Plasma metabolic profiling in male patients with newly-diagnosed type 2 diabetes

: Association with inflammatory and oxidative stress markers and arterial stiffness

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Graduate Program in Science for Aging

Aging and Clinical Nutrition

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: Association with inflammatory and oxidative stress markers and arterial stiffness

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저보다 먼저 이 과정에 들어와서 이 과정을 저에게 처음 소개해주어 강남미즈메디 병원에 재직 중이던 저를 이종호 교수님과 보스턴 Interleukin

Genetics.Co 연구그룹과 골다공증 연구에 함께 참여하도록 한 계기가 된 의대생 때부터 늘 학문과 신앙의 열정이 가득한 좋은 친구 김경철 선생님에게도 감사를 드립니다. 논문 작성에 많은 자문과 실제적인 도움을 주신 김지영 교수님께도 감사 드립니다. 함께 수업을 듣고 조금 먼저 이 과정을 거쳐간 경험자로서, 편안하게 여러 질문에 응해주신 국립 암센터 여현양 박사께도 감사 드립니다. 많은 세미나 일정으로 바쁘신 중에도 논문 관련 많은 도움을 주신 베를린 공대 김민경 박사님께도 감사 드립니다. 논문제본 과정을 도와준 서우주님께도 감사 드립니다.

끝으로 사랑하는 가족, 착한 동생 영화와 대영이. 이제는 때로는 조언자로, 존재자체로 늘 격려와 힘이 되는 아낌없이 주어도 아깝지 않은 든든한 형제들에게 무한한 사랑과 고마움을 전합니다. 일평생 각자 공직자로서, 교육자로서 정직하고 청렴하게 살아오신 아버지, 어머니. 삶 자체로서 모범이 되셨고, 나이와 상관없이 새로운 경험과 도전을 두려워하지 않으시는 열정과 부지런함은 늘 안주하려는 저를 돌아보게 합니다. 제가 지나온 모든 과정과정마다 어머니의 기도가 함께 한 것을 알기에 감사합니다 라는 말만으로는 늘 부족함을 느낍니다. 무뚝뚝한 아들로 평소에 말로는 잘 표현 못하지만 늘 마음속에 있는 인사를 드리며 글을 맺을까 합니다. 아버지, 어머니 감사합니다. 사랑합니다.

지금까지 은혜로 인도하신 하나님께 감사와 찬양, 영광을 드리며, 아버지, 어머니께 이 논문을 바칩니다.

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ABSTRACT

Plasma metabolic profiling in male patients with newly-diagnosed type 2 diabetes: Association with inflammatory and oxidative stress markers and arterial stiffness

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This study investigated whether circulating metabolic intermediates are associated with inflammation, oxidative stress, and arterial stiffness in newly-diagnosed type 2 diabetes men. newly-diagnosed type 2 diabetes men (n=26) and age- and body mass index-matched healthy men (n=27) were included. We measured inflammatory and oxidative markers and arterial stiffness by brachial-ankle pulse wave velocity (ba-PWV). Metabolomic profiling was analyzed with UPLC/Q-TOF MS. Diabetes patients showed higher levels of glucose, triglyceride, oxidized-LDL, hs C-reactive

protein, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), homeostasis model assessment-insulin resistance, urinary 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$), and ba-PWV than healthy men. In plasma, 19 metabolites including leucine, lysine and phenylalanine , 8 acylcarnitines, 6 lyso-phosphatidylcholines (lysoPCs) and 2 lyso-phosphatidylethanolamines (lysoPEs) significantly increased in diabetes patients, whereas serine and lysoPE (C18:1) decreased. Decanoyl carnitine, lysoPCs (C14:0, C16:1, C18:1, and C22:6), and lysoPE (C18:1) were major plasma metabolites that distinguished healthy and diabetic men. Decanoyl carnitine positively correlated with oxidized-LDL, 8-epi-$PGF_{2\alpha}$, IL-6, and TNF-α, and ba-PWV. Ba-PWV correlated positively with lysoPCs C14:0 and C16:1, and negatively with lysoPE C18:1. 8-epi-$PGF_{2\alpha}$ correlated positively with lipoprotein-associated phospholipaseA_2, ba-PWV and lysoPCs (C14:0 and C16:1). Metabolic analysis revealed that circulating intermediate metabolites are closely associated with inflammation, oxidative stress, and arterial stiffness in early diabetes.

Key words: intermediate metabolites, UPLC/Q-TOF MS, inflammation, oxidative stress, arterial stiffness, early diabetes

1. INTRODUCTION

With people in both developed and developing countries becoming increasingly less active and more obese, the incidence of type 2 diabetes is increasing at an alarming rate. In recent decades, Koreans increased the percentage of fat calories in their diet from 7.2% in 1969 to 18.5% in 2007 (1), and the mortality rate from diabetes has increased rapidly from 7.4% in 1988 to 22.9% in 2007 (2). Metabolomic studies have shown many changes in the human plasma metabonome associated with insulin resistance, type 2 diabetes, and glucose challenge-induced metabolic responses from prediabetes (3-6). More recently, a metabonomic study of biochemical changes was performed with the serum of type 2 diabetes patients after diabetes medications (7). However, these studies were aimed at developing methods for diabetes diagnosis or studying treatment effects. Similar studies focused solely on the Korean population still need to be performed, especially with the dynamic lifestyle changes evident in modern society. A comprehensive understanding of the metabolic changes, as well as the differences in inflammatory markers, oxidative markers, and arterial stiffness, between early diabetic and healthy subjects also remains relatively unstudied.

In this study, the plasma metabonomic characteristics of age- and body mass index (BMI)-matched Korean men with normal fasting glucose levels and men newly-diagnosed with type 2 diabetes were studied using a metabonomics approach

based on the combination of the ultra performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS) and multivariate data analysis. Additionally, inflammatory and oxidative markers, and arterial stiffness measured by brachial-ankle pulse wave velocity (ba-PWV) were analyzed. The aim of this study was to determine whether circulating metabolic intermediates are related to inflammation, oxidative stress, and arterial stiffness in men with newly-diagnosed type 2 diabetes.

2. BACKGROUND

2.1. Type 2 Diabetes in Korea

Type 2 diabetes mellitus (T2DM) is a major health problem affecting more than 170 million people worldwide, with a major increase expected in Asia in the next 20 years, as indicated by the predicted doubling of the diabetic populations in India and China (8,9). In the USA, more than 13% of adults over the age of 20 years have been diagnosed with T2DM (10). The prevalence of T2DM in Korea rapidly increasing from less than 1% in 1960 to 6-9% in late 1990s (11,12). The rapid rise in the prevalence of T2DM in Korea may be related to an increasingly Westernized diet, decreased physical activity, an increasing obese population, and genetic background

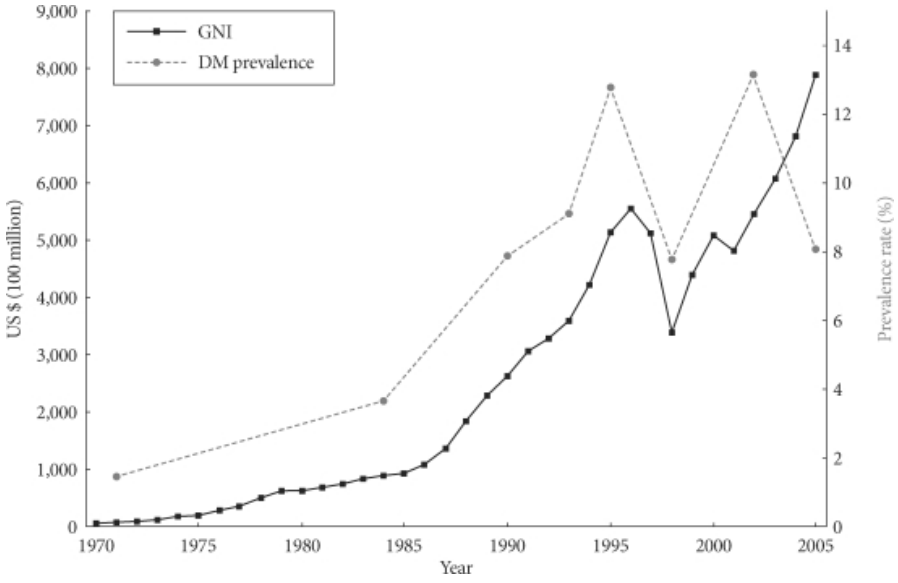


Fig. 1 The prevalence of diabetes and gross national income (GNI) (14)

(13). The putative risk factors for T2DM in the Korean population include increasing age, urban living, female gender, obesity, smoking, family history of diabetes, impaired liver function, metabolic syndrome, elevated blood pressure, and increased triglycerides. Cho et al showed the rise in the prevalence of T2DM in Korea correlates with economic growth (Fig.1) (14).

2.2. Type 2 diabetes : stimuli and signaling

Type 2 diabetes is a multifactorial disease based on environmental factors and genetic background. In many patients, diabetes is asymptomatic for a long period and the patient is not aware of the disease. Type 2 diabetes is the most prevalent and serious metabolic disease all over the world, and its hallmarks are pancreatic beta-cell dysfunction and insulin resistance. Under diabetic conditions, chronic hyperglycemia and subsequent augmentation of reactive oxygen species (ROS) deteriorate beta-cell function and increase insulin resistance which leads to the aggravation of type 2 diabetes (Fig. 2) (15).

The JNK pathway is activated by various factors such as ROS, ER stress, free fatty acids (FFAs), and inflammatory cytokines such as TNF-α and is involved in the development of insulin resistance found in type 2 diabetes. It has been also shown that the IkappaB kinase β (IKK) pathway is also activated by such factors and is involved in the development of insulin resistance. Therefore, it is likely that activation of stress signaling is involved in the development of insulin resistance (Fig. 3) (15).

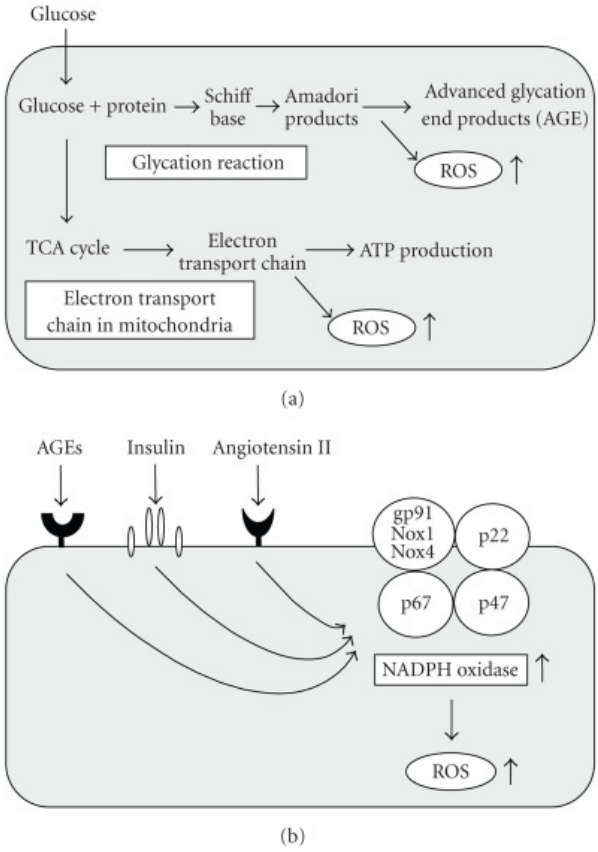


Fig. 2. Augmentation of ROS by various pathways under diabetic conditions (15)

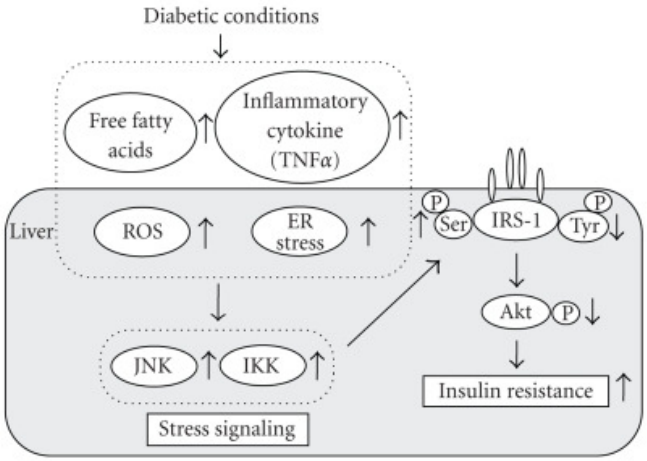


Fig. 3. Role of ROS in the development of insulin resistance in type 2 diabetes (15)

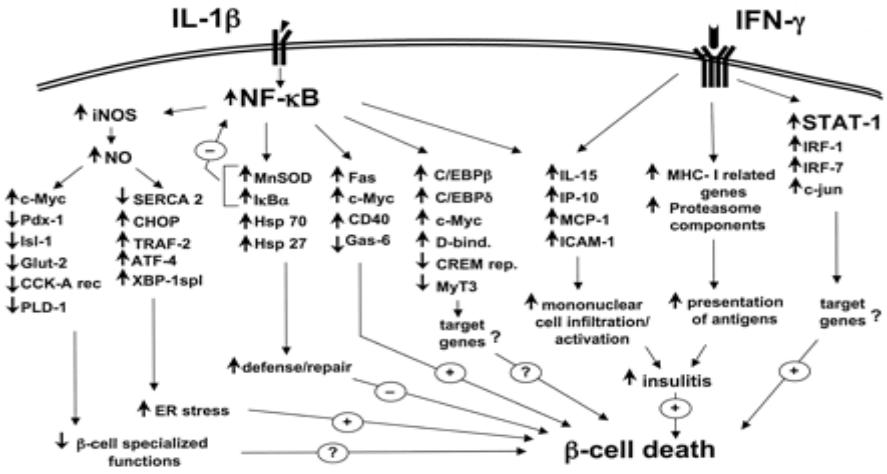


Fig. 4. The transcription factor and gene networks in cytokine induced ß-cell death (16)

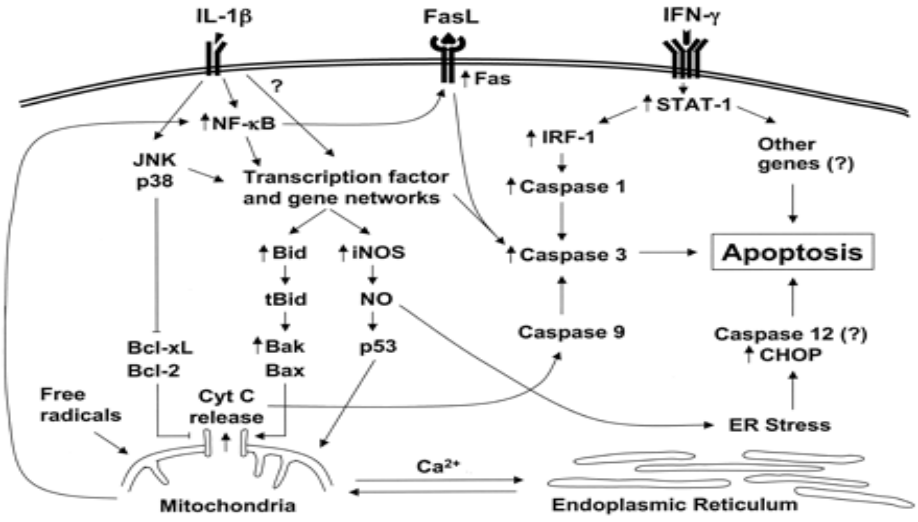


Fig. 5. Proposed model for the different pathways contributing to the execution of cytokine-induced ß-cell death (16)

Pancreatic beta-cells are sensitive to a number of proapoptotic stimuli. Thus, apoptosis is an important part of the physiological neonatal remodeling of the

endocrine pancreas, and a number of pathological stimuli involved in type 1 and type 2 diabetes have been shown to elicit beta-cell apoptosis. Factors of relevance to type 1 diabetes include proinflammatory cytokines, nitric oxide, and reactive oxygen species as well as Fas ligand. Free fatty acids, glucose, sulfonylurea, and amylin cause beta-cell apoptosis in vitro suggest that programmed cell death may also be involved in the pathogenesis of type 2 diabetes. Furthermore, there is evidence favoring a convergence in signaling pathways toward common effectors of beta-cell apoptosis elicited by stimuli implicated in the pathogenesis of type 1 and type 2 diabetes. immunological, inflammatory, and metabolic signals cause beta-cell apoptosis(Fig. 4. 5.) (16-22).

In type 2 diabetes, the metabolic derangement is associated with production of inflammatory mediators in insulin-sensitive tissues leading elevated levels of circulating inflammatory mediators such as IL-6 and TNF. Further glucose has been suggested to induce beta-cell apoptosis via the induction of beta-cell synthesis of IL-1 which via autocrine action may elicit signaling cascades analogous to those seen in beta-cell destruction in type 1 diabetes (23). The blood or urine glucose and HbA1C are the only laboratory markers for diagnosis of diabetes. Therefore, the potential biomarker(s) for early detection and/or prevention of diabetes are strongly required. There are some studies to find out new biomarkers of diabetes by new technologies. Metabolomic methodologies including nuclear magnetic resonance (NMR) and mass spectrometry (MS)-based metabolic profiling technologies are applied to diabetes research (24-26). There is abundant evidence that glucotoxicity and lipotoxicity

contribute to impaired β-cell function in type 2 diabetes. Interestingly, amino acid (AA) derangement is also a characteristic part of the diabetic state. The effects of AA on pancreatic β-cell function have been widely explored. Wang TJ et al reported five branched-chain and aromatic amino acids had highly significant association with future diabetes: isoleucine, leucine, valine, tyrosine and phenylalanine (27). Huffman KM et al reported a factor containing large neutral amino acids was inversely related to insulin sensitivity and a factor containing fatty acids was inversely related to the acute insulin response to glucose (28). Mochida et al reported the plasma concentrations of valine, leucine, isoleucine, as well as the total branched chain amino acids, alanine, citrulline and proline, were significantly higher in the diabetic mice (29). Liu et al reported the clonal b-cell line INS-1E cells were differentially regulated (≥1.5- or ≤ -1.5-fold) by Pro (295 transcripts), Hcy (301 transcripts), and Leu (701 transcripts). It appeared that Hcy effects changes opposite to those induced by Leu and/or Pro (30).

The clinical spectrum is very wide in patients with diabetes. Weir GC et al proposed that there are five stages in the progression of diabetes (Fig. 6), each of which is marked by important changes in β-cell mass, phenotype, and function. Although conventionally thought of as a continuum, we make the case that each has its own important characteristics (31). Stage 1 is best described as compensation: insulin secretion increases to maintain normal glucose levels in the face of insulin resistance resulting from obesity, physical inactivity, and genetic predisposition. Stage 2 occurs when glucose levels rise to levels of ~5.0–6.5 mmol/l (89–116 mg/dl)—a

stable state of β-cell adaptation. Stage 3 is an unstable period of early decompensation in which glucose levels rise relatively rapidly to stage 4, which is characterized as stable decompensation. Finally, there is the severe decompensation of stage 5 that represents profound β-cell failure with progression to ketosis. Further studies are needed to interpret different clinical spectrums of patients with T2DM.

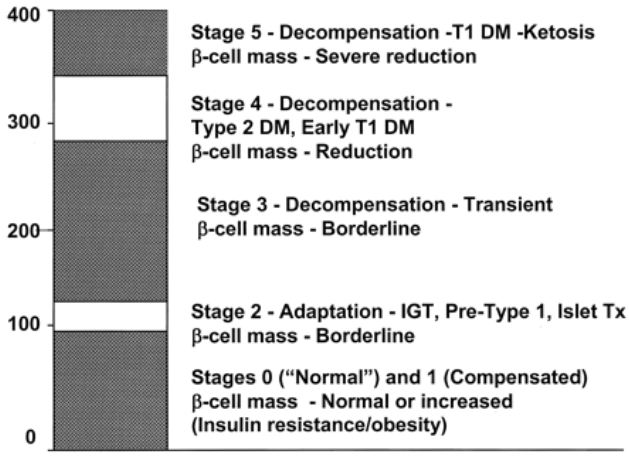


Fig. 6. Schema of five stages of progression of diabetes (31)

2.3. Type 2 diabetes and Atherosclerosis

Diabetes impairs endothelium-dependent (nitric oxide–mediated) vasodilation before the formation of atheroma (Fig. 7) (32-33). A number of fundamental mechanisms contribute to the decreased bioavailability of endothelium-derived nitric oxide in diabetes. Hyperglycemia inhibits production of nitric oxide by blocking eNOS (Nitric Oxide Synthase) activation and increasing the production of reactive oxygen species, especially superoxide anion (O^{2-}), in endothelial and

vascular smooth muscle cells (34). Superoxide anion directly quenches nitric oxide by forming the toxic peroxynitrite ion,(35) which uncouples eNOS by oxidizing its cofactor, tetrahydrobiopterin, and causes eNOS to produce $O^{2.}$ Other common abnormalities in type 2 diabetes also decrease endothelium-derived nitric oxide. Insulin resistance leads to excess liberation of free fatty acids from adipose tissue (36), which activate the signaling enzyme protein kinase C, inhibit phosphatidylinositol-3 (PI-3) kinase (an eNOS agonist pathway), and increase the production of reactive oxygen specieic (37) Production of peroxynitrite decreases synthesis of the vasodilatory and antiplatelet prostanoid prostacyclin (38). Thus, as nitric oxide bioavailability progressively decreases, concomitant increases in peroxynitrite further impair production of subsidiary vasodilators.

In addition to reducing ambient concentrations of nitric oxide, diabetes increases the production of vasoconstrictors, most important, endothelin-1, which activates endothelin-A receptors on vascular smooth muscle cell to induce vasoconstriction (Fig. 7). In addition to its modulation of vascular tone, endothelin-1 increases renal salt and water retention, stimulates the renin-angiotensin system, and induces vascular smooth muscle hypertrophy (39). Endothelin-1 activity may rise in response to insulin-mediated increases in gene expression and receptor formation, stimulation of the receptor for advanced glycation end products, and increased gene transcription induced by oxidized low-density lipoprotein (LDL) cholesterol (40-42).

Diabetes increases other endothelium-derived vasoactive substances such as

vasoconstrictor prostanoids and angiotensin II, and investigation into their pathophysiological relevance in diabetes continues (43, 44). Migration of T-cell lymphocytes and monocytes into the intima participates integrally in atherogenesis. T cells secrete cytokines that modulate lesion formation (45). Monocytes, upon reaching the subendothelial space, ingest oxidized LDL via scavenger receptors and become foam cells. Localized accumulation of foam cells leads to formation of fatty streaks, the hallmark of early atherosclerotic lesions (46). Diabetes augments these pathologic processes.

Hyperglycemia via decreased nitric oxide, increased oxidative stress, and receptor for advanced glycation end products activation increases the activation of the transcription factors nuclear factor κB and activator protein 1 (Fig. 7). These factors regulate the expression of the genes encoding a number of mediators of atherogenesis; for example, leukocyte-cell adhesion molecules on the endothelial surface, leukocyte-attracting chemokines, such as monocyte chemoattractant proteins that recruit lymphocytes and monocytes into the vascular wall, and proinflammatory mediators found in atheroma, including interleukin 1 and tumor necrosis factor (47-49). Lipid abnormalities commonly found in diabetes, such as increased very low-density lipoprotein (VLDL) and excess free fatty acid liberation, also increase endothelial nuclear factor κB and subsequent cell adhesion molecule and cytokine expression (50).

In addition to enhancing the initiation of atherogenesis, diabetes promotes plaque instability and clinical sequelae. Diabetic endothelial cells elaborate

cytokines that decrease the de novo synthesis of collagen by vascular smooth muscle cells (51). Diabetes also enhances the production of matrix metalloproteinases that lead to breakdown of collagen (52). Collagen confers mechanical stability to the plaque's fibrous cap. When collagen breakdown increases and synthesis decreases, plaques may rupture more readily, a trigger to thrombus formation. Finally, an important modulator of the severity of plaque rupture is the extent of vascular occlusion by thrombus formation. In diabetes, endothelial cells increase production of tissue factor, the major procoagulant found in atherosclerotic plaques along with alterations in soluble coagulation and fibrinolytic factors (Fig. 7) (53).

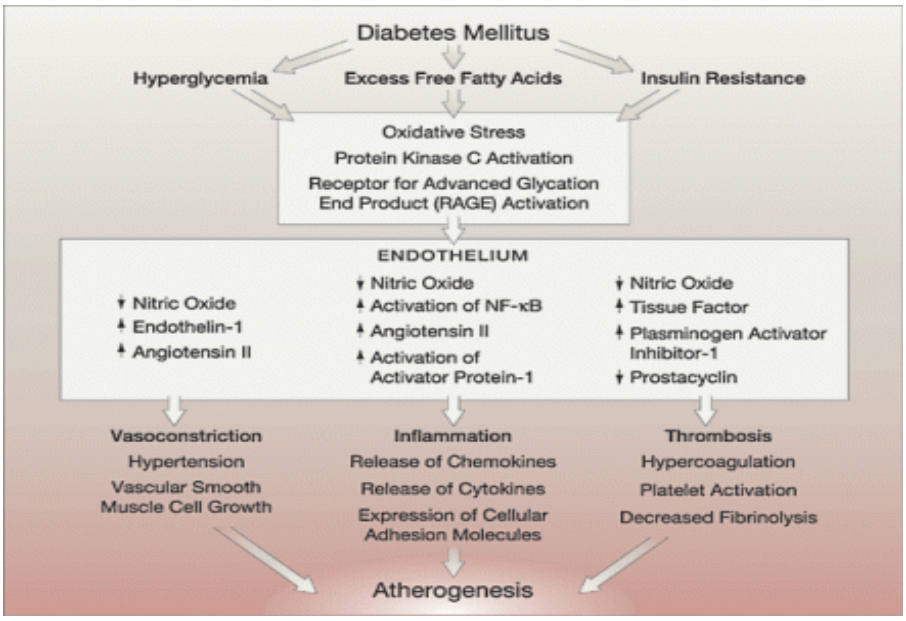


Fig. 7. The pathogenesis of Atherogenesis in Diabetes (53)

ROS are produced by various pathways under diabetic conditions and involved in the development of atherosclerosis in various aspects. Hyperglycemia

induces ROS through activation of the glycation reaction and electron transport chain in mitochondria. Also, advanced glycosylation end products (AGEs), insulin, and angiotensin II induce ROS through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Increased ROS are involved in the development of atherosclerosis in various aspects. (Fig. 8)(54)

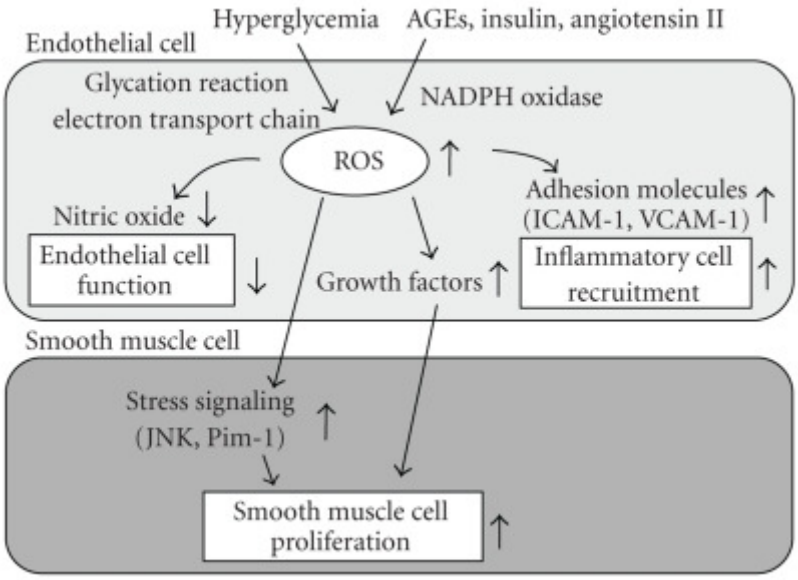


Fig. 8. Role of ROS in the development of atherosclerosis (54)

2.4. Atherosclerosis and Biomarkers

A biomarker is defined as a substance used as an indicator of a biological state. Through various techniques it is objectively measured and used to assess normal or pathogenic biological processes, or of pharmacologic responses to a therapeutic intervention. By definition it is critical that this diagnostic tool be relied upon clinically to improve accuracy of diagnosis, delineate disease subtypes, monitor

disease progression, or improve prognostication and risk assessment. In addition, a biomarker may demonstrate treatment efficacy when used with disease modifying therapies, such as lipid lowering drugs.

2.4.1. hs-CRP

CRP protein is an acute phase reactant discovered by Tillett and Francis at Rockefeller University in 1930 in the blood of patients with pneumonia (55), was crystallized in 1947, and today, over 60 years later, there is still controversy about its physiology and applications in biomedicine. CRP protein is a pentraxin comprised of 5 subunits (56) (Fig. 9) which is primarily synthesized in the liver, and plays an active part in regulating the innate immune system. Since CRP mRNA levels rise in adipose tissue as CRP expression is enhanced in vitro by interleukin-6 (IL-6), adipose cells also have some ability to synthesize CRP. Innate immunity and adaptive immunity significantly modulate atherosclerosis (57-59), with both pro- and anti-atherogenic potential (60,61).

CRP binding offers a glimpse into teleological function. CRP binds to phosphocholine, commonly found in cell membranes as well as in bacterial and fungal polysaccharides. Phosphocholine is used by the placenta and nematodes to cloak them from their host immune system. Binding to complement C1q complex and factor H, CRP assists in human complement activity to promote antigen presentation and phagocytosis (62), essentially functioning as an innate opsonin. In addition, CRP binds directly with phagocyte Fcγ receptors(63), literally becoming the interface

between innate immunity, C1q on complement, and inflammation, Fcγ on macrophages. Details of CRP binding to its ligands and their implications have been recently reviewed (64). As an acute phase reactant during infections or diffuse tissue destruction, CRP concentrations may increase 50,000-fold quickly (65). A constant potent stimulus may sustain high circulating CRP concentrations, depending upon synthetic capacity; the half-life of CRP in the circulation is about 19 hours (66). Hepatic synthesis is driven at the transcriptional level by IL-6, largely expressed by macrophages, T cells, and adipocytes (67. 68). Several upstream cytokines also regulate hepatic CRP release. In part because there is overlap in signaling, plasma CRP values are related to other inflammatory markers. However, despite a plethora of research, the precise role(s) of CRP remain unclear.

Since CRP binds to LDL and can be identified within atherosclerotic plaque (69, 70), is involved with inflammatory atherogenic processes (71-82), is elevated in patients with acute (ACS) and chronic coronary syndromes (83-86) with unfavorable plaque compositions in these syndromes (87) and is associated with complications of heart failure (88) a causal role in atherothrombotic disease has long been suspected. At this time, causality remains unproven (72, 89, 90). One intriguing property of CRP is the associated inhibition of endothelial nitric oxide synthase in vitro (91) and the relationship to impaired vasoreactivity and hypertension in such models (92). Overall, the evidence for a role of CRP in endothelial cell dysfunction and monocyte activation in metabolic syndrome is approaching 53% in some populations (excluding those with diabetes) (94) and the importance of endothelial dysfunction in

pathogenesis is now unassailable (95).

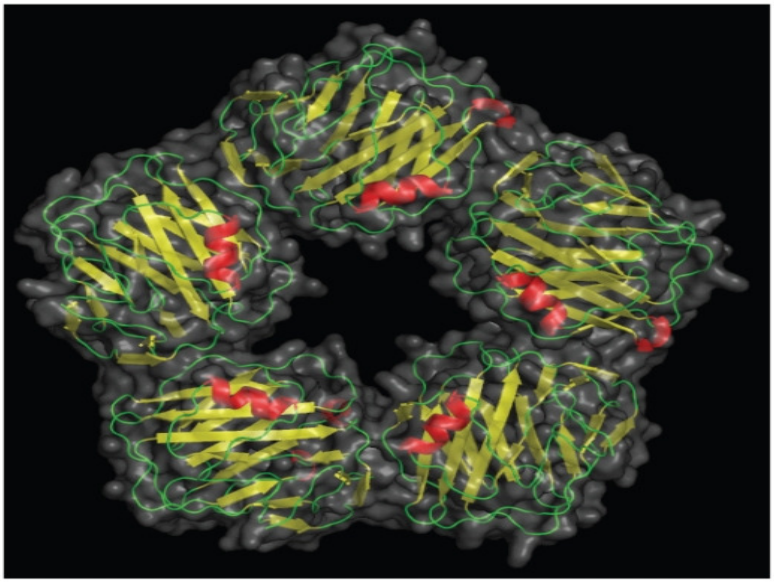


Fig. 9. A picture of C-reactive protein (CRP) (56)

2.4.2. Lp-PLA_2

Lipoprotein-associated phospholipase A2 (Lp-PLA_2) is a novel biomarker of vascular-specific inflammation that provides information about atherosclerotic plaque inflammation and stability. Elevated levels of serum Lp-PLA_2 are indicative of rupture prone plaque and a strong independent predictor of cardiovascular risk, including coronary artery disease, MI, and stroke (96. 97). Lp-PLA_2 is associated clinically with increased CHD risk, and there is a large body of published evidence from epidemiologic studies addressing the relationship of Lp-PLA_2 and risk of cardiovascular disease (98-100).

Biologically, Lp-PLA_2 is a vascular-specific proinflammatory enzyme that operates physiologically in the arterial intima (Fig. 10) (101). Lp-PLA_2 localizes to

atherosclerotic plaque, particularly in plaques with a necrotic core and in ruptured plaques (102). High levels of Lp-PLA_2 are found in rupture prone plaques, and it appears Lp-PLA_2 is released from these plaques into the circulation. Lp-PLA_2 is primarily produced by macrophages and then bound to various lipoproteins, including the ApoB portion of low-density lipoprotein (LDL) and lipoprotein(a) (103). Staining of coronary and carotid tissue demonstrates the presence of Lp-PLA_2 in the thin fibrous cap of rupture-prone plaques, but not in the early-stage plaques (102, 104). Coronary and carotid tissue concentrations of Lp-PLA_2 are notably very high in the rupture-prone shoulder region of thin fibrous cap atheromas, and histopathologic stains reveal that Lp-PLA_2 co-localizes with macrophages and oxidized LDL in atherosclerotic coronary and carotid plaques (105).

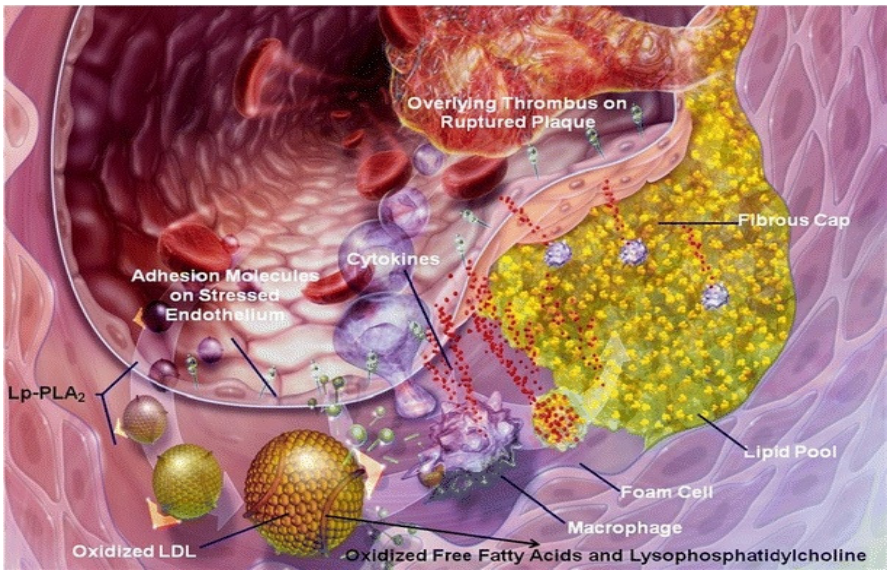


Fig. 10. Lipoprotein-associated phospholipase A2 and atherogenesis (101)

Lp-PLA_2 hydrolyzes phospholipids on oxidized LDL particles in the

subendothelial space (Fig. 11) (101). Lp-PLA_2 hydrolyzes the center (sn-2) ester bond of phospholipids which yields oxidized fatty acids and lysophosphatidylcholine (lysoPC), a molecule with a range of potentially atherogenic effects, including chemoattraction of monocytes, increased expression of adhesion molecules, and inhibition of endothelial nitric oxide production (106. 107). In this manner, a vicious cycle is set up that leads to recruitment of monocytes to the intima, where they differentiate to become macrophages, and, ultimately, foam cells, while at the same time locally producing more Lp-PLA_2. Furthermore, lysoPC has been found to be cytotoxic to vascular smooth muscle cells and can induce local production of matrix metalloproteinases (MMP's), which can thin the fibrous cap and destabilize the architectural integrity of an atheromatous plaque through destruction of the collagen matrix, increasing its propensity to rupture (108).

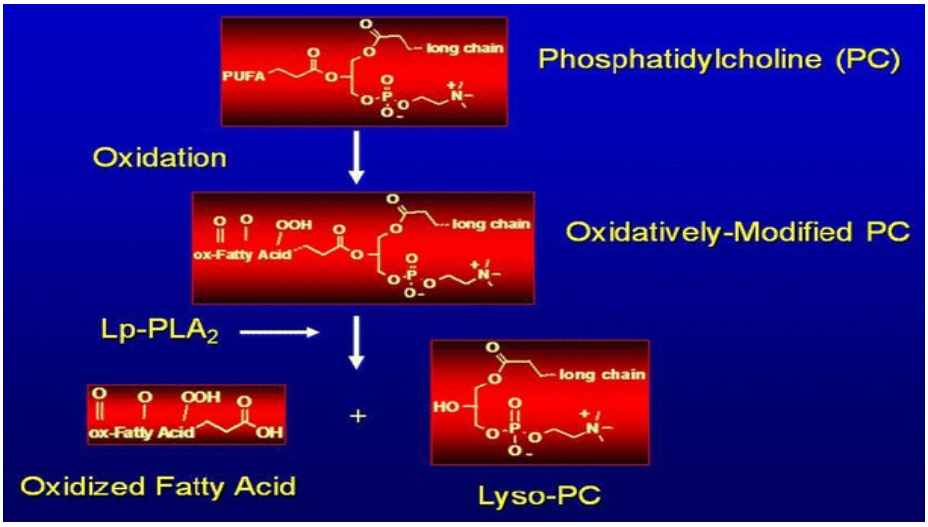


Fig. 11. Lp-PLA2 hydrolyzes oxidized LDL to release proinflammatory lipids (101)

In terms of its utility as a circulating biomarker, Lp-PLA_2 produced by activated

macrophages and foam cells reenters the bloodstream and can be quantitatively measured. As reported by Lavi et al (103), Lp-PLA_2 blood concentrations sampled simultaneously in the human coronary sinus demonstrated a net increase in Lp-PLA_2 levels as blood traverses the coronary vascular bed from individuals with significant atherosclerotic plaque. However, when no coronary plaque is present, a decrease in Lp-PLA_2 levels is found. This study also showed that the lysoPC produced by Lp-PLA_2-mediated hydrolysis of oxidized LDL is highly associated with coronary artery endothelial dysfunction. Lp-PLA_2 is a marker of vascular-specific inflammation and reflects the presence of rupture prone plaque. It is an independent predictor of cardiovascular risk, over and above traditional risk factors. However, tests for other robust biomarkers may be clinically useful in an additive manner. For example, it has been shown that while these markers are independent predictors of risk, Lp-PLA_2 added to hs-CRP provides significantly more risk assessment information over hs-CRP alone. With regard to hs-CRP as a standalone test, the measured values are quite variable, requiring several independent measurements over the course of time to confirm the level, since general inflammation, infection and adiposity could be driving the value, while Lp-PLA_2 has high specificity and low biovariability. Lp-PLA_2 activity and mass each showed continuous associations with risk of coronary heart disease, similar in magnitude to that with non-HDL cholesterol or systolic blood pressure in this population. Associations of Lp-PLA_2 mass and activity are not exclusive to vascular outcomes, and the vascular associations depend at least partly on lipids (Fig. 12, 13) (109).

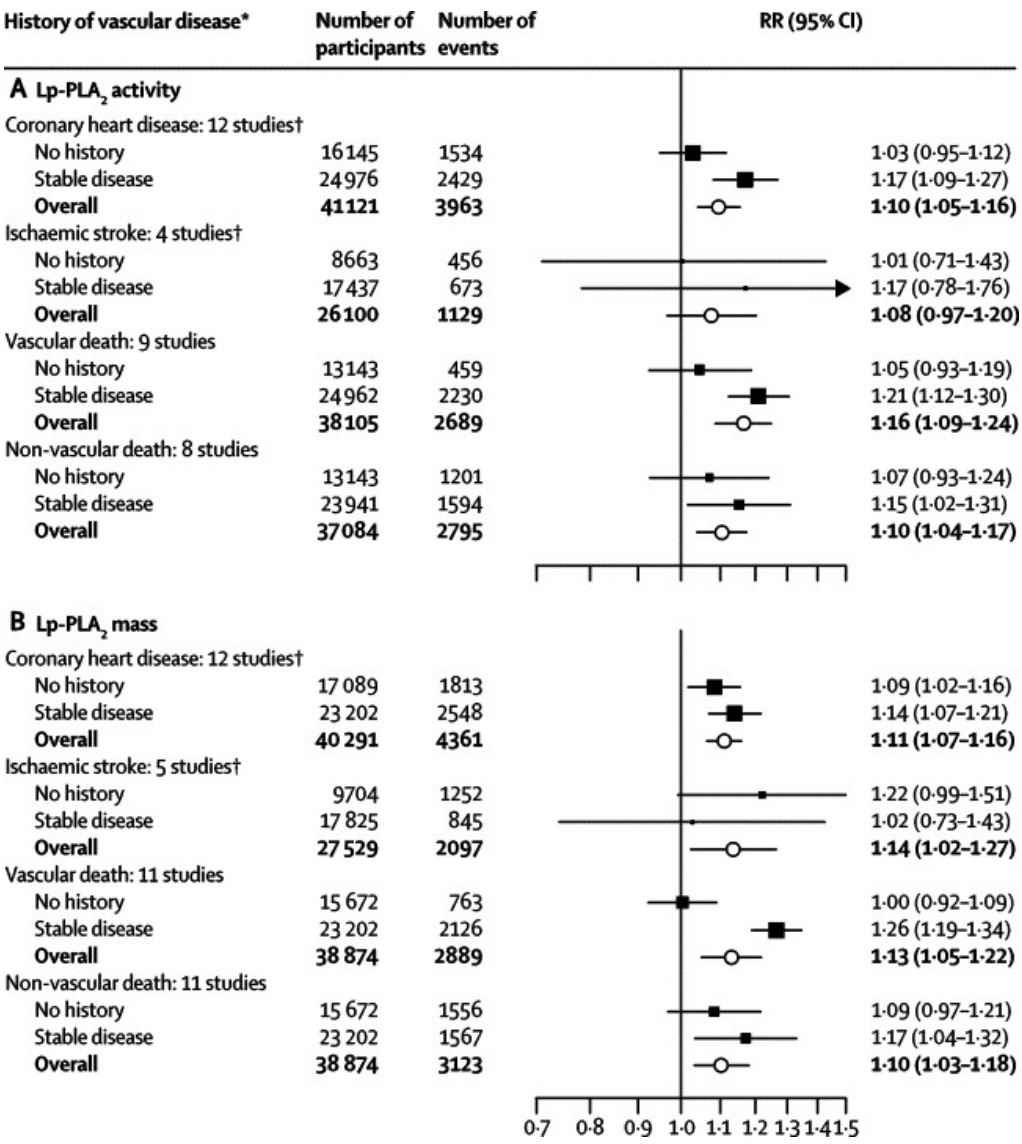


Fig. 12. Risk ratios for coronary heart disease, ischaemic stroke, and vascular and non-vascular mortality (109)

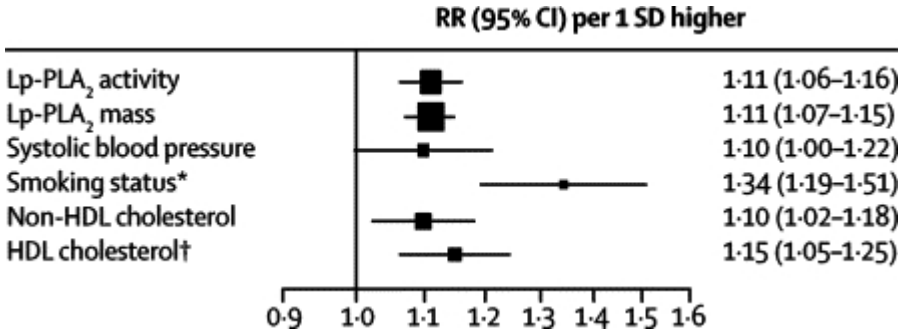


Fig. 13. Adjusted risk ratios for coronary heart disease per 1 SD higher baseline Lp-PLA2 activity, mass, and several conventional risk factors (109)

Consensus expert panel recommendations advocate for the measurement of Lp-PLA_2 as an adjunct to traditional risk factor assessment to improve the prediction of cardiovascular risk (110). It is recommended that Lp-PLA_2 testing be performed in patients at moderate risk, those defined as apparently healthy with two or more traditional risk factors or a 10-year Framingham risk score of 10–20%, as well as high risk, those with established coronary heart disease (CHD) or CHD risk equivalents, who will benefit from more aggressive lifestyle changes and lipid-modifying therapies. The Lp-PLA_2 cut point, or clinical decision threshold, for risk reassignment is >200 ng/mL. The consensus panel agreed to this threshold based upon review of Lp-PLA_2 studies (111-113) which demonstrated a sufficient increase in the risk of cardiovascular events above this level to warrant more aggressive patient management. Patients at moderate risk with Lp-PLA_2 levels >200 ng/mL are reclassified as having high CHD risk status, while those persons with known CHD or CHD risk equivalent and elevated levels of Lp-PLA_2 are reclassified as very high risk (Fig. 14)(101).

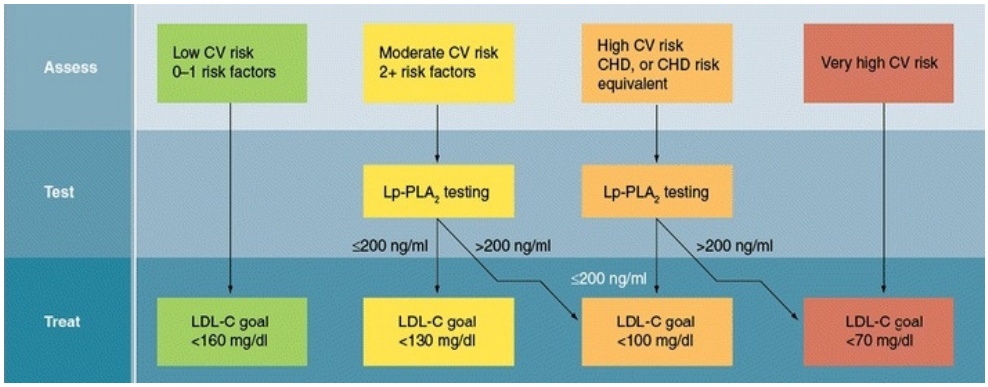


Fig. 14. Algorithm for Lp-PLA2 screening and utility for refining cardiovascular risk estimation (101)

It is not recommended that Lp-PLA_2 be measured in patients at low risk for cardiovascular disease (one or fewer risk factors). Patients with two or more risk factors (10 year Framingham risk score of 10–20%) are optimal candidates for Lp-PLA_2 screening. If the serum level of this enzyme is <200 ng/mL, then their level of risk requires no further adjustment. However, if the serum level of Lp-PLA_2 is >200 ng/mL, then the patient is reassigned to high risk status and the LDL-C and non-HDL-C targets are adjusted to <100 mg/dL and <130 mg/dL, respectively. Among patients who are high risk (established CHD, diabetes mellitus, abdominal aortic aneurysm, peripheral vascular disease, symptomatic carotid artery disease, or a 10 year Framingham risk score >20%), consideration can be given to further refining risk estimation with an Lp-PLA_2 measurement. If the patient's serum Lp-PLA_2 measurement >200 ng/mL, then the patient can be reclassified as very high risk, and the LDL-C and non-HDL-C targets should be <70 mg/dL and <100 mg/dL, respectively. (110).

2.4.3. oxidized LDL

Modified LDL (ie, oxidized LDL [oxLDL]) has been shown to contribute to atherosclerosis development, and that has prompted attempts to use circulating oxLDL as a disease biomarker. Santos et al examined the relationship of human immunoglobulin G (IgG) anti-oxLDL antibodies with cardiovascular disease risk markers in stable individuals and in patients with acute coronary syndrome. They observed that acute inflammatory and metabolic conditions decrease titers of human antibodies of IgG class against oxLDL. They also suggested that circulating anti-oxLDL antibodies could be associated with a protective role in atherosclerosis (114).

2.5. Lipidomics and Metabolomics

2.5.1 Lipidomics and Molecular Lipids

The recent development of mass spectrometry-driven lipid analysis approaches called “lipidomics” has made it possible to resolve complex lipidomes to their molecular lipid species level at high-throughput and quality required for analyses of clinical cohorts (115-117). As a result of the high sensitivity and selectivity of the methods, a lipidome-wide analysis of minute sample amounts has become feasible. Present technologies are capable of identifying lipids with different sum compositions (ie, phosphatidylcholine [PC] 34:1) (118), but more important is the identification of molecular lipid species, (eg, PC 16:0/18:1) (119). In the latter analysis, information of the type of fatty acids and their positions attached to the glycerol backbone making up the particular PC molecule is retrieved. Furthermore, in

the same analysis, it is now possible to simultaneously determine the double bond positions of the fatty acids, thus providing information of structurally defined molecular lipid species (eg, PC 16:0/18:1n9)(120). Although similar information can be obtained by traditional lipid analysis approaches, such as thin-layer chromatography in combination with gas chromatography, not only does it require much larger sample amounts and more laborious sample preparation, but it does not deliver the molecular lipid species.

There are cases where molecular lipids require preseparation prior to the mass spectrometric analysis. In such circumstances, targeted lipidomics is preferred for enabling the reliable retrieval of high qualitative data. In targeted lipidomics, lipid extracts are separated by liquid chromatography (LC), and eluted lipid species are monitored by online mass spectrometry (121, 122). Multiple reaction monitoring (MRM) analysis offers both the high selectivity and sensitivity necessary for quantification of low abundant lipid species (123, 124). In an optimized setting, the MRM approach can offer the highest detection sensitivity possible to date. However, due to the narrow time window of eluting peaks, especially when using ultra high-performance LC (UHPLC), only a restricted number of MRM transitions are feasible when high qualitative and quantitative data are called for. Thus, targeted lipidomics is best suited for absolute quantification of only a select number of molecular lipids that can be controlled by appropriate lipid standards to minimize possible errors arising from changes in the lipid ionization efficiency.

Despite the fact that lipidomics is thought of as another “omics” technology,

the type of data and information it delivers is considerably different from transcriptomics and proteomics. Measurement of lipid concentrations provides phenotypic information about a state of an organism determined by genetic regulation, functionality of protein machinery, environmental factors, and diet. When a disease or condition is studied, transcriptomics and proteomics will indicate what genes or proteins are affected. Lipidomic measurements, in turn, will bring to light the consequences expressed by lipid concentration changes in biofluid and tissue. Thus, lipidomics provides a readout that is closely connected to the cause of the disease (eg, accumulation of fatty substance in the arteries). Detailed lipidomic information will also be useful in tailoring proper treatments to the right patients.

Lipidomics has also proven to be an efficient way for detecting adverse drug actions. So far, drug-induced liver and muscle toxicities have been demonstrated in plasma lipidomic profiles. The PC to phosphatidylethanolamine (PE) ratio has been observed to reflect hepatocyte membrane fluidity changes, allowing a sensitive measure for liver toxicity (125). Also, by taking advantage of the molecular lipid information, the authors found new insight into the mechanisms behind the membrane fluidity alterations. Laaksonen R et al reported that a distinct plasma lipid pattern can be used to monitor statin-induced gene expression changes in human skeletal muscle and, thus, can detect statin-induced muscle toxicity (126).

Currently, lipidomics attempts to characterize structurally defined molecular lipids from a single analysis (127) and localize lipids in tissues such as aortic wall (128) and arterial plaques (129). Maturation of lipidomics and evolution of new lipid-labeling

techniques will not only improve lipidome characterization, but also identify the deleterious molecular lipids involved in the dysfunction, where this occurs inside the organism and at what rate. Thus, lipidomics is a prerequisite for advancing the cardiovascular disease (CVD) biology and promoting the discovery of cardiovascular disease biomarkers and new drug targets in coming years.

2.5.2. Metabolomics

The term metabolomics (and the related term metabonomics) was coined at the end of the 1990s to describe the development of approaches which aim to measure all the metabolites that are present within a cell, tissue or organism during a genetic modification or physiological stimulus (Fig. 15) (130-132). Metabolites are small molecules that participate in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell. The term metabolome, derived from the word genome, refers to the complete set of metabolites in an organism and its organelles (131, 133) or the total complement of metabolites in a cell (134). In this way, metabolomics and metabonomics refer to the use of analytical methods to identify and quantify all metabolites in a biological system, as well as the monitoring of changes in the metabolome of a biofluid, cell culture or tissue sample following perturbation (132, 133).

It has already been used to identify the function of genes, describe the effects of toxicological, pharmaceutical, nutritional and environmental interventions, and to build integrated databases of metabolite concentrations across human and research

animal populations. Metabolomics provides nutrition with an invaluable tool for determining the distributions of metabolite concentrations in humans, the relationship of these metabolite concentrations to disease, and the extent to which nutrition can modulate metabolite concentrations (135).

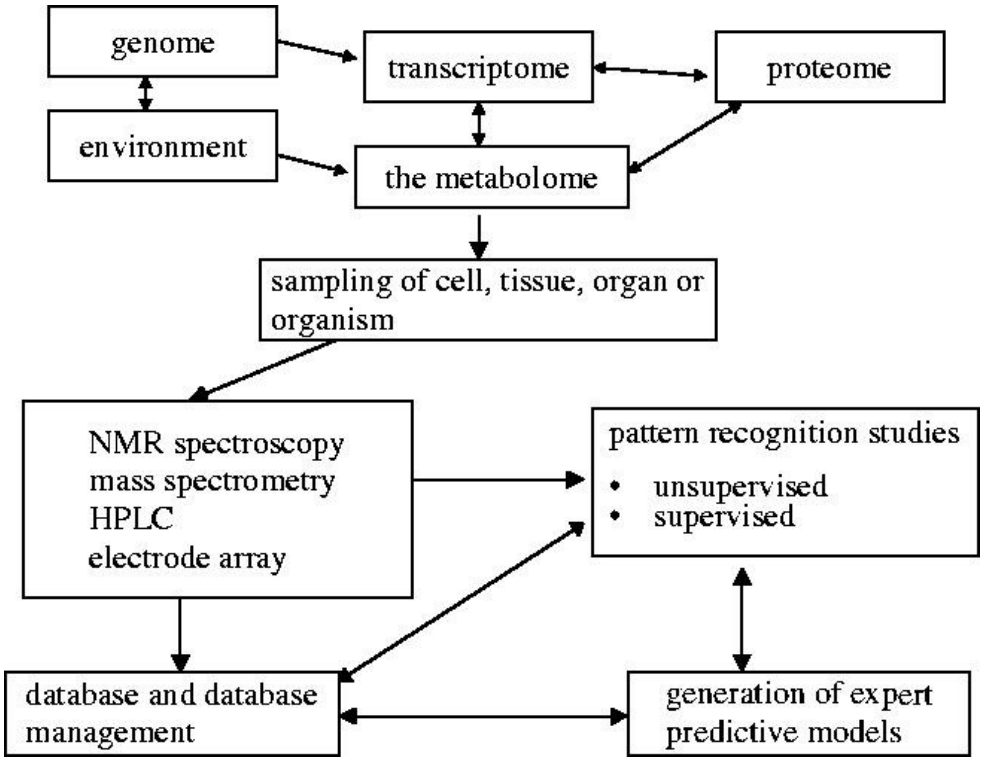


Fig. 15. A schematic view of metabolomics N (140)

Metabolomics involves the identification and quantification of metabolites present in a biological system. Three different approaches can be used: metabolomic fingerprinting, metabolic profiling, and metabolic footprinting,

There are several analytical strategies that can be used to analyse the metabolome (136), such as nuclear magnetic resonance (NMR)(137), Fourier transformation

infrared spectroscopy (FT-IR)(138. 139), and mass spectrometry (MS) coupled to separation techniques such as 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 (fig. 16) (140). However, because of the very diverse range of metabolites found within the cell, in conjunction with the large dynamic range of metabolite concentrations, none of these technologies, even when used in conjunction with one another, can be relied upon to generate a complete metabolomic description of even the simplest organism.

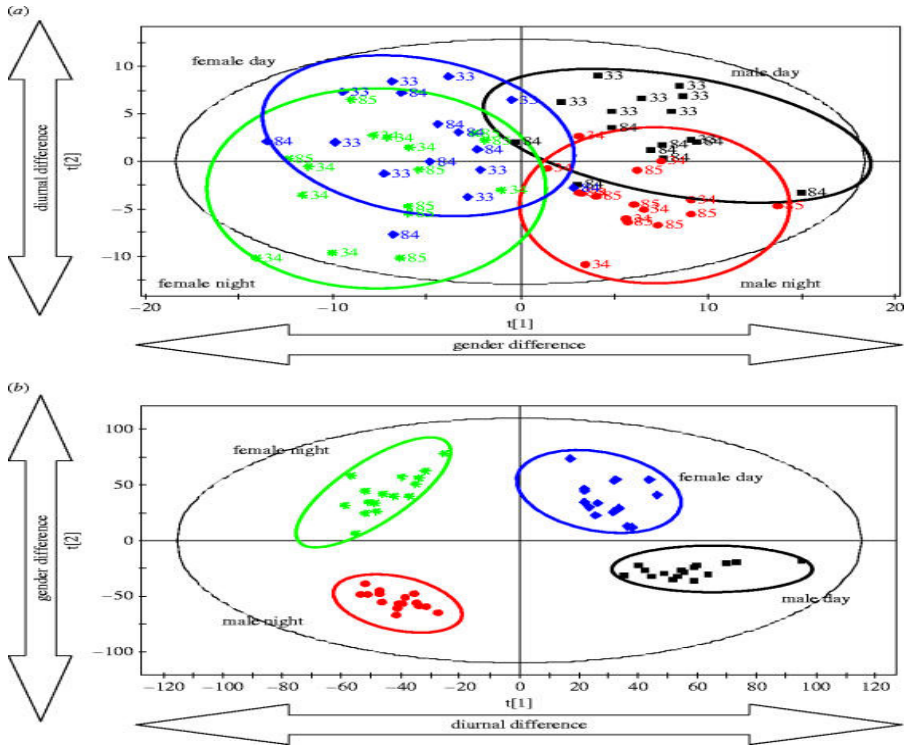


Fig. 16. A comparison of NMR and LC–MS analysis of urinary metabolite profiles (140)

High field ^{1}H NMR is one of the preferred platforms for urine and plasma analysis (141, 142), as it is a nondestructive technique that does not require prior separation of the analytes and it provides detailed information on molecular structure. For example, the capacity to predict the occurrence of exercise-induced ischemia in patients with suspected CAD was investigated by NMR blood analysis, demonstrating lactate, glucose, lipids, and long-chain fatty acids to be the main metabolites involved (143). Xanthine and ascorbate were proposed as possible markers of plaque formation in an atherosclerotic mouse model (144) and lipoprotein subclasses can now be analyzed by a commercial NMR-based protocol called NMR LipoProfile (145, 146). However, one of the main limitations of NMR is the poor sensitivity, although this can be improved enormously when it is combined with mass spectrometry.

Mass spectrometry (MS) is a rapidly growing technology for the comprehensive profiling of small molecules (metabolites) and proteins (147, 148). Indeed, gas chromatography/mass spectrometry (GC-MS) (149), liquid chromatography/mass spectrometry (LC-MS) (150), and capillary electrophoresis /mass spectrometry (CE-MS) (151) are the most powerful techniques for metabolite separation and analysis. GC-MS provides an extraordinary resolution, permitting the separation of structurally similar compounds that would otherwise be very difficult to separate by HPLC. However, this technique requires the analyte to be volatile and thermally stable. In some cases, a chemical derivatization step is required prior to the chromatographic separation in order to render polar metabolites volatile. Some of the metabolites best suited for GC-MS include fatty acids, organic acids, steroids, di-

glycerides, sugars and sugar alcohols.

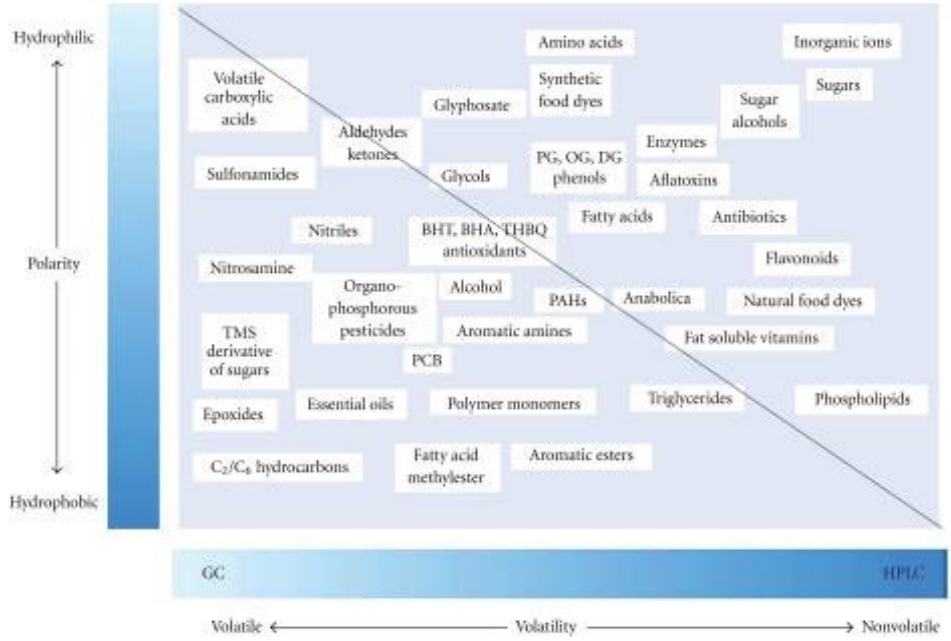


Fig. 17. Suitability of gas and liquid chromatography for metabolomic analysis based on metabolite polarity. Courtesy of Agilent Technologies (152)

For those metabolites that are not volatile and which cannot be derivatized, LC is the separation technique of choice. Thus, LC-MS can analyze a much wider range of chemical species (polar and nonpolar metabolites) with ample selectivity and sensitivity. Apart from reversed phase chromatography (RP-LC), which is widely used in metabolomics applications, hydrophilic interaction chromatography (HILIC) is a complementary approach suitable for very polar metabolites (nonvolatile). Indeed, the metabolites suited to analysis by GC or HPLC can be represented according to their polarity (Fig. 17) (152). Similarly, capillary electrophoresis (CE) can be coupled to a mass spectrometer (MS), with the particular advantage of improving the

resolution of separation as narrower peaks than with LC are obtained. Accordingly, different approaches have been described in combination with ion trap (IT), triple quadrupole (QQQ), time of flight (TOF), and Q-TOF instruments. The main advantage of QQQ and Q-TOF instruments is that they provide the possibility of identifying the compounds by tandem MS/MS analyses. In order to obtain a full overview of the detectable molecules, electrospray ionization (ESI) should be performed in both positive and negative modes on the same sample.

In principle, metabolites can be measured in several body fluids or tissues, although plasma and urine are the most commonly used biological matrices in cardiovascular research due to their availability and clinical relevance as a source of potential biomarkers. Sample pretreatment varies depending on the analytical platform chosen (Fig. 18) (153), Metabolites from frozen tissue samples can be extracted and simultaneously fractionated by treating the ground tissue with mixtures of organic solvents, such that molecules are extracted in different fractions according to their polarity. If a biological fluid is the starting material (urine, serum, plasma), metabolite fractions are usually obtained after proteins are removed by precipitation. The crude or diluted sample can then be injected directly, although matrix effects causing ion suppression should be expected. If analyzed by LC-MS(/MS), it may be desirable to preconcentrate (e.g., by lyophilisation) or fractionate the sample prior to chromatographic separation. In case of GC-MS(/MS), preconcentration can be performed by solid phase microextraction (SPME) with or without head space (HS) procedures, which are particularly useful when analysing volatile organic compounds

(VOCs). For CE analysis, the salt content should be minimized in the sample.

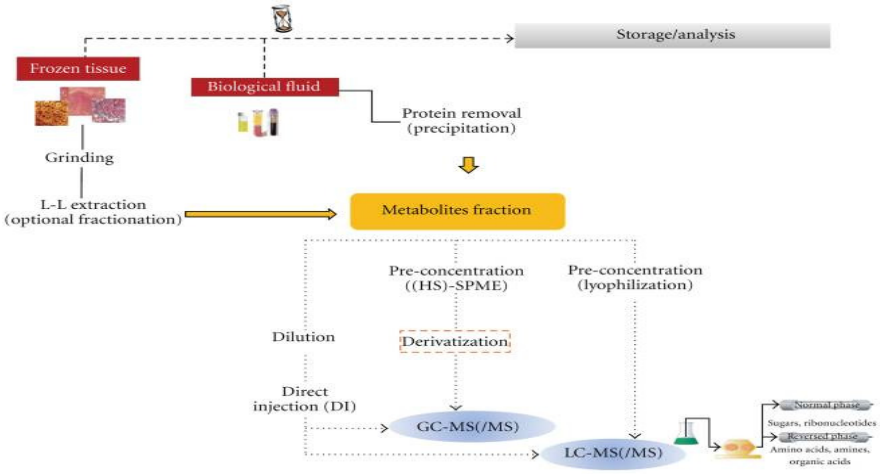


Fig. 18. Schematic view of the sample pretreatment for metabolomic analysis of frozen tissue or biological fluid prior to GC-MS or LC-MS analysis (153)

2.5.3. Metabolites : New biomarkers

Chen X et al showed that palmitate significantly contributes to atherosclerosis development via targeting apoptosis and inflammation pathways (154). von Zur Muhlen C et al analyzed urine proteom of 38 patients with coronary artery disease (CAD) and showed a combination of 17 urinary polypeptides allowed separation of both groups in the test set with a sensitivity of 81%, a specificity of 92%, and an accuracy of 84%. Sequencing of urinary marker peptides identified fragments of collagen alpha1 (I and III) demonstrated to be expressed in atherosclerotic plaques of human aorta (155).

Recently, non-hypothesis-driven metabolomic screenings have been used for the identification of novel CVD biomarkers. Zhang et al (156) utilized an ultra-fast

liquid chromatography coupled with ion trap time-of-flight mass spectrometry (UFLC/MS-IT-TOF) to study plasma and urine metabolic profiles of atherosclerosis rats. 12 metabolites in rat plasma and 8 metabolites in urine were identified as potential biomarkers. Concentrations of leucine, phenylalanine, tryptophan, acetylcarnitine, butyrylcarnitine, propionylcarnitine and spermine in plasma and 3-O-methyl-dopa, ethyl N2-acetyl-L-argininate, leucylproline, glucuronate, t6A N(6)-(N-threonylcarbonyl)-adenosine and methyl-hippuric acid in urine decreased in atherosclerosis rats. Their observations suggest that abnormal metabolism of phenylalanine, tryptophan, bile acids, and amino acids might be related to atherosclerosis development. In addition, Zha et al (157) identified a metabolic fingerprint of 21 compounds in hamsters that could be a potential marker for the development of atherosclerosis. Do Yup Lee et al (158) identified twenty-one compounds as markers involved in the development to atherosclerosis.

2.5. Arterial stiffness and ba-PWV

Pulse Wave Velocity (PWV) is now widely used to evaluate arterial stiffness in clinical situation. It is an easy and convenient clinical diagnostic tool for the patient. PWV correlates with other markers reflecting the severity of atherosclerosis such as ultrasound examination of carotid artery. Kim et al reported subjects with metabolic syndrome had significantly higher arterial pulse wave velocity than subjects without metabolic syndrome, ba-PWV is also known positively correlated with HbA1c and duration of diabetes (159). Ohnishi H et al showed there was a significant positive

correlation between fasting blood sugar (FBS) and mean right/left ba-PWV (Fig. 19). There was also a significant positive correlation between HbA1c and mean right/left ba-PWV (Fig. 19). A comparison of the values of ba-PWV in the three groups showed that the ba-PWV value increased with increasing plasma glucose level (Fig. 20). Significant differences were found between the ba-PWV values in the normal and impaired fasting glucose (IFG) groups (1,518 vs. 1,673 cm/s, $P = 0.01$) and in the normal and diabetic groups (1,518 vs. 1,771 cm/s, $P < 0.0001$). The results of multiple regression analysis showed that FBS was closely related to ba-PWV, as well as to age and systolic blood pressure (SBP), suggesting that FBS is independently related to ba-PWV (160).

In addition, several studies demonstrated that increased PWV is a predictor of future cardiovascular events in patients with either hypertension or diabetes mellitus. Thus, PWV is thought to be applicable as a marker relating with the severity of atherosclerosis and/or predicting future atherosclerotic cardiovascular events. Age and blood pressure is a major determinant of PWV. Anti-hypertensive medication or statins improve PWV, but it has not been fully evaluated whether these improvement reflect the improvement of prognosis. Therefore, the significance of PWV as a surrogate marker for the treatment of atherosclerotic cardiovascular risk has not been fully established. Carotid-femoral PWV is used as a conventional method. Recently, more simple method: brachial-ankle PWV is available in clinical settings (161).

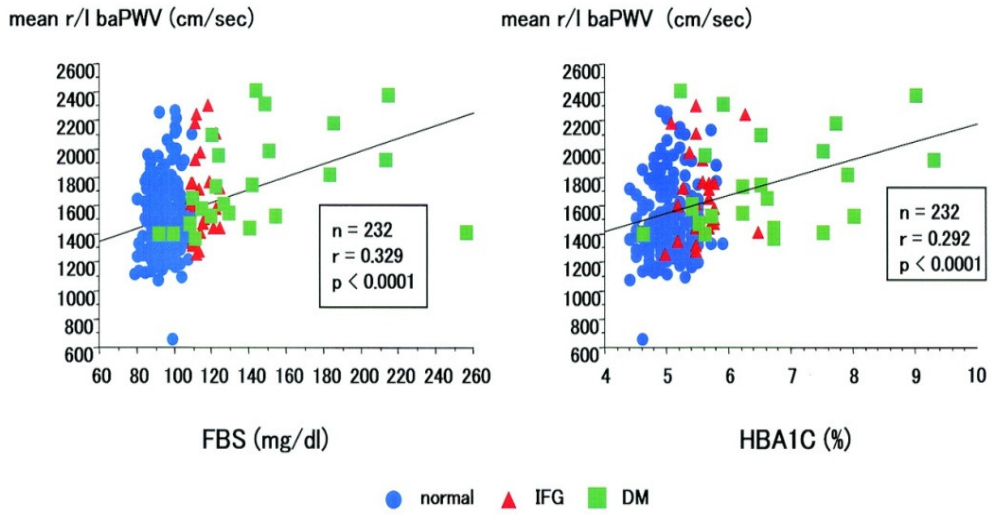


Fig. 19. Correlation between FBS, HbA1c and ba-PWV (160)

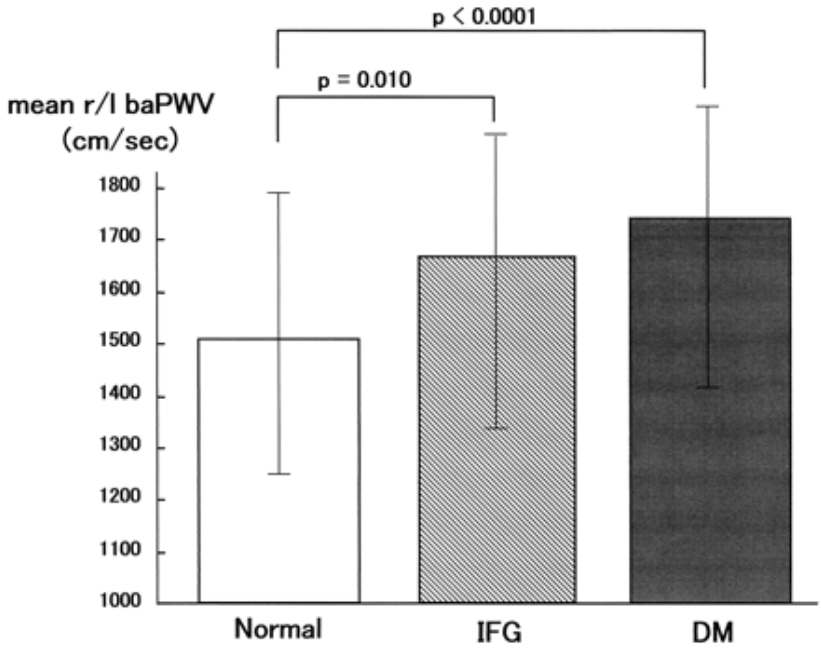


Fig. 20. A comparison of the values of ba-PWV in the three groups (160)

Carotid-femoral PWV is most commonly measured by applanation tonometry, particularly in Europe, but this method is critically dependent upon the accurate placing of transducers over the arteries and is both time-consuming and complex. Brachial-ankle PWV is a suitable screening method because of its technical simplicity and shorter measurement time.

The PWV of the brachial artery (ba) and the ankle was measured by applying air pressure with the aid of a volume plethysmograph. However, brachial-ankle PWV includes peripheral component of artery in its assessment of arterial stiffness, and it has not been fully concluded that brachial-ankle PWV has a similar potential as a marker using the management of atherosclerotic cardiovascular disease as carotid-femoral PWV. Many following studies by using ba-PWV were done. Tsuchikura S et al showed ba-PWV is an index of arterial stiffness showing similar characteristics to those of aortic PWV (162). Some studies were done to know relation ba-PWV and clinical markers. Andoh N et al reported baPWV is significantly associated with serum levels of hs-CRP in Japanese men (163), Saijo et al reported ba-PWV is associated not only with conventional cardiovascular risk factors but also with new risk factors, such as inflammation, gamma-glutamyltransferase, chronic kidney disease, and psychosocial factors (164). Miyaki K et al reported linear increase in ba-PWV occurred with increasing MS components after adjustment for potential confounding factors (165). Imanishi R et al reported high ba-PWV was shown to be a good independent predictor for the presence of CAD in men (166). However, a suitable cut-off value has yet to be established in each country. Yamashina

A et al suggested a cut-off value of 14.0 m/s to screen subjects, especially in middle-aged ones, of either gender in Japan (167). Aso K et al compared ba-PWV and carotid PWV (from heart to carotid) to study the relation of these two types of PWVs to diabetic complications in patients with type 2 diabetes mellitus. The ba-PWV was determined by oscillometrically measuring the pulse volume record at the upper arm and ankles. The carotid PWV was measured tonometrically. The correlations of these variables with albuminuria, peripheral neuropathy, coefficient of variation of R-R intervals (CV R-R) on the electrocardiogram at rest, and retinopathy were examined by logistic regression analysis. After adjustment for age, systolic blood pressure, and duration of diabetes, logistic regression analysis showed that ba-PWV was directly related to the frequencies of albuminuria, decreased CV R-R, peripheral neuropathy, and retinopathy. In contrast, carotid PWV did not significantly correlate with any diabetic complications. Oscillometrically determined ba-PWV was related to the risk of diabetic microvascular disease in patients with type 2 diabetes mellitus and suggested to be useful for assessing risk factors of diabetic complications (168).

However ba-PWV has some limitations. Ba-PWV is influenced by blood pressure. With the ba-PWV technique, blood pressure compensation is not carried out. Furthermore, the pulse pressure is measured by air pressure; therefore any stimulus that exerts pressure on an artery may influence these results. Due to these reasons, a cardie-ankle vascular index (CAVI) has been proposed in which the pressure wave form indicating the closing of the aortic valve appears in the form of an arterial pressure wave after a fixed delay time (169).

3. MATERIALS and METHODS

3.1. Subjects

Study participants included 53 men between 35 and 65 years of age. Participants were recruited during routine check-ups at a health promotion center at the National Health Insurance Corporation Ilsan Hospital. Male patients with newly-diagnosed type 2 diabetes were defined as those with a fasting serum glucose concentration ≥126 mg/dL. Twenty-six men meeting this criterion and 27 age- and BMI-matched healthy men with normal fasting glucose levels (<126 mg/dL) were included. Exclusion criteria included any diagnoses of vascular, renal, liver, or acute or chronic inflammatory disease. Patients taking any drugs or supplements were also excluded. Before participation, the purpose of the study was carefully explained to all patients, and informed consent was obtained. The study protocol was approved by the Institutional Review Board of Yonsei University.

3.2. Anthropometric parameters, blood pressure, and blood collection

Body weights and heights were measured in the morning unclothed and without shoes, and used to calculate BMI (kg/m^2). Waist and hip circumferences were measured with a tape measure horizontally at the umbilicus with participants in the standing position after normal expiration and used to calculate the waist-to-hip ratio (WHR). Blood pressures (BPs) were measured in the left arm of seated participants with an automatic BP monitor (TM-2654, A&D, Tokyo, Japan) after a 20-min rest. After a 12-h fasting period, venous blood specimens were collected in EDTA-treated

or plain tubes, centrifuged to yield plasma or serum, and stored at -70°C until analysis.

3.3. Assessment of dietary intake

Dietary intake was assessed with a 24-h recall method and semi-quantitative food frequency questionnaire. Dietary energy values and nutrient content were calculated using the Computer Aided Nutritional analysis program (CAN-pro 3.0. Korean Nutrition Society, Seoul, Korea). Total energy expenditure (TEE) was calculated from activity patterns, including basal metabolic rate (BMR), physical activity for 24 h and specific dynamic actions of food. The BMR of each participant was calculated with the Harris-Benedict equation.

3.4. Serum glucose concentration, insulin concentration, and homeostasis model assessment-insulin resistance (HOMA-IR)

Fasting glucose levels were measured using a glucose oxidase method with a Beckman Glucose Analyzer (Beckman Instruments, Irvine, CA). Insulin levels were measured by radioimmunoassay using commercial kits from Immuno Nucleo Corporation (Stillwater, MN). Insulin resistance (IR) was calculated by the homeostasis model assessment (HOMA) using the following equation: HOMA-IR = [fasting insulin (μIU/mL) × fasting glucose (mmol/L)] /22.5.

3.5. Serum lipid profile

Fasting serum concentrations of total cholesterol and triglyceride were measured

using commercially available kits on a Hitachi 7150 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). After precipitation of serum chylomicrometers using dextran sulfate magnesium, LDL and HDL cholesterol concentrations in the supernatants were measured using an enzymatic method. LDL cholesterol was indirectly estimated in participants with serum triglyceride concentrations <400 mg/dL using the Friedewald formula: LDL cholesterol = total cholesterol - [HDL cholesterol + (triglyceride/5)].

3.6. Lipid peroxidation: plasma MDA, oxidized LDL, and urinary 8-epi-$PGF_{2\alpha}$

Plasma malondialdehyde (MDA) was measured from thiobarbituric acid-reactive substances (TBARS Assay Kit, Zepto-Metrix Co., Buffalo, NY). Assays were read using a Victor2 plate reader (Perkin-Elmer Life Sciences, Turku, Finland) at 540 nm. Plasma oxidized LDL (ox-LDL) was measured using an enzyme immunoassay (Mercodia, Uppsala, Sweden), and the resulting color reaction was read at 450 nm on a Victor2 plate reader. Urine was collected in polyethylene bottles containing 1% butylated hydroxytoluene after a 12-h fast. Bottles were immediately covered with aluminum foil and stored at -70°C until analyzed. 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) was measured using an enzyme immunoassay (BIOXYTECH Urinary 8-epi-$PGF_{2\alpha}$ Assay kit, OXIS International, OR). The resulting color reaction from the enzyme immunoassay was read at 650 nm on a $Victor^2$ plate reader. Urinary creatinine was determined by the alkaline picrate (Jaffe) reaction. Urinary 8-epi-$PGF_{2\alpha}$ concentrations were expressed as pmol/mmol creatinine.

3.7. Inflammatory marker: serum hs-CRP, IL-1ß, IL-6, and TNF-α concentrations

Serum high sensitivity C-reactive protein (hs-CRP) concentrations were measured with an Express^{+} autoanalyzer (Chiron Diagnostics Co., Walpole, MA) using a commercially available, high-sensitivity CRP-Latex (II) X2 kit (Seiken Laboratories Ltd., Tokyo, Japan). Serum interleukin (IL)-1ß, IL-6, and tumor necrosis factor-alpha (TNF-α) concentrations were measured with a Bio-plex human cytokine panel (Bio-Rad, Hercules, CA) using a Fluorokine MultiAnalyte Profiling (MAP) cytokine multiple kits and analytes (R&D Systems, Minneapolis, MN).

3.8. Plasma Lp-PLA$_2$ activity and adiponectin concentration

The activity of lipoprotein-associated phospholipase A2 (Lp-PLA$_2$), also known as platelet-activating factor acetylhydrolase, was measured using a previously described modified method (170). Plasma adiponectin concentrations were measured using an enzyme immunoassay (Human Adiponectin ELISA kit, B- Bridge International Inc., CA). Assays were read using a Victor2 plate reader at 450 nm, and wavelength correction was set to 540 nm.

3.9. Brachial-ankle pulse wave velocity (ba-PWV)

Ba-PWV was measured using an automatic waveform analyzer (model VP-1000; Nippon Colin Ltd., Komaki, Japan) using a previously described methods (171). Participants were examined in the supine position after 5 min-bed rest.

Electrocardiogram electrodes were placed on both wrists, and a microphone for the phonogram was placed on the left edge of the sternum. ba-PWVs were recorded using a semiconductor pressure sensor (1200-Hz sample acquisition frequency) and calculated using the equation: (La−Lb)/ΔTba. The distance from the suprasternal notch to the elbow (La) and to the ankle (Lb) were calculated by: La=[0.2195×height of subject (cm)]−2.0734 and Lb=[0.8129×height of subject (cm)]+12.328. The time interval between the arm and ankle distance (ΔTba) was defined as the pulse transit time between brachial and tibial arterial pressure waveforms. 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, $p<0.001$).

3.10. Fatty acid composition in serum phospholipids

Total lipids were extracted with chloroform:methanol (2:1, v/v) as described by Folch et al (172). Phospholipids were methylated as described by Lepage and Roy after separation using thin-layer chromatography (173). Fatty acid methyl esters were analyzed by gas chromatography (HP 7890A, Agilent Technologies, Santa Clara, CA). Fatty acids were identified by comparing their retention times with those of standard fatty acid methyl esters. Percentages of individual fatty acids were calculated according to the peak areas relative to the total area (total fatty acid area was set at 100%).

3.11. Metabolic profiling of plasma samples by UPLC/Q-TOF MS analysis

3.11.1. Sample preparation and analysis

Prior to analysis, 400 μL extract mix solution (acetonitrile:methanol:ethanol=1:1:1, v/v/v) was added to 100 μL plasma. After shaking, the mixture was centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was evaporated to dryness under nitrogen, dissolved in 80% methanol, and transferred into a vial. A 10-μL sample was injected into the UPLC/Q-TOF MS (Waters, Milford, MA). The plasma extract was injected into an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm; Waters) in line with the UPLC system and equilibrated with water containing 0.1% formic acid. Samples were eluted by an acetonitrile gradient containing 0.1% formic acid at a flow rate of 0.4 mL/min for 25 min, and metabolites were separated by UPLC, analyzed, and assigned by Q-TOF MS. The Q-TOF was operated in ESI-positive mode. The capillary and sampling cone voltages were set at 3 kV and 30 V, respectively. The desolvation flow was set to 700 L/h at a temperature of 250°C, and the source temperature was set to 120°C. MS data were collected in the range of m/z 50-1000 with a 0.2-s scan time and 0.02-s interscan delay time. MS/MS spectra of metabolites were obtained by a collision energy ramp from 10-30 eV. The accurate mass and composition for the precursor and fragment ions were calculated and sequenced by MassLynx (Waters) incorporated in the instrument.

3.11.2. Data processing and identification of metabolites

All MS data-related information including retention times, m/z, and ion intensities was extracted by MarkerLynx software (Waters) incorporated in the instrument, and the resulting MS data were assembled into a matrix. The following MarkerLynx parameters were set: peak width at 5%, height 1 s, intensity threshold 120 counts, mass window 0.04 amu, retention time window 0.15 min, noise elimination level 6, and mass tolerance 0.04 Da. Peak integration was performed using Apex Track integration. Metabolites were identified with the Chemspider (www.chemspider.com) and Human Metabolome (www.hmdb.ca) databases. Identified compounds were matched by authentic standards based on both retention time and mass spectra. Authentic standards were purchased from Sigma Chemical (St. Louis, MO), Crystal Chem (Chicago, IL), and Avanti Polar Lipids (Alabaster, AL**).** ***MS/MS fragmentation*** data of identified compounds were obtained by collision energy ramp from 10-30 eV and matched to authentic standards.

3.12. Statistical Analysis

Statistical analyses were performed with SPSS ver12.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to determine the normality of the distribution, and skewed variables were logarithmically transformed for statistical analysis. For descriptive purposes, mean values are presented using untransformed values. Results are expressed as the mean ± standard error (SE). A two-tailed P value < 0.05 was considered statistically

significant. Pearson's correlation coefficients were used to examine the relationships between variables. Differences in clinical variables, including mass intensities of plasma metabolites between the two groups, were tested by independent t-test with the Mann-Whitney U-test. In addition, multivariate statistical analysis was performed using SIMCA-P^+ software version 12.0 (Umetrics, Umeå, Sweden). Cross-validation with seven cross-validation groups was used throughout to determine the number of principal components. Partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling the discrimination between the diabetes and control subjects by visualizing the score plot or S-plot using the first and second PLS components. 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 data in the training set are mathematically reproduced, varies between 0 and 1, with 1 indicating a model with a perfect fit. Furthermore, models with Q^2Y greater ≥ 0.5 are considered to have good predictive capability (174).

4. RESULTS

4.1. Clinical characteristics and nutrient intake

Clinical characteristics and estimates of daily nutritional intake of healthy men with normal fasting serum glucose levels and men with newly-diagnosed type 2 diabetes are shown in Table 1. No significant differences were observed in age, BMI, smoking, alcohol consumption, serum levels of insulin, total cholesterol, LDL cholesterol, HDL-cholesterol, and serum GPT. Men with newly-diagnosed type 2 diabetes showed significantly higher percentages of body fat ($P=0.008$), larger waist circumferences ($P=0.031$), higher serum glucose levels ($P<0.001$), HOMA-IR ($P<0.001$), higher serum triglyceride levels ($P=0.001$), and lower GPT levels than healthy men. Additionally, diabetes patients showed a trend toward higher levels of serum free fatty acids ($P=0.089$). There was no significant difference between the groups with respect to total energy intake (TEI), total energy expenditure (TEE), or the proportion of caloric intake from macronutrients; however, the ratio of TEI to TEE was significantly higher in diabetes patients than healthy men ($P=0.031$).

Table 1. Clinical characteristics and nutrient intake of healthy men and men with newly-diagnosed type 2 diabetes

	Healthy men (n=27)	Diabetes patients (n=26)	P
Age (yrs)	50.0 ± 1.27	49.2 ± 1.40	0.728
Body mass index (kg/m^2)	24.6 ± 0.40	25.1 ± 0.35	0.364
Body fat (%)	21.0 ± 0.88	23.9 ± 0.67	0.008
Waist (cm)	86.2 ± 1.01	89.0 ± 0.74	0.031
[1]WHR	0.91 ± 0.01	0.92 ± 0.01	0.314
Cigarette smoker, n (%)	9 (47.4)	10 (52.6)	0.779
Alcohol drinker, n (%)	18 (43.9)	23 (56.1)	0.099
Systolic BP (mmHg)	120.2 ± 2.17	132.4 ± 2.44	0.001
Diastolic BP (mmHg)	73.8 ± 1.67	82.5 ± 1.67	0.001
Glucose (mg/dL)[ʃ]	91.0 ± 1.21	165.2 ± 7.21	<0.001
Insulin (μU/mL)[ʃ]	8.34 ± 0.48	8.95 ± 0.82	0.993
[2]HOMA-IR[ʃ]	1.88 ± 0.12	3.69 ± 0.42	<0.001
Free fatty acid (uEq/L)[ʃ]	483.2 ± 37.3	634.1 ± 54.2	0.089
Triglyceride (mg/dL)[ʃ]	102.0 ± 10.3	169.0 ± 15.7	0.001
Total cholesterol (mg/dL)	194.3 ± 4.80	189.3 ± 5.69	0.373
LDL cholesterol (mg/dL)	121.0 ± 5.73	111.7 ± 3.83	0.227
HDL cholesterol (mg/dL)	52.8 ± 2.77	48.0 ± 2.52	0.196
GOT	24.0 ± 1.00	21.9 ± 1.29	0.046
GPT	23.0 ± 1.68	25.8 ± 2.11	0.344
Estimates of daily nutrient intakes[†]			
Total energy intake (kcal/d)	2452.2 ± 26.2	2521.1 ± 38.9	0.200
Carbohydrate (g/d)	379.7 ± 4.23	392.0 ± 5.57	0.105
Protein (g/d)	106.7 ± 1.87	107.7 ± 2.64	0.993
Fat (g/d)	58.4 ± 0.83	58.5 ± 1.50	0.644
Alcohol (g/d)	13.3 ± 2.60	19.9 ± 4.11	0.387
Total energy expenditure (kcal/d)	2512.7 ± 31.2	2525.1 ± 36.5	0.908
[3]TEI/TEE	1.01 ± 0.01	1.05 ± 0.01	0.031

Data are mean ± SE. [ʃ] Tested by logarithmic transformation. [1]Waist-to-hip ratio. [2]HOMA-IR = {fasting insulin (μU/mL) x fasting glucose (mmol/L)}/22.5. [3]total energy intake/total energy expenditure. Tested by independent *t*-test with the Mann-Whitney U-test. [†]Nutrient intake, obtained from weighed food records and calculated using the database of the computerized Korean food code.

4.2. Lipid peroxides, inflammatory markers, and arterial stiffness

Compared to healthy men, men with newly-diagnosed type 2 diabetes showed higher plasma levels of ox-LDL (P=0.021) and MDA (P=0.028); higher urinary 8-epi-$PGF_{2\alpha}$ levels (P=0.001); higher serum levels of hs-CRP (P=0.027), IL-6 (P=0.011), and TNF-α (P=0.031); and lower serum adiponectin levels (P<0.001) (Table 2). Diabetes patients also showed a trend toward higher serum Lp-PLA_2 activity (P=0.083) and significantly higher ba-PWV than healthy men (P<0.001).

Table 2. Markers of lipid peroxides, inflammation, and brachial-ankle pulse wave velocity

	Healthy men (n=27)	Diabetes patients (n=26)	P
Oxidized LDL (U/L)[$]	44.9 ± 3.66	55.9 ± 3.59	0.021
MDA (nmol/mL)[$]	9.80 ± 0.44	11.8 ± 0.91	0.028
8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)[$]	1135.9 ± 54.7	1650.9 ± 110.2	0.001
Lp-PLA_2 activity (nmol/mL/min)	34.7 ± 2.23	39.9 ± 2.18	0.083
hs-CRP (mg/dL)[$]	0.53 ± 0.09	1.03 ± 0.17	0.027
IL-1B (ρg/mL)[$]	1.06 ± 0.11	1.11 ± 0.11	0.426
IL-6 (ρg/mL)[$]	2.68 ± 0.17	5.04 ± 1.48	0.011
TNF-a (ρg/mL)[$]	10.1 ± 0.64	13.2 ± 1.41	0.031
Adiponectin (μg/mL)[$]	5.62 ± 0.50	3.10 ± 0.27	<0.001
baPWV (cm/sec)[$]	1258.2 ± 34.9	1519.6 ± 41.1	<0.001

Data are mean ± SE.

[$] Tested by logarithmic transformation.

Tested by independent *t*-test with the Mann-Whitney U-test.

4.3. Fatty acid composition of serum phospholipids

Compared to healthy men, men with newly-diagnosed type 2 diabetes showed higher proportions of dodecanoic acid (12:0, P=0.032) and myristic acid (14:0, P=0.001) in serum phospholipids (Table 3). Diabetic men also showed a tendency toward lower levels of γ-linolenic acid (18:3ω6, P=0.057) and a trend toward higher α-linolenic acid levels (18:3ω3, P=0.067) in serum phospholipids. There were no significant differences between the two groups in δ-9 (18:1ω9/18:0), δ-9 (16:1ω7/16:0), δ-6 (18:3ω6/18:2ω6), and δ-5 (20:4ω6/20:3ω6) desaturase activities in serum phospholipids.

Table 3. Fatty acid composition in serum phospholipids of healthy men and men with newly-diagnosed type 2 diabetes

	Healthy men (n=27)	Diabetes patients (n=26)
Dodecanoic acid (c12:0) §	0.21 ± 0.01	0.39 ± 0.07*
Myristic acid (c14:0) §	0.42 ± 0.02	0.69 ± 0.07**
Palmitic acid (c16:0)	34.7 ± 0.58	33.5 ± 1.04
Stearic acid (c18:0) §	18.0 ± 0.34	18.9 ± 0.70
Palmitoleic acid (c16:1) §	0.66 ± 0.05	0.70 ± 0.06
Oleic acid (c18:1 ω9)	6.94 ± 0.32	7.19 ± 0.42
Oleic acid (c18:1 ω7)	1.78 ± 0.09	1.61 ± 0.08
Linoleic acid (c18:2 ω6)	13.1 ± 0.44	11.9 ± 0.63
γ-linolenic acid (c18:3 ω6) §	0.17 ± 0.01	0.15 ± 0.03
Eicosadienoic acid (c20:2 ω6) §	0.41 ± 0.05	0.56 ± 0.17
Dihomo-γ-linolenic acid (c20:3 ω6)	1.50 ± 0.08	1.59 ± 0.11
Arachidonic acid (c20:4 ω6)	4.90 ± 0.30	4.87 ± 0.35
α-linolenic acid (c18:3 ω3) §	0.15 ± 0.01	0.19 ± 0.02
Eicosapentaenoic acid (c20:5 ω3) §	1.16 ± 0.11	1.39 ± 0.17
Docosahexaenoic acid (c22:6 ω3) §	2.85 ± 0.21	2.98 ± 0.29
δ-9 desaturase (18:1 ω9/18:0)	0.388 ± 0.02	0.393 ± 0.03
δ-9 desaturase (16:1 ω7/16:0)	0.019 ± 0.00	0.022 ± 0.00
δ-6 desaturase (18:3 ω6/18:2 ω6)	0.013 ± 0.00	0.013 ± 0.00
δ-5 desaturase (20:4 ω6/20:3 ω6)	3.456 ± 0.25	3.116 ± 0.18

Data are mean ± SE. § Tested by logarithmic transformation.

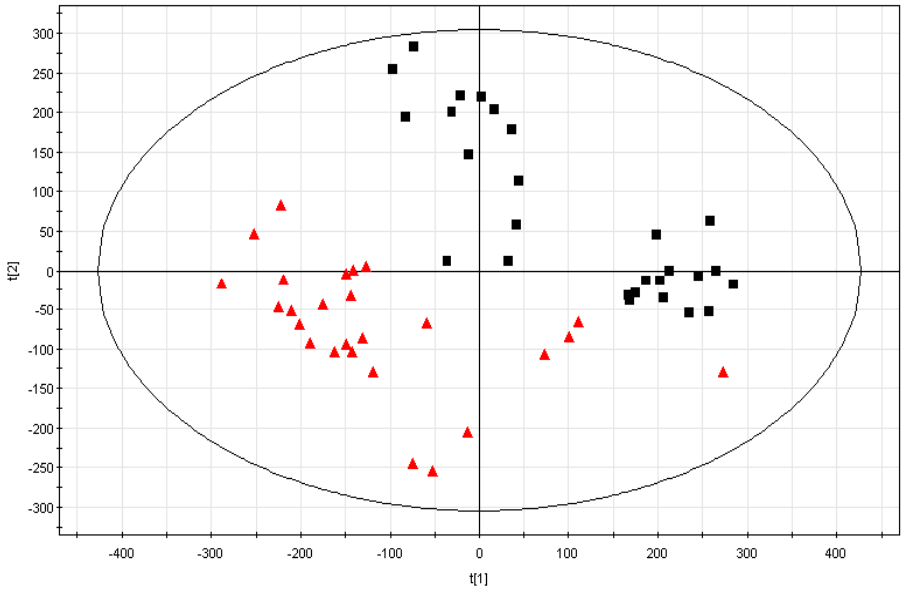
Tested by independent *t*-test with the Mann-Whitney U-test. *P<0.05, **P<0.01

4.4. Plasma metabolic profiling based on UPLC-Q-TOF MS

4.4.1. Non-targeted metabolomics pattern analysis

To explain the maximum separation between defined class samples in the dataset, the 382 variables obtained from the plasma of healthy men and men with newly-diagnosed diabetes were analyzed by PLS-DA. The PLS-DA score plot (Fig. 1a) distinguished men with newly-diagnosed diabetes and healthy men along the axes corresponding to the first two PLS components. Our model describes 57% of the variation in X (R^2X=0.570) and 80% of the variation in the response Y (class) (R^2Y=0.800), and predicts 75.2% of the variation in the response Y (Q^2Y=0.752) for the two-component model. The permutation test with a permutation number of 200 was performed and indicated a R^2 intercept value of 0.249 and a Q^2 intercept value of -0.297. The score plot indicated that diabetes patients were clearly separated from the healthy group, and the S-plot (Fig. 1b) indicated that each metabolite contributed to this distinction between diabetes patients and healthy controls along the axes corresponding to the covariance [p] and reliability correlation [p(corr)].

a)



b)

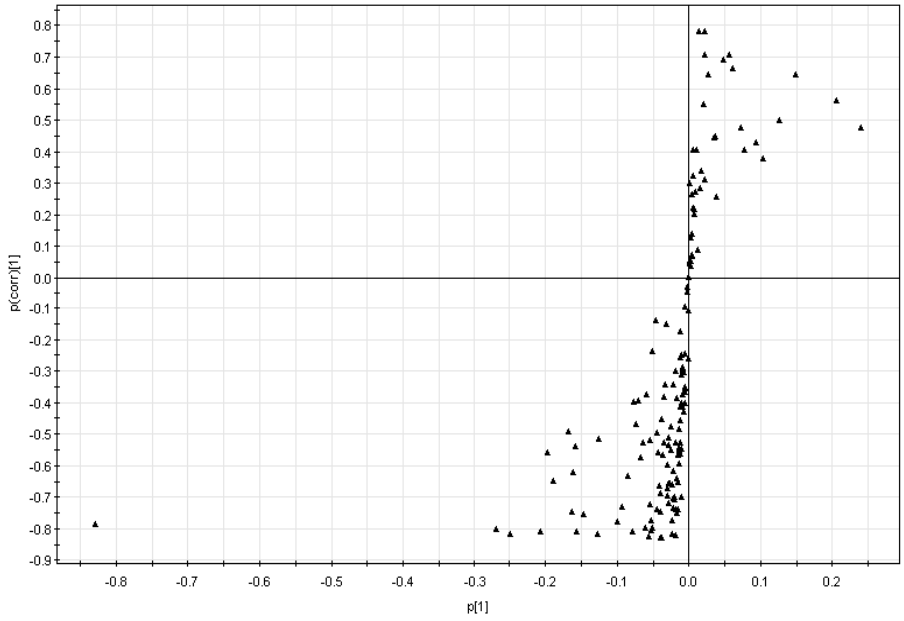


Fig. 21. (a) Score plots from PLS-DA models classifying men with newly-diagnosed type 2 diabetes (▲) and healthy men with normal fasting serum glucose levels (■). (b) S-plot for covariance [p] and reliability correlation [p(corr)] from PLS-DA models.

4.4.2. Identification and fold change levels of plasma metabolites

The normalized intensities of whole metabolites detected by UPLC-Q-TOF (382 metabolites in plasma) were statistically analyzed by nonparametric t test. All metabolites were significantly affected by the new onset of type 2 diabetes; however, few metabolites could be identified. The results assigned by UPLC-Q-TOF are shown in Table 4.

In plasma, 19 metabolites, including 3 amino acids (leucine, lysine, and phenylalanine), 8 acylcarnitines (propionyl-, octanoyl-, decanoyl-, palmityl-, heptadecanoyl-, linoleyl- and vaccenyl-carnitines), 6 lyso-phosphatidylcholines (lysoPCs) (14:0, 16:1, 18:1, 18:3, 20:5 and 22:6), and 2 lyso-phosphatidylethanolamines (lysoPEs) (18:2 and 22:6) were significantly increased in diabetes patients, whereas 2 metabolites, serine and lysoPE 18:1, were decreased. Decanoyl carnitine; lysoPCs containing C14:0, C16:1, C18:1, and C22:6; and lysoPE containing C18:1 with variable importance in the projection (VIP) values greater than 1.0, which indicates high relevance to the difference between sample groups, were major plasma metabolites contributing to the discrimination between healthy and diabetic men on the PLS-DA score plot (Table 4). Although oleamide was not significantly increased in diabetic men (P=0.124), oleamide with a VIP value of 6.952 was the most important plasma metabolite for evaluating the difference between healthy and diabetic men.

Table 4. Identification and fold change levels of plasma metabolites of healthy men and men with newly-diagnosed type 2 diabetes

No	Identity	Mass	Elemental composition (mDa)	Fold change (vs healthy me n) [a]	P [b]	VIP
1	L-Leucine	132.1025	$C_6H_{13}NO_2$ (0.0)	1.55	<0.001	0.164
2	L-Lysine	147.1134	$C_6H_{14}N_2O_2$ (0.2)	2.33	<0.001	0.406
3	L-Phenylalanine	166.0868	$C_9H_{11}NO_2$ (4.0)	1.89	<0.001	0.501
4	L-Serine	106.0504	$C_3H_7NO_3$ (7.7)	0.30	<0.001	0.203
5	Propionyl carnitine	218.1392	$C_{10}H_{19}NO_4$ (0.1)	1.55	0.002	0.082
6	Octanoyl carnitine	288.2175	$C_{15}H_{29}NO_4$ (0.5)	4.77	<0.001	0.682
7	Decanoyl carnitine	316.2488	$C_{17}H_{33}NO_4$ (0.5)	3.71	<0.001	1.122
8	Dodecanoyl carnitine	344.2801	$C_{19}H_{37}NO_4$ (0.2)	3.90	<0.001	0.273
9	Palmityl carnitine	400.3427	$C_{23}H_{45}NO_4$ (0.7)	4.78	<0.001	0.489
10	Heptadecanoyl carnitine	414.3583	$C_{24}H_{47}NO_4$ (4.4)	3.39	0.001	0.435
11	Linoleyl carnitine	424.3427	$C_{25}H_{45}NO_4$ (2.5)	2.01	0.006	0.108
12	Vaccenyl carnitine	426.3583	$C_{25}H_{47}NO_4$ (1.6)	5.13	<0.001	0.657
13	LysoPC(14:0)	468.3090	$C_{22}H_{46}NO_7P$ (3.0)	2.25	<0.001	1.144
14	LysoPC(16:0)	496.3403	$C_{24}H_{50}NO_7P$ (0.2)	1.04	0.545	1.927
15	LysoPC(16:1)	494.3247	$C_{24}H_{48}NO_7P$ (0.8)	2.13	<0.001	1.580
16	LysoPC(18:0)	524.3716	$C_{26}H_{54}NO_7P$ (1.1)	0.90	0.051	1.872
17	LysoPC(18:1)	522.3560	$C_{26}H_{52}NO_7P$ (0.4)	1.19	0.008	1.699
18	LysoPC(18:2)	520.3403	$C_{26}H_{50}NO_7P$ (0.3)	1.11	0.200	1.251
19	LysoPC(18:3)	518.3247	$C_{26}H_{48}NO_7P$ (0.7)	1.69	0.047	0.367
20	LysoPC(20:2)	548.3716	$C_{28}H_{54}NO_7P$ (2.0)	0.87	0.262	0.077
21	LysoPC(20:4)	544.3403	$C_{28}H_{50}NO_7P$ (0.6)	1.17	0.062	0.569
22	LysoPC(20:5)	542.3247	$C_{28}H_{48}NO_7P$ (0.0)	1.85	0.001	0.377
23	LysoPC(22:6)	568.3403	$C_{30}H_{50}NO_7P$ (0.3)	1.46	0.002	2.033
24	LysoPE(18:1)	480.3090	$C_{23}H_{46}NO_7P$ (0.2)	0.55	0.003	1.799
25	LysoPE(18:2)	478.2934	$C_{23}H_{44}NO_7P$ (0.2)	1.99	<0.001	0.255
26	LysoPE(22:6)	526.2934	$C_{27}H_{44}NO_7P$ (0.1)	2.04	<0.001	0.549
27	Oleamide	282.2797	$C_{18}H_{35}NO$ (0.5)	1.30	0.124	6.952

[a] calculated by the mean of intensity of each metabolite from T2DM by the mean of intensity of each metabolite from healthy men. [b] tested by independent *t*- test with the Mann-Whitney U-test.

4.5. Relationship of major plasma metabolites with lipid peroxides, inflammatory markers, arterial stiffness, and fatty acid compositions of serum phospholipids

Correlation analysis was performed for the major plasma metabolites as determined by UPLC-Q-TOF. Decanoyl carnitine positively correlated with ox-LDL (r=0.285, P=0.047), 8-epi-$PGF_{2\alpha}$ (r=0.317, P=0.036), IL-6 (r=0.403, P=0.009), TNF-α (r=0.328, P=0.036), and ba-PWV (r=0.548, P=0.001). Ba-PWV correlated positively with lysoPC C14:0 (r=0.543, P=0.001) and lysoPC C16:1 (r=0.559, P=0.001), and negatively with lysoPE C18:1 (r=-0.444, P=0.011). 8-epi-$PGF_{2\alpha}$ positively correlated with lysoPC C14:0 (r=0.439, P=0.003) and lysoPC C16:1 (r=0.352, P=0.019), as well as Lp-PLA_2 (r=0.313, P=0.044) and ba-PWV (r=0.409, P=0.034). LysoPC containing C22:6 positively correlated with IL-6 (r=0.324, P=0.039). Oleamide positively correlated with TNF-α (r=0.328, P=0.036) and oleic acid (C18:1ω9) (r=0.291, P=0.05) in serum phospholipid. LysoPCs containing C14:0, C16:1, and C18:1 positively correlated with the proportions of myristic acid (C14:0) (r=0.427, P=0.003), palmitoleic acid (C16:1) (r=0.415, P=0.005), and oleic acid (C18:1ω9) (r=0.376, P=0.011) in serum phospholipids, respectively. There was a positive correlation between lysoPC C22:6 and lysoPE C22:6 (r=0.518, P<0.001), and between lysoPC C18:2 and lysoPE C18:2 (r=0.310, P=0.024).

5. DISCUSSION

Significant differences were demonstrated between the metabolic profiles, including lysoPCs, lysoPEs, acylcarnitines, and amino acids, of healthy men with normal fasting serum glucose levels and men with newly-diagnosed type 2 diabetes. These results are in agreement with the previous finding of different compositions of the phospholipid species, including PEs and lysoPCs, as potential biomarkers for classifying type 2 diabetes patients from healthy adults (175). In this study, diabetes patients showed a significant increase in six lysoPCs (14:0, 16:1, 18:1, 18:3, 20:5, and 22:6). LysoPCs containing C14:0, C16:1, C18:1, and C22:6 with VIP values >1.0 were the major plasma metabolites contributing to the discrimination of healthy and diabetic men on the PLS-DA score plot. LysoPC has been shown to increase endothelial permeability and to contribute to the loss of vascular integrity in inflammatory conditions (176). Compared to healthy men, newly-diagnosed type 2 diabetes patients also showed higher concentrations of TNF-α, IL-6, and hs-CRP. Higher arterial stiffness assessed by ba-PWV was also identified in diabetes patients and represents a composite risk factor to identify patients with early atherosclerotic change (177). In this study, ba-PWV correlated positively with levels of hs-CRP, IL-6, lysoPC C14:0, lysoPC C16:1, and ox-LDL.

Oxidized phospholipids in LDL particles are hydrolyzed by phosholipase A_2 (PLA_2), including Lp-PLA_2 and secretory PLA_2 (sPLA_2), and produce bioactive oxidized free fatty acids and lysoPCs (178). Stafforini et al (179) showed that the

secreted form of Lp-PLA_2 released F_2-isoprostanes, the end-products of lipid oxidation, from the sn-2 position of PC with high affinity. In the sn-1 position of the phospholipid, a saturated fatty acid (SFA) or monounsaturated fatty acid (MUFA) predominates while in the sn-2 position, a MUFA or polyunsaturated fatty acid (PUFA) predominates (180). Similar to previous findings (181. 182), this study also showed a positive correlation between Lp-PLA_2 activity and urinary 8-epi-$PGF_{2\alpha}$ concentrations. In addition, compared to healthy men, newly-diagnosed type 2 diabetes patients showed significantly higher ox-LDL and 8-epi-$PGF_{2\alpha}$ levels and a trend toward increased Lp-PLA_2 activity. Furthermore, a positive relationship between 8-epi-$PGF_{2\alpha}$ and lysoPC C14:0, lysoPC C16:1, and ba-PWV identified in this study may indicate that increased production from Lp-PLA_2-catalized PC hydrolysis during increased LDL-oxidation closely relates to arterial stiffness in men with newly-diagnosed type 2 diabetes. In fact, lysoPC is reported to constitute only 1-5% of the total PC content of non-ox-LDL; however, as much as 40-50% of PC contained within LDL molecules is converted to lysoPC during LDL oxidation (183).

LysoPC, representing 5-20% of total plasma phospholipids (184), is also formed by the lecithin cholesterol acyltransferase (LCAT) in plasma (185). Human LCAT releases lysoPCs C20:4 and C22:6 from the sn-1 position of PC (186). In plasma, up to 80% of lysoPC is found in the non-lipoprotein fraction, where albumin is considered the main lipid binding protein (187). Unsaturated lysoPC was associated mainly with albumin rather than lipoproteins (188). In this study, lysoPCs containing C14:0, C16:1, and C18:1 positively correlated with the proportions of myristic acid

(C14:0), palmitoleic acid (C16:1), and oleic acid (C18:1ω9) in serum phospholipids, respectively. In addition, a positive correlation between lysoPC C22:6 and lysoPE C22:6, and between lysoPC C18:2 and lysoPE C18:2, in this study could reflect the common origin involved in determining fatty acid composition (180). On the other hand, oleic acid in serum phospholipids and lysoPC C18:1 did not correlate with lysoPE C18:1, and diabetes patients showed a significant decrease in lysoPE (18:1), an intermediate in the biosynthesis of oleoylethanolamine (189). Oleoylethanolamine binds to peroxisome proliferator-activated receptor (PPAR) α and PPARγ, inhibiting feeding behavior (190).

Higher total body fat percentages and central fat gain in men with diabetes compared with healthy men of similar age and BMI, might be partly due to the long-term effects of slightly higher caloric intake than caloric expenditure. High-calorie diets lead to de novo synthesis of several fatty acids, including the SFAs myristic acid (14:0) and palmitic acid (16:0), and the MUFAs 16:1 and 18:1 (191). With respect to individual fatty acids, a prospective study showed that high proportions of saturated fatty acids 16:0 and 18:0 in plasma phospholipids are associated with increased incidence of type 2 diabetes (192). Diabetes patients in this study showed higher proportions of 12:0 and 14:0 in serum phospholipids than healthy men. In addition, diabetes patients with increased central adipose tissue also showed a significant increase in the HOMA-IR index and triglyceride levels. Hyperlipidemia is detectable even before the appearance of fasting or postprandial hyperglycemia, suggesting that the altered lipid metabolism occurs long before type 2 diabetes fully develops (193).

A prevailing model for the development of diet-induced IR holds that mitochondrial fatty acid oxidation is inadequate to process the large fat load, thus leading to accumulation of lipid-derived metabolites such as diacyglycerols and ceramide (5). In this study, oleamide was the most important plasma metabolite for distinguishing between healthy and diabetic men, although oleamide was not significantly increased in diabetes patients.

Recently, Koves et al (194, 195) found that chronic exposure of muscle to elevated lipids in vitro, or in vivo as a consequence of overnutrition, resulted in increased expression of genes related to fatty acid β-oxidation. However, incomplete metabolism of fatty acids in the β-oxidation pathway results in broad-scale increase of plasma acylcarnitines (196). Diabetes patients in this study showed significant increases in acylcarnitines (propionyl-, octanoyl-, decanoyl-, palmityl-, heptadecanoyl-, linoleyl-, and vaccenyl-carnitines). Specifically, short chain acylcarnitines, including propionyl carnitine, reflect the propionyl CoA pool that is a byproduct of branched-chain amino acid (BCAA) catabolism. The observed significant increases in leucine, lysine, and phenylalanine, and decreases in serine in diabetes patients men are in agreement with the previous finding that BCAA-related factors, including leucine/isoleucine, valine, phenylalanine, and tyrosine, are strongly and positively associated with the HOMA-IR index (182, 197). Huffman et al (4) also showed that insulin sensitivity is associated negatively with the large neutral amino acids glutamate and glutamine, and positively associated with glycine and serine. Elevated amino acid concentrations have been suggested to produce IR by disrupting

insulin-mediated glucose uptake pathways, resulting in reduced glucose uptake and glycogen synthesis (198).

This study has several limitations. The moderate sample size, as well as the cross-sectional and observational nature of this study, may limit the applicability of our findings. We evaluated associations rather than prospective predictions; thus, the causal relationship of the identified biomarkers and the exact internal mechanisms of the changes of the metabolites in newly-diagnosed type 2 diabetes remain unclear. In addition, many markers were detected by UPLC-MS, but most remain unidentified. Despite these limitations, our findings indicate that metabolic processes that have evolved to sustain demands with a low-calorie diet produce many of the aberrant characteristics of type 2 diabetes when metabolic fuels are in excess relative to caloric expenditure (4).

The present study revealed a cluster of newly-diagnosed type 2 diabetes-associated changes in metabolites, including 6 lysoPC species (C14:0, C16:1, C18:1, C18:3, C20:5, and C22:6), 3 lysoPE species (C18:1, C18:2, and 22:6), 8 acylcarnitines (propionyl-, octanoyl-, decanoyl-, palmityl-, heptadecanoyl-, linoleyl-, and vaccenyl-carnitine), and 4 amino acids (leucine, lysine, phenylalanine, and serine) using a UPLC-Q-TOF MS-based metabolomic strategy and multivariate data analysis. These metabolites were closely associated with inflammatory and oxidative markers, as well as arterial stiffness. Therefore, differences in these metabolic profiles between the healthy men with normal fasting glucose levels and men with newly-diagnosed type 2 diabetes may provide a better understanding of the metabolic, inflammatory,

oxidative, and arterial stiffness changes involved in early diabetes, which may, in turn, improve future clinical diagnosis and treatment.

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국문요약

초기 제 2 형 당뇨병 남자 환자군의 혈장 대사 프로파일링 : 염증성 지표 및 산화 스트레스와 동맥경화도의 관계

본 논문에서는 초기 제 2형 당뇨병 환자에서의 혈장 대사체들과 염증, 산화 스트레스, 혈관 경직도와의 상관관계에 대해 연구하였다. 초기 제 2형 당뇨병 남자 환자 26명과 나이와 체질량지수가 일치하는 건강한 남자 27명을 대상으로 염증성 지표, 산화 지표들과 혈관 경직도를 측정하는 지표인 상완-발목 맥파전파속도 (ba-PWV)를 측정하였다. 대사체 profile 들은 UPLC/Q-TOF MS 로 분석하였다. 당뇨병 환자군에서 대조군보다 혈당, 중성지방, oxidized LDL, hs-CRP, IL-6, TNF-α, 인슐린 저항성, urinary 8-epi-prostaglandin F2α (8-epi-PGF2α) 와 상완-발목 맥파전파속도 (ba-PWV)가 높게 나타났다. 또한 당뇨병 환자군에서 leucine, lysine and phenylalanine , 8 acylcarnitines, 6 lyso-phosphatidylcholines (lysoPCs) and 2 lyso-phosphatidylethanolamines (lysoPEs) 을 포함하는 19개의 대사체가 유의하게 높게 나타났고, serine and lysoPE (C18:1) 은 낮게 관찰되었다. 당뇨병 환자군과 정상 대조군을 구분짓는 주요 대사체는 Decanoyl carnitine, lysoPCs (C14:0, C16:1, C18:1, and C22:6) lysoPE (C18:1) 이었다. Decanoyl carnitine 은 oxidized-LDL, 8-epi-PGF2α, IL-6, and TNF-α, ba-PWV 과 양의 상관관계를

보였다. Ba-PWV 는 lysoPCs C14:0, C16:1 과는 양의 상관관계를 lysoPE C18:1 과는 음의 상관관계를 보였다. 8-epi-PGF2α 는 lipoprotein-associated phospholipaseA2, ba-PWV lysoPCs (C14:0 and C16:1) 과 양의 상관관계를 보였다. 본 연구를 통해 초기 당뇨병 환자에서 혈중 대사체들이 염증, 산화스트레스, 혈관 경직도와 밀접하게 연관되어 있음을 알 수 있다.

핵심이 되는 말 : 초기 제 2 형 당뇨병, 대사체 profile, UPLC/Q-TOF MS, 염증성 지표, 산화 스트레스, 혈관 경직도, 상완-발목 전파속도