±25.4	<0.001	0.067	
Follow-up∮	1306.1±50.6		1403.3±31.4		0.008	
Change	-79.9±13.7		104.0±13.0		<0.001	<0.001
White blood cells (×10^9/L)						

Baseline∮	6.71±0.56	0.107	5.67±0.29	0.314	0.246	
Follow-up∮	5.74±0.25		5.81±0.24		0.936	
Change	-0.97±0.50		0.14±0.26		0.064	0.210
hs-CRP (mg/dL)						
Baseline∮	1.84±0.51	0.107	0.71±0.11	0.111	0.042	
Follow-up∮	0.73±0.09		0.94±0.13		0.250	
Change	-1.11±0.53		0.23±0.17		0.025	0.321
Serum IL-6 (pg/mL)						
Baseline∮	5.40±0.82	0.029	4.28±0.60	0.753	0.173	
Follow-up∮	3.37±0.43		4.94±0.78		0.222	
Change	-2.03±1.01		0.67±0.57		0.044	0.031
[2]MDA (nmol/mL)						
Baseline∮	10.2±0.46	0.019	9.68±0.42	<0.001	0.522	
Follow-up∮	11.6±0.55		13.1±0.72		0.148	
Change	1.42±0.54		3.46±0.67		0.031	0.037
Oxidized LDL (U/L)						
Baseline∮	33.6±2.08	0.006	34.3±2.08	0.001	0.972	
Follow-up∮	41.4±2.07		44.7±3.21		0.488	
Change	7.81±2.66		10.4±2.60		0.972	0.396

sICAM-1 (pg/mL)						
Baseline	181.7±10.6	1.000	201.7±10.6	0.570	0.122	
Follow-u	187.6±15.2		197.8±12.1		0.651	
Change	5.91±10.7		-3.96±5.81		0.715	0.399
sVCAM-1 (pg/mL)						
Baseline	698.6±74.4	0.255	660.5±61.5	0.108	0.895	
Follow-up	755.5±104.5		745.6±85.8		0.919	
Change	56.9±66.8		85.1±65.4		0.988	0.778
P-selectin (pg/mL)						
Baseline	36.3±2.92	0.215	32.2±1.87	0.005	0.284	
Follow-up	33.8±3.06		39.5±3.04		0.246	
Change	-2.53±2.34		7.33±2.67		0.006	0.024

Mean ± S.E. P^a-values derived from paired *t*-test with the Wilcoxon signed rank test; P^b-values derived from independent *t*-test with the Mann-Whitney U-test; P^c-values derived after adjusting for baseline values; [1]ba-PWV = brachial-ankle pulse wave velocity; [2]MDA = malondialdehyde

5. DISCUSSION

Using a metabolomics approach based on UPLC/Q-TOF MS, we identified 15 endogenous metabolites that showed age-related changes in middle-aged men. Among these metabolites, lysoPCs containing C16:0, C18:0, C18:2, C20:4, and C20:5 and linoleyl carnitine were the two major metabolites contributing to the discrimination between the baseline and 3-year follow-up. Various species of lysoPC are defined by fatty acid chain length and degree of saturation, which may translate into different physical and biological properties.[29] Although lysoPC levels obviously increase in aging rats,[30] this important issue is largely unexplored in humans. In our study, among the six major metabolites identified by mass spectrometric analysis, lysoPC 16:0 was found to be the most important plasma metabolite for evaluating aging-related changes. Additionally, the changes in lysoPC 16:0 levels were strongly positively correlated with the changes in levels of MDA, hs-CRP, IL-6, and ba-PWVs. This result suggests that age-related increases in lysoPC 16:0 levels in middle-aged men could contribute to lipid peroxidation, the activation of a proinflammatory phenotype, and arterial stiffness. Furthermore, increases in lysoPC 16:0 levels from the baseline to the 3-year follow-up data were significantly greater in subjects with increased ba-PWVs. PWV is an

established index of arterial stiffness,[31] and ba-PWVs show similar characteristics to those of central aortic PWV.[32]

Arterial stiffness, one of the most significant manifestations of vascular aging,[33,34] can result in increased systolic blood pressure.[35,36] This is a condition that can worsen with age, even in healthy individuals without CVD.[5] In addition, the presence of CVD risk factors such as obesity may accelerate the vascular changes that result in arterial stiffening.[5] In our study, men with increased ba-PWVs also showed significant increases in abdominal obesity. Additionally, changes in WHR, MDA, IL-6, and P-selectin levels were significantly different between subjects with decreased and increased ba-PWVs. Although the mechanisms underlying arterial stiffening remain to be elucidated, the fact that changes in ba-PWV between the baseline and 3-year follow-up data were positively correlated with the changes in ox-LDLs and MDA levels suggest that oxidative stress conditions, such as age-related increases in these oxidants, could play an important role, in part, in accelerating arterial stiffness. Recently, Brinkley et al.[3] suggested that ox-LDL levels may be related to the pathogenesis of arterial stiffness, independent of other CVD risk factors.

Ox-LDLs, which contain lysoPCs, not only reside in atherosclerotic lesions,[37] but also circulate in the blood stream.[38] In plasma, a small

population of lysoPCs is associated with ox-LDLs, and is implicated as a critical factor in the atherogenic activity of ox-LDLs.[39] A saturated fatty acid or a monounsaturated fatty acid predominates in the sn-1 position of the phospholipid.[40] The generation of free radicals as a result of oxidative stress can activate phospholipase A_2,[30] which hydrolyzes phosphatidylcholine (PC) to produce lysoPCs.[41] Production of α-palmitoyl-lysoPC (C16:0) can stimulate endothelial cells to express adhesion molecules and release cytokines.[42,43-47] In fact, IL-6 was found to be induced by α-palmitoyl-lysoPC treatment in human umbilical vein endothelial cells. In line with this result, our study showed a significant age-related increase in both ox-LDL and lysoPC 16:0 levels and a positive relationship between the changes in lysoPC 16:0 and IL-6 levels.

LysoPCs, representing 5%−20% of the total plasma phospholipids,[48] are also formed by the action of lecithin cholesterol acyltransferase (LCAT) in plasma.[49] Human LCAT releases lysoPC 20:4 and 22:6 from the sn-1 position of PC.[50] In plasma, up to 80% of the lysoPC is found in the non-lipoprotein fraction, in which albumin is considered to be the main lipid-binding protein.[51] Unsaturated lysoPCs are mainly associated with albumin rather than lipoproteins.[52] In our study, we observed a positive relationship between the

changes in levels of lysoPC 16:0 and those of lysoPCs containing C18:0, C18:2 and C20:5, which could reflect an alternative source of lysoPC C16:0 production in addition to ox-LDLs.

6. CONCLUSIONS

In our study, a large number of metabolite markers were detected by UPLC-MS; however, most of these metabolite markers remain unidentified. Unlike gas chromatography-mass spectrometry, for which large databases exist, the use of liquid chromatography-mass spectrometry-based techniques for metabolomics research is still in its infancy, and the databases of endogenous biomolecules have not yet been constructed.[53] Despite this limitation, using an UPLC-Q-TOF MS-based metabolomics strategy and multivariate data analysis, our study identified a cluster of age-associated changes in plasma metabolites that included six major metabolites: lysoPCs containing C16:0, C18:0, C18:2, C20:4, and C20:5, and linoleyl carnitine. Among these six major metabolites, lysoPC 16:0 served as the most important plasma metabolite for evaluating age-related differences between the baseline

and 3-year follow-up data. Additionally, the changes in lysoPC 16:0 levels were positively correlated with the changes in levels of lipid peroxides, proinflammatory markers, and ba-PWV. These results suggest that increases in lysoPC 16:0 can be explored further as a potential marker for lipid peroxidation, the activation of a proinflammatory phenotype, and arterial stiffness related to aging.

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국문 요약

중년층 남성에게서 노화와 중간 지질 대사체, 염증반응, 산화 스트레스 지표와 동맥경화도의 변화와의 연관성

본 연구는 지난 3년간 건강한 정상체중의 34세~55세 남성 57명을 대상으로 노화와 혈액 내를 순환하는 대사체, 염증성 지표, 그리고 산화 지표와 혈관 경직도와의 상관관계를 알아보고자 하였다. 혈관 경직도는 상완-발목 맥파 전파속도(brachial-ankle pulse wave velocity, ba-PWV)를 이용하여 측정 하였다. 대사체 profile들은 UPLC/Q-TOF MS를 사용하여 분석하였다. 3년 후 HDL-Cholesterol의 수치는 감소하였고, malondialdehyde(MDA)와 oxidized-LDL의 수치는 증가하였다.

15개의 lipid 중에 노화에 따른 차이를 보여주는 주요 대사체는 lyso-phosphatidylcholines (C16:0, C18:0, C18:2, C20:4, and

C20:5)와 linoleyl carnitine 이었다. 그 중 특히 LysoPC16:0 (variable importance in the projection [VIP] value: 6.2029) 은 가장 유의적인 차이를 보여주는 중요한 대사체였다. LysoPC16:0은 MDA (r=0.413), high-sensitivity C-reactive protein (r=0.509), Interleukin6 (r=0.497), ba-PWV (r=0.283) 와 양의 상관관계를 보였다. ba-PWV는 염증지표, 산화지표의 변화와 양의 상관관계를 보였다. 한편 3년간 혈관경직도가 증가한 집단과 감소한 집단으로 나누어 각각을 비교했을 때 WHR과 lysoPC16:0 수치, ba-PWV, IL-6, MDA, P-selectin에서 유의적인 차이를 보였다.

본 결과는 노화에 따른 lysoPC16:0의 증가는 지질 산화와 염증반응지표, 혈관경직도에 기여할 수 있다는 것을 시사하였다.

핵심이 되는 말: 노화에 따른 변화, 중간 대사체, 염증성 지표, 산화 스트레스, 혈관 경직도