Macrophage Elovl6 Deficiency Ameliorates Foam Cell Formation and Reduces Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice

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Objective—Elovl6, a long-chain fatty acid elongase, is a rate-limiting enzyme that elongates saturated and monounsaturated fatty acids and has been shown to be related to obesity-induced insulin resistance via modification of fatty acid composition. In this study, we investigated the roles of Elovl6 in foam cell formation in macrophages and atherosclerosis in mice.

Methods and Results—To investigate the roles of Elovl6 in macrophages in the progression of atherosclerosis, we transplanted bone marrow cells of wild-type or Elovl6$^{-/-}$ mice into irradiated LDL-R$^{-/-}$ mice that were fed a western diet. Aortic atherosclerotic lesion areas and infiltration of macrophages were significantly smaller in Elovl6$^{-/-}$ bone marrow cells-transplanted LDL-R$^{-/-}$ mice than in wild-type. Accumulation of esterified cholesterol on exposure to acetylated-LDL was less severe in peritoneal macrophages from Elovl6$^{-/-}$ mice than those from wild-type. Cholesterol efflux and expression of cholesterol efflux transporters were increased in Elovl6$^{-/-}$ macrophages, although no difference in uptake of acetylated-LDL was found between the two groups. On analysis of fatty acid composition of the esterified cholesterol fraction in macrophages, n-6 polyunsaturated fatty acids were decreased by absence of Elovl6.

Conclusion—These findings suggest that Elovl6 in macrophages may contribute to foam cell formation and progression of atherosclerosis. (***Arterioscler Thromb Vasc Biol.* 2011;31:1973-1979.**)

Key Words: Elovl6 ■ atherosclerosis ■ macrophage ■ fatty acid composition

Elongation of very long-chain fatty acids family member 6 (Elovl6, also known as LCE, FACE, and MASR) belongs to a highly conserved family of endoplasmic reticulum enzymes that consists of at least 7 fatty acid elongases in mice and humans. Elovl6 specifically catalyzes the elongation of saturated fatty acids and monounsaturated fatty acids (MUFA) with 12, 14, and 16 carbons, thereby converting palmitate (C16:0) and palmitoleate (C16:1n-7) to stearate (C18:0) and vaccinate (C18:1n-7). These fatty acids are important constituents of triglycerides, esterified cholesterol, and phospholipids.

See accompanying article on page 1939

Elovl6 is expressed in essentially all tissues, though high levels of it are found in the adrenal gland, liver, white adipose tissue, brain, testis, and skin, where lipogenesis and steroidogenesis are active.[1–3] Expression of Elovl6 is directly regulated by sterol regulatory element-binding protein-1a, -1c, and -2, as well as other lipogenic enzymes such as fatty acid synthase (FAS) and stearoyl coenzyme A desaturase (SCD)-1.[2–6] Dietary n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoate and docosahexaenoate acid, cause profound suppression of Elovl6 expression.[3,7]

Absence of Elovl6 increases levels of palmitate and palmitoleate but reduces levels of stearate and oleate (C18:1n-9), and it provides protection from obesity-induced hyperinsulinemia, hyperglycemia, and hyperleptinemia despite the development of obesity and hepatosteatosis.[8] Inhibition of Elovl6 is thus a potential therapeutic target for ameliorating insulin resistance, diabetes, and cardiovascular risk. A major unanswered question is whether inhibition of this elongase will lead to reduced susceptibility to atherosclerosis.

The differentiation of macrophages into lipid-laden foam cells is a critical event in the early stages of atherosclerosis. Traditionally, it has been assumed that the uptake of oxidized LDL by macrophages and the subsequent accumulation of esterified cholesterol formed via catalysis by acyl-coenzyme A:cholesterol acyltransferase (ACAT) is largely responsible for this process. Although free cholesterol, a substrate for ACAT, is supplied from the intracellular cholesterol pool,

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little is known concerning the source and pathways involved in the supply of fatty acids as precursors for fatty acyl-CoA, another substrate of ACAT. Oleate, linoleate (C18:2n-6), palmitate, and palmitoleate were identified as the major fatty acids found in triglycerides and esterified cholesterol fractions of human fatty streak lesions. In particular, because oleate is the preferred fatty acid substrate of ACAT,[9] we considered the possibility whether inactivation of Elovl6 in macrophages affects foam cell formation and the development of atherosclerosis.

To test whether Elovl6 in macrophages modulates susceptibility to atherosclerosis, we performed bone marrow (BM) transplantation from Elovl6 deficient mice into LDL-R–deficient mice. Our findings demonstrated that the absence of Elovl6 in macrophages increased cholesterol efflux, and that this was associated with significant suppression of atherosclerosis in LDL-R–deficient mice.

Materials and Methods

Animal Procedures

Elovl6 knockout mice (Elovl6$^{-/-}$ mice, C57BL/6 background) and low-density lipoprotein receptor knockout mice (LDL-R$^{-/-}$ mice, C57BL/6 background) prepared as described previously were used in this study.[8,10] The Elovl6$^{-/-}$ mice were crossed with the LDL-R$^{-/-}$ mice to produce LDL-R$^{-/-}$/Elovl6$^{-/-}$ mice. Animals received a standard laboratory rodent chow diet or a western diet containing 21% (w/w) fat and 0.15% (w/w) cholesterol (D12079B; Research Diets Inc., NJ). All animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulations of Animal Experiments and were approved by the Animal Experiment Committee of the University of Tsukuba.

BM Transplantation

Donor BM cells were extracted from the tibias and femurs of male wild-type or Elovl6$^{-/-}$ mice with RPMI1640 medium containing 10 U/mL heparin and 2% fetal bovine serum. Male LDL-R$^{-/-}$ mice were lethally irradiated with a single dose of 900 rads using a MBR-1520R (Hitachi Medical Corporation, Tokyo, Japan). After irradiation, LDL-R$^{-/-}$ recipient mice were injected with 5×10^6 BM cells via the tail vein. Recipient mice were provided with neomycin- and polymyxin- (Wako Pure Chemicals, Osaka, Japan)supplemented water for 1 week before and 4 weeks after transplantation. Mice were fed a regular chow diet for the first 4 weeks after BM transplantation and then switched to a western diet for 16 weeks.

The hematologic chimerism of LDL-R$^{-/-}$ mice was determined in genomic DNA from blood by polymerase chain reaction (PCR) at 16 weeks post-transplantation. The primers used to detect the Elovl6 gene were described previously.[8]

Plasma Analysis

Plasma samples were collected via orbital bleeding in irradiated LDL-R$^{-/-}$ recipient mice transplanted with BM cells isolated from wild-type or Elovl6$^{-/-}$ mice. Plasma lipids (total cholesterol, triglycerides, free fatty acids, and phospholipids) and glucose levels were measured using commercially available kits (Wako Pure Chemicals). In addition, plasma hematologic parameters (hemoglobin, red blood cells, and white blood cells) were measured in other irradiated LDL-R$^{-/-}$ recipient mice.

Measurement of Atherosclerotic Lesions

Atherosclerotic lesions were analyzed as previously described.[11] In brief, mice were euthanized and their hearts and aortas were isolated. The degree of atherosclerosis was assessed by determining lesion sizes on both pinned-open aortas and serial cross-sections through the aortic root. The aorta was opened longitudinally along the ventral midline from the iliac arteries to the aortic root. After branching vessels were removed, the aorta was pinned out flat on a black rubber board. The lesions were stained with Sudan IV for 15 minutes, destained with 70% ethanol, and then fixed in 4% phosphate-buffered formalin. Aortic images were analyzed with Adobe Photoshop 7 software (Adobe Systems Incorporated, CA). Values are the percentage of the aortic surface covered by lesions. The hearts were perfused with 10% formalin and were fixed. The basal half of the hearts was embedded in Tissue-Tek OCT compound (Sakura Finetek Japan Co., Tokyo, Japan), and serial sections were captured using a Cryostat microtome. The sections were stained with Oil-Red O (0.3% in 60% isopropyl alcohol) and counterstained with hematoxylin.

Immunohistochemical Analyses of Atherosclerotic Lesions

To detect macrophage infiltration in atherosclerotic lesions, we first incubated sections with primary antibody to mouse macrophage marker F4/80 (1:20; Serotec, Raleigh, NC) for 2 hours. After washes, the sections were incubated with biotinylated anti-rat antibody for 30 minutes at room temperature and then with avidin-biotin peroxidase complex (Vector Laboratories Inc., CA) for 30 minutes. Finally, the sections were developed with DAB (Wako Pure Chemicals) and counterstained with methylgreen.

Mouse Peritoneal Macrophages

Macrophages were obtained by peritoneal lavage from wild-type or Elovl6$^{-/-}$ mice 4 days after intraperitoneal injection of thioglycollate (Difco Laboratories Inc., Detroit, MI). Cells were plated on 24-well plates or 6-cm dishes at a density of 1.5×10^6 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) with penicillin-streptomycin and 2% fetal bovine serum. Cells were incubated in an atmosphere of 5% CO_2 at 37°C.

Foam Cell Formation Assays

Mouse peritoneal macrophages (MPM) were incubated with 100 μg/mL acetylated-LDL (acLDL) (Biomedical Technologies Inc., Stoughton, USA) in DMEM medium containing 0.2% fatty acid-free bovine serum albumin (Sigma-Aldrich Japan, Tokyo, Japan) for 48 hours. Cells were extensively washed, fixed with 10% formalin, and stained with Oil-Red O. Cellular lipids were extracted with hexane/isopropyl alcohol on exposure to acLDL for 24 or 48 hours, and cellular cholesterol contents (total cholesterol, esterified cholesterol, and free cholesterol) were measured as previously described.[12] For pretreatment of long chain unsaturated fatty acids, MPM were treated with medium alone, 50 μmol/L oleate (C18:1 n-9), or linoleate (C18:2 n-6) (Sigma-Aldrich Japan) in DMEM medium containing 0.2% bovine serum albumin for 24 hours before incubation with acLDL.

Uptake of 1,1′-dioctadecyl-3,3,3′, 3′–tetramethylindocarbocyanice (Dil)-acLDL

Fluorescent Dil-labeled acLDL was prepared according to a method described elsewhere.[13] MPM were incubated with 100 μg/mL Dil-acLDL for 3 hours at 37°C. After the cells were washed, Dil was extracted with isopropyl alcohol, and relative fluorescence intensity was determined at 524 nm (excitation) and at 567 nm (emission). Experiments were performed 3 times, and representative values are shown as means ± SEM.

Cholesterol Efflux Measurements

MPM were loaded with 1.2 μCi/mL (1,2,$-^3$H)-cholesterol and 100 μg/mL acLDL in DMEM medium containing 0.2% bovine serum albumin for 24 hours at 37°C. Cells were then incubated with DMEM medium containing 0.2% bovine serum albumin supplemented or not with 50 μg/mL of high-density lipoprotein (HDL, Biomedical Technologies Inc.). Medium and cell lysates were collected after 48 hours incubation and radioactivity was determined by liquid scintillation counting. Cholesterol efflux was expressed as

the percentage of radioactivity released into the medium relative to total radioactivity.

Quantitative Real-Time PCR

Total RNA was isolated using Sepasol Reagent (Nacalai Tesque, Kyoto, Japan), and was reverse-transcribed using the ThermoScript RT-PCR System (Applied Biosystems Japan, Tokyo, Japan). Quantitative real-time PCR was performed using SYBR Green Dye (Applied Biosystems Japan) in an ABI Prism 7300 PCR instrument (Applied Biosystems Japan) as previously described.[12] Expression levels were normalized to GAPDH expression. The primers in this study were shown in Supplemental Table I, available online at http://atvb.ahajournals.org.

Fatty Acid Composition of Esterified Cholesterol Fraction in MPM

Total lipids in acLDL-treated MPM were extracted according to Bligh-Dyer's procedure and esterified cholesterol was separated on 500 mg silica columns (Discovery DSC-NH2, Sigma-Aldrich Japan) exactly as described.[14] After saponification, the fatty acids in each sample were methyl-esterified and the relative abundance of each fatty acid was quantified by gas chromatography.[15]

Statistics

All values are expressed as the mean ± SEM. The significance of differences between means was determined using Student *t* test with SAS software (SAS Institute Inc, NC).

Results

Deficiency of Elovl6 in Macrophages Improves Development of Atherosclerosis in LDL-R$^{-/-}$ Mice

To assess the biological roles of Elovl6 in macrophages in the development of atherosclerotic lesions in vivo, irradiated LDL-R$^{-/-}$ mice, which represented an established animal model for the development of atherosclerosis, were transplanted with BM cells isolated from wild-type or Elovl6$^{-/-}$ mice. Successful reconstitution of recipients with cells of donor origin after BM transplantation was confirmed by PCR-assisted amplification of the Elovl6 gene (Supplemental Figure I). To induce atherosclerotic lesion development, we fed these transplanted mice a western diet for 16 weeks, and the degree of atherosclerosis was determined either by quantifying Sudanophilic surface lesions in pinned-out whole aortas or by quantifying Oil-Red O-stained lesions in cross-sections from the aortic root. Deficiency of Elovl6 in hematologic cells did not alter body weight, serum lipid levels, glucose levels, or hematologic parameters (Supplemental Tables II and III). Atherosclerotic lesions emerged in both groups with the western diet regimen for 16 weeks. En face analysis demonstrated that aortic surface lesion areas in LDL-R$^{-/-}$ mice transplanted with BM cells isolated from Elovl6$^{-/-}$ mice were significantly smaller than in those transplanted with BM cells isolated from wild-type mice (42% decrease, $P<0.05$) (Figure 1A and 1B). Consistent with aortic surface lesion area, the decrease in cross-sectional lesion area in LDL-R$^{-/-}$ mice transplanted with BM cells isolated from Elovl6$^{-/-}$ mice was also significant on quantification of Oil Red O-stained aortic root sections (32% decrease, $P<0.05$) (Figure 1C and 1D). In addition, we quantified macrophage contents in cross-sections from the aortic root by immunohistochemical analysis using

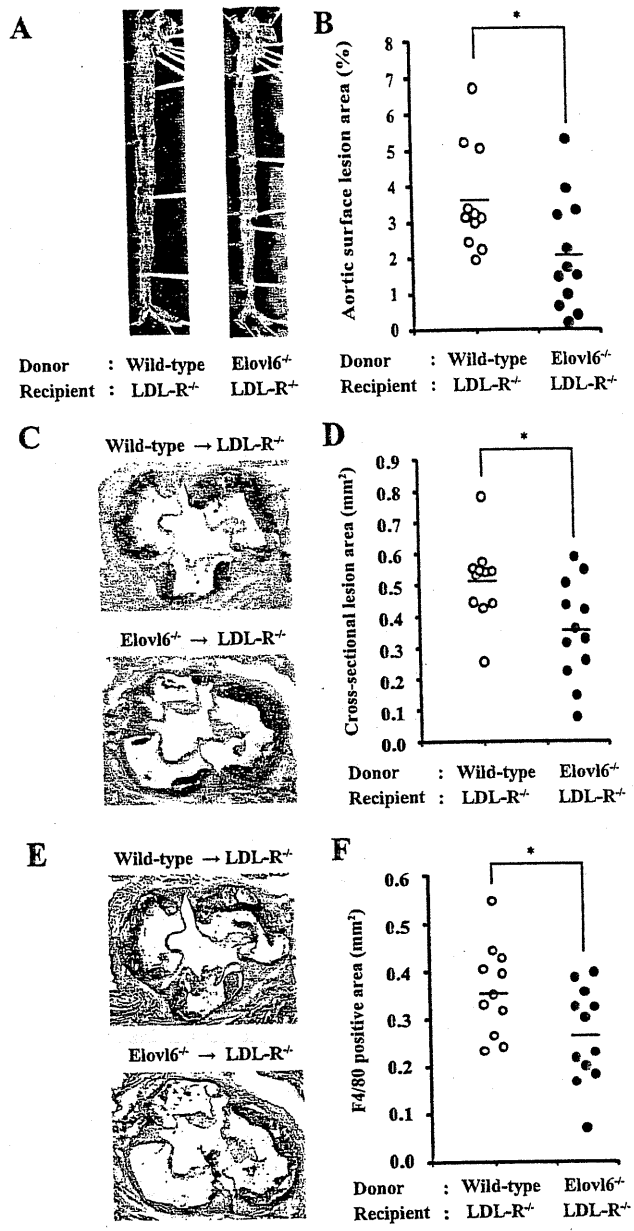

Figure 1. Atherosclerotic lesion development in LDL-R$^{-/-}$ mice transplanted with bone marrow cells isolated from wild-type or Elovl6$^{-/-}$ mice. A, Representative images of pinned Sudan IV stained aortas en face. B, Quantification of the surface area occupied by lesions. C, Representative photomicrographs of aortic root cross-sections stained with Oil-Red O. D, Quantification of the area occupied by lesions using cross-sections of aortic roots. E, Representative photomicrographs of aortic root cross-sections stained with F4/80 as a macrophage marker. F, Quantification of F4/80-positive area using cross-sections of aortic roots. Bar represent the mean value (n=11 to 12 per group). *$P<0.05$ vs the respective wild-type.

F4/80 as a macrophage marker. Similarly, macrophage-infiltrated areas in aortic roots were decreased in LDL-R$^{-/-}$ mice transplanted with BM cells isolated from Elovl6$^{-/-}$ mice compared with in those transplanted with BM cells isolated from wild-type mice (27% decrease, $P<0.05$) (Figure 1E and 1F). Taken together, these findings indicate that BM transplantation from Elovl6$^{-/-}$ mice attenuated the development of atherosclerotic lesions.

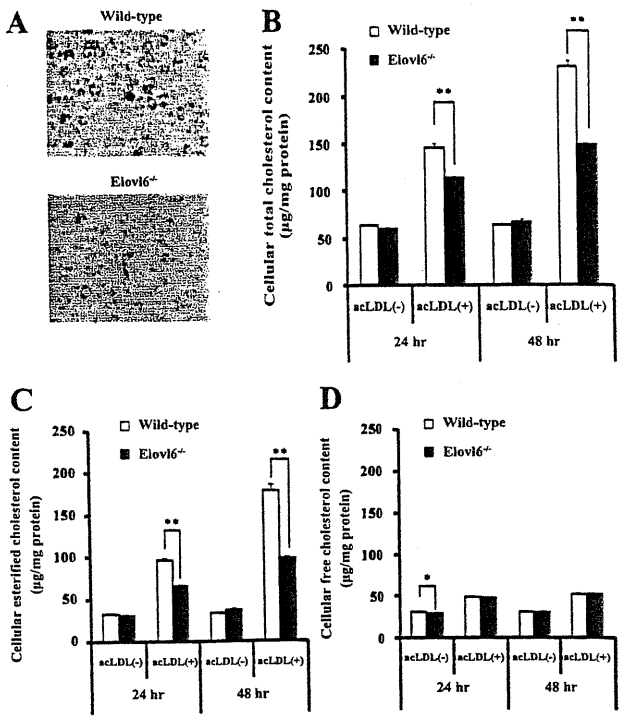

Figure 2. Foam cell formation in peritoneal macrophages isolated from wild-type or Elovl6$^{-/-}$ mice. A, Representative photomicrographs of macrophages stained with Oil-Red O. Macrophages were incubated with acLDL (100 μg/mL) for 48 hours. B–D, Cellular levels of total cholesterol (B), esterified cholesterol (C), and free cholesterol (D) in macrophages incubated with or without acLDL (100 μg/mL) for 24 or 48 hours. Values are means ± SEM (n=3 per group). *P<0.05, **P<0.01 vs the respective wild-type.

Elovl6 Deficiency in MPM Suppresses Lipid Accumulation on Exposure to AcLDL

To examine whether macrophage Elovl6 deficiency had an impact on foam cell formation, MPM isolated from wild-type or Elovl6$^{-/-}$ mice were incubated with acLDL. We also detected lipid accumulation in these cells by Oil-Red O staining or by extraction of cholesterol. Deficiency of Elovl6 was confirmed by expression of Elovl6 mRNA (Supplemental Figure II). As shown by Oil-Red O staining, cellular lipid accumulation was obviously lower in Elovl6$^{-/-}$ MPM than wild-type MPM (Figure 2A). With acLDL loading, cellular total cholesterol contents in MPM increased in time-dependent fashion. The increase in cellular total cholesterol content was significantly lower in Elovl6$^{-/-}$ MPM than in wild-type (Figure 2B). Cellular esterified cholesterol accumulation in Elovl6$^{-/-}$ MPM was suppressed by 45% at 48 hours after acLDL addition, whereas cellular-free cholesterol content was not affected by genotype (Figure 2C and 2D). Thus, the differences in cellular total cholesterol content between both wild-type and Elovl6$^{-/-}$ macrophages mainly resulted from changes in esterified cholesterol content.

In addition, to examine whether responses of wild-type and Elovl6$^{-/-}$ MPM were same as those of LDL-R$^{-/-}$ and LDL-R$^{-/-}$/Elovl6$^{-/-}$ MPM, we detected lipid accumulation in MPM incubated with acLDL. In LDL-R$^{-/-}$ background MPM, deficiency of Elovl6 similarly suppressed accumulation of cellular total and esterified cholesterol (Supplemental Figure IIIA and IIIB). These findings suggested that the reduction of lipid accumulation by Elovl6 deficiency in MPM was not affected by LDL-R deficiency.

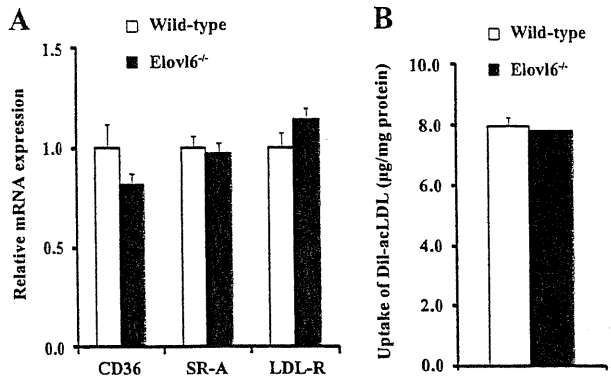

Figure 3. Cholesterol influx analysis in peritoneal macrophages isolated from wild-type or Elovl6$^{-/-}$ mice. A, Expression of lipid transporter mRNA involved in cholesterol influx in macrophages before incubation with acLDL. Values are means ± SEM (n=3 per group). B, Uptake of DiI-labeled acLDL in macrophages. Macrophages were incubated with DiI-labeled acLDL (100 μg/mL) for 3 hours, and fluorescence intensity was analyzed by fluorescent microscopy. Values are the means ± SEM of results of 3 representative independent experiments (n=3 dishes per group). SR-A indicates scavenger receptor-A.

Cholesterol Influx into MPM

To study the impact of Elovl6 on cholesterol influx into MPM, we examined the expression profiles of lipid transporters involved in cholesterol influx. The expression of genes encoding CD36, scavenger receptor-A, or LDL-R was not affected by Elovl6 deficiency in acLDL-untreated condition (Figure 3A). In agreement with this observation, the uptakes of DiI-labeled acLDL by MPM were similar for the 2 genotypes (Figure 3B).

Cholesterol Efflux from MPM

Cholesterol efflux from foam cells preloaded with radiolabeled cholesterol and acLDL was evaluated. In contrast to cholesterol influx, cholesterol efflux from foam cells was significantly increased in Elovl6$^{-/-}$ MPM compared with wild-type MPM, and these effects were more pronounced in the presence of HDL in medium (Figure 4A). Furthermore, we investigated the expression profiles of lipid transporters involved in cholesterol efflux after incubation with or without acLDL. The expressions of ATP-binding cassette, subfamily A, member 1 (ABCA1), ATP-binding cassette, subfamily G, member 1 (ABCG1), and scavenger receptor-B1, which are involved in cholesterol efflux from macrophages as components of reverse cholesterol transport, were markedly increased in Elovl6$^{-/-}$ MPM compared with wild-type MPM after incubation with acLDL (Figure 4B). Taken together, these results suggested that macrophage Elovl6 deficiency suppressed foam cell formation due to increase in cholesterol efflux.

Fatty Acid Composition of Esterified Cholesterol Fraction in MPM

To estimate the impact of Elovl6 deficiency on fatty acid composition in MPM, the esterified cholesterol fraction was separated and the various fatty acid contents were measured.

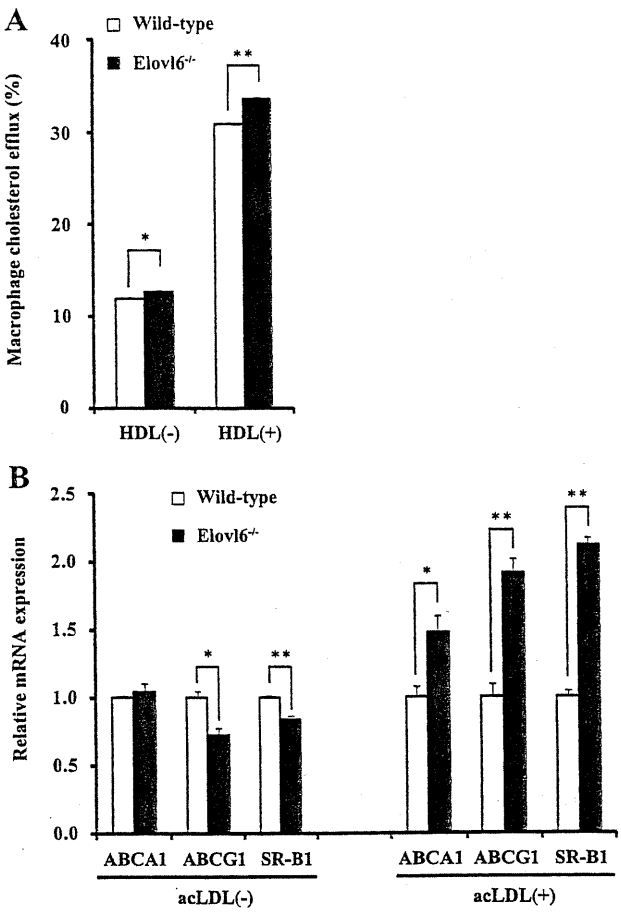

Figure 4. Cholesterol efflux analysis in peritoneal macrophages isolated from wild-type or Elovl6$^{-/-}$ mice. A, HDL-mediated cholesterol efflux. Macrophages were loaded with acLDL (100 μg/mL) in the presence of (1,2,-^{3}H)-cholesterol and treated with medium alone or with HDL (50 μg/mL) for 48 hours. Cholesterol efflux was expressed as the percentage of radioactivity released into the medium relative to total radioactivity. Values are means ± SEM (n=3 per group). B, Expression of lipid transporter mRNA involved in cholesterol efflux in macrophages treated with medium alone or with acLDL (100 μg/mL) for 48 hours. Values are means ± SEM (n=3 per group). *P<0.05, **P<0.01 vs the respective wild-type. SR-B1 indicates scavenger receptor-B1.

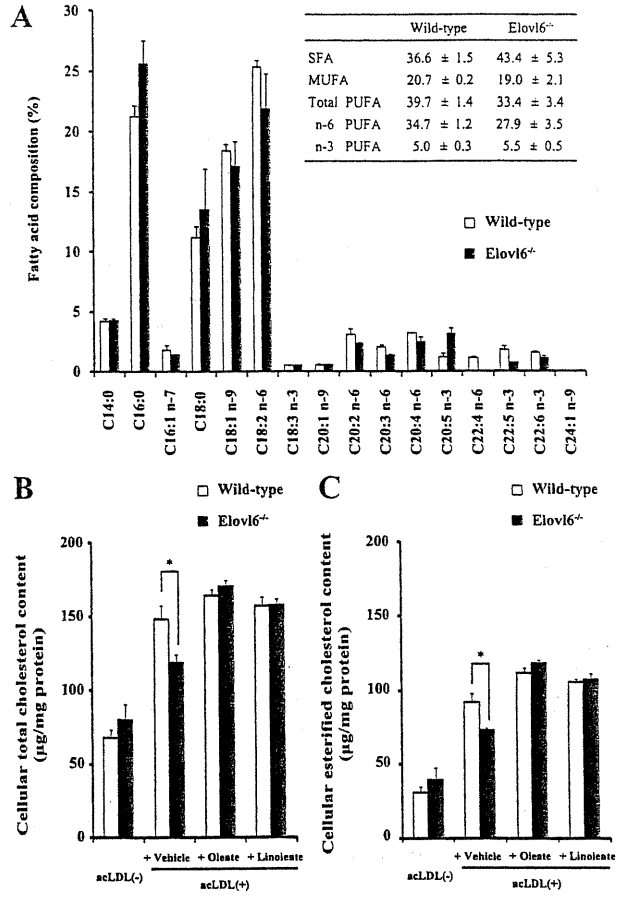

Figure 5. Differences in cellular fatty acid composition in peritoneal macrophages isolated from wild-type or Elovl6$^{-/-}$ mice. A, Fatty acid composition (% of total) of esterified cholesterol fraction in macrophages. Macrophages were incubated with acLDL (100 μg/mL) for 48 hours. The fatty acid composition of cellular esterified cholesterol was analyzed by gas chromatography. Values are means ± SEM (n=3 per group). Total of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), total polyunsaturated fatty acids (PUFA), n-6 PUFA, and n-3 PUFA fatty acid composition (%) were shown in table. B, C, Macrophages were pretreated with medium alone, oleate (50 μmol/L), or linoleate (50 μmol/L) for 24 hours and then incubated with acLDL (100 μg/mL) for 48 hours. Cellular levels of total cholesterol (B) and esterified cholesterol (C) were measured by the above procedure. Values are means ± SEM (n=3 to 6 per group). *P<0.05 vs the respective wild-type.

Although no statistically significant differences were found between the 2 groups, there were trends toward slight decrease in MUFA, whereas saturated fatty acid levels tended to be increased, accompanied by decreases in PUFA, specially n-6 PUFA, in Elovl6$^{-/-}$ MPM (Figure 5A). Furthermore, we investigated the expression profiles of lipogenic enzymes after incubation with acLDL. The expression of FAS was significantly higher, and the expressions of SCD-1 and ACC-1 tended to be higher in Elovl6$^{-/-}$ MPM compared with wild-type MPM (Supplemental Figure IV).

Exogenous Long-Chain Unsaturated Fatty Acids Normalize the Reduction of Cholesterol Contents in Elovl6$^{-/-}$ MPM

To determine whether exogenous long-chain unsaturated fatty acids influence cholesterol accumulation in MPM, oleate (C18:1 n-9) and linoleate (C18:2 n-6) were administered before incubation with acLDL. Pretreatment with oleate and linoleate normalized the reduction of cellular cholesterol accumulation in Elovl6$^{-/-}$ MPM (Figure 5B and 5C). These findings suggested that the reduction of long-chain unsaturated fatty acids by Elovl6 deficiency played an unknown role in the decrease in foam cell formation.

Elovl6 Deficiency in BM Cells and MPM Did Not Alter Inflammatory Cytokines, Chemokines, and Adhesion Molecules

We investigated the expression of genes encoding inflammatory cytokines, chemokines, and adhesion molecules in the aorta of LDL-R$^{-/-}$ mice transplanted with BM cells isolated from wild-type or Elovl6$^{-/-}$, which were fed a western diet. The expressions of genes encoding inflammatory cytokines (IL-1β, TNF-α, IL-6), chemokines (MCP1, CCR2), and

adhesion molecules (VCAM, ICAM) were almost not affected by Elovl6 deficiency in BM cells (Supplemental Figure VA). In agreement with these results, the expression of genes encoding inflammatory cytokines and chemokines was similar in Elovl6$^{-/-}$ MPM and wild-type MPM with incubation with acLDL (Supplemental Figure VB). Taken together, these findings suggested that the reduction of atherosclerosis by Elovl6 deficiency was due to some other mechanism and was not involved in inflammatory responses, chemotaxis, or adhesion molecule function as modulated by cholesterol loading.

Discussion

Accumulation of esterified cholesterol is a characteristic of macrophage foam cells, which are central to the development of atherosclerotic plaque.[16,17] In the process of foam cell formation, cholesterol incorporated from modified LDL and taken up was re-esterified mainly by endogenous fatty acyl-CoA rather than exogenous fatty acids from modified LDL.[9,18–20] It can be speculated that the fatty acids themselves and their regulation of fatty acid homeostasis play important roles in foam cell formation in addition to regulation of cholesterol content. One of the means of regulation of intracellular fatty acid levels and compositions is fatty acid biosynthesis by lipogenic enzymes, such as FAS, Elovl6, and SCD-1. FAS generates palmitate (C16:0),[21] Elovl6 elongates saturated fatty acids and MUFA with C12–16, and SCD-1 synthesizes MUFA, mainly palmitoleate (C16:1 n-7) and oleate (C18:1 n-9).[22] We found that expression of these enzymes tended to be increased in the aorta of atherosclerotic mice, though there were no significant differences (Supplemental Figure VI). The lipogenic enzymes in vessels and macrophages could be involved in the development of atherosclerosis. The roles of macrophage SCD-1 and FAS in atherosclerosis have been already reported. Macrophage SCD-1 deficiency did not alter atherosclerotic lesion sizes in LDL-R$^{-/-}$ mice and had no effect on cholesterol efflux from macrophages.[23] In contrast, macrophage FAS deficiency decreased atherosclerotic lesions in apoE-deficient mice.[24] In the present study, Elovl6-deficient hematopoietic cells derived from BM in LDL-R$^{-/-}$ mice ameliorated lipid accumulation on the aorta and the infiltration of macrophages, without differences in serum lipid parameters, aortic inflammatory cytokines, chemokines, or adhesion molecules from wild-type. These findings imply that the suppression of de novo fatty acid biosynthesis or change in long-chain fatty acid composition affects atherosclerosis.

The findings of the present study of macrophages from Elovl6$^{-/-}$ mice imply roles of Elovl6 in foam cell formation. It was reported that foam cell formation in FAS-deficient macrophages was diminished due to both increased cholesterol efflux and decreased uptake of oxidized LDL.[24] In contrast, Elovl6 deficiency in MPM also suppressed the cellular accumulation of esterified cholesterol after incubation of acLDL, although uptake of acLDL as well as expression of related transporters did not change and expression of FAS was enhanced, suggesting that amelioration of foam cell formation by Elovl6 deficiency might proceed via mechanisms different from those in the case of FAS deficiency. Therefore, suppression could be at the level of esterification. Because oleate, the major esterified cholesterol in foam cells, is a better substrate for ACAT than palmitate and miristate, inhibition of conversion from palmitate to stearate resulting in decreased endogenous MUFA and increased endogenous saturated fatty acids could lead to reduction in esterified cholesterol. The enhancement of expression of lipogenic enzymes in Elovl6$^{-/-}$ MPM might be compensated for the unbalanced fatty acid composition. Furthermore, in Elovl6$^{-/-}$ MPM, n-6 PUFA levels were decreased in the esterified cholesterol fraction, although in FAS-deficient MPM, levels of palmitate and stearate, which are products of the FAS reaction, and oleate were decreased and major PUFA levels were not changed.[24] In foam cells, cholesteryl linoleate and cholesteryl arachidonate are along with cholesteryl oleate also major components of esterified cholesterol.[9] One possible reason for amelioration of foam cell formation by Elovl6 deficiency is the suppression of esterification of cholesterol by decrease in n-6 PUFA levels. Although the causes of the decreases in n-6 PUFA in the esterified cholesterol fraction in Elovl6-deficient MPM are currently unclear, pretreatment with MUFA and n-6 PUFA compensated for the reduction of cellular cholesterol content in Elovl6$^{-/-}$ macrophages. The present findings suggest that the intracellular fatty acid composition that Elovl6 regulates is a novel determinant of the amount of esterified cholesterol.

Another new finding of the present study is activation of cholesterol efflux from Elovl6-deficient foam cells. Reduction of esterified cholesterol accompanied increased free cholesterol, which could lead to enhanced cholesterol efflux from foam cells. Increased free cholesterol was associated with activated gene expression of LXRα target genes such as ABCA1, ABCG1, and scavenger receptor-B1.[25,26] The enhancement of expression of these lipid transporters in Elovl6$^{-/-}$ MPM might be related to LXRα activation via increases in cellular cholesterol content after acLDL addition.[25,26] Though we did not detect increase in free cholesterol in Elovl6$^{-/-}$ MPM, cellular cholesterol levels might be increased with shorter incubation times. In addition, it is known that unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ABCA1.[27,28] Decrease in MUFA and PUFA may increase cholesterol efflux via increasing ABCA1. In contrast, cholesterol uptake in Elovl6$^{-/-}$ MPM was not altered, unlike the suppression of cholesterol uptake in FAS-deficient MPM. The main transporter involved in cholesterol influx, CD36, is a peroxisome proliferator-activated receptor-α target gene,[29] which is activated by various fatty acids such as palmitate. In FAS-deficient MPM, decrease in palmitate levels in addition to those of oleate was observed, suggesting greater suppression in peroxisome proliferator-activated receptor-α regulation. It may be that Elovl6 deficiency is directly linked to the cholesterol efflux genes by an unknown mechanism.

In conclusion, we showed that Elovl6 plays a crucial role in the development of both foam cells and atherosclerotic lesions. These findings increase understanding of the pathophysiology of atherosclerosis and provide further evidence of the importance of fatty acid quality in the development of atherosclerosis.

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Disclosures

None.

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HbA_{1c} 5·7–6·4% and impaired fasting plasma glucose for diagnosis of prediabetes and risk of progression to diabetes in Japan (TOPICS 3): a longitudinal cohort study

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Summary

Background The clinical relevance of the diagnostic criteria for prediabetes to prediction of progression to diabetes has been little studied. We aimed to compare the prevalence of prediabetes when assessed by the new glycated haemoglobin A_{1c} (HbA_{1c}) 5·7–6·4% criterion or by impaired fasting glucose, and assessed differences in progression rate to diabetes between these two criteria for prediabetes in a Japanese population.

Methods Our longitudinal cohort study included 4670 men and 1571 women aged 24–82 years without diabetes at baseline (diabetes was defined as fasting plasma glucose ≥7·0 mmol/L, self-reported clinician-diagnosed diabetes, or HbA_{1c} ≥6·5%) who attended Toranomon Hospital (Tokyo, Japan) for a routine health check between 1997 and 2003. Participants with a baseline diagnosis of prediabetes according to impaired fasting glucose (fasting plasma glucose 5·6–6·9 mmol/L) or HbA_{1c} 5·7–6·4%, or both, were divided into four groups on the basis of baseline diagnosis of prediabetes. Rate of progression to diabetes was assessed annually.

Findings Mean follow-up was 4·7 (SD 0·7) years. 412 (7%) of 6241 participants were diagnosed with prediabetes on the basis of the HbA_{1c} 5·7–6·4% criterion. Screening by HbA_{1c} alone missed 1270 (61%) of the 2092 prediabetic individuals diagnosed by a combination of impaired fasting glucose and HbA_{1c} 5·7–6·4%. Overall cumulative probability of progression to diabetes did not differ significantly between participants with prediabetes discordantly diagnosed by either HbA_{1c} or impaired fasting glucose alone (incidence was 7% for HbA_{1c} alone [n=412 individuals and 30 incident cases] and 9% for impaired fasting glucose alone [n=1270, 108 cases]; log-rank test, p=0·3317). Multivariate-adjusted hazard ratios for incident diabetes were 6·16 (95% CI 4·33–8·77) for those diagnosed with prediabetes by impaired fasting glucose alone and 6·00 (3·76–9·56) for diagnosis by HbA_{1c} alone, and were substantially increased to 31·9 (22·6–45·0) for diagnosis by both impaired fasting glucose and HbA_{1c} compared with normoglycaemic individuals.

Interpretation Diagnosis of prediabetes by both the new HbA_{1c} criterion and impaired fasting glucose identified individuals with an increased risk of progression to diabetes. Although the new HbA_{1c} criterion identified fewer individuals at high risk than did impaired fasting glucose, the predictive value for progression to diabetes assessed by HbA_{1c} 5·7–6·4% was similar to that assessed by impaired fasting glucose alone. The two tests used together could efficiently target people who are most likely to develop diabetes and allow for early intervention.

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See Comment page 104

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Introduction

In prediabetes, blood glucose concentrations are higher than normal, but are not high enough for diagnosis of diabetes. The disorder is thought to place individuals at high risk of future diabetes, according to the American Diabetes Association (ADA).[1] ADA guidelines suggest targeting of individuals identified as having prediabetes for early intervention.[1] A new criterion has been proposed for the diagnosis of prediabetes: glycated haemoglobin A_{1c} (HbA_{1c}) 5·7–6·4%. However, the performance of HbA_{1c} as a screening test for identification of prediabetic individuals has been controversial.[2–6] Many individuals who were diagnosed as having prediabetes on the basis of impaired fasting glucose are reclassified as not having the disorder when the new HbA_{1c} 5·7–6·4% criterion is used; thus, screening by HbA_{1c} alone might miss a large number of prediabetic individuals.[2–5] The new criterion's performance in detection of prediabetic individuals differs according to ethnic origin,[4,5] and more evidence of its usefulness in non-western populations is needed.[4,6]

Few studies[7,8] have longitudinally compared the difference in progression rate to diabetes after diagnosis of prediabetes with the HbA_{1c} 5·7–6·4% criterion or by impaired fasting glucose, or established which criterion for prediabetes is clinically relevant for prediction of progression. Whether introduction of the new HbA_{1c} criterion in addition to assessment of fasting glucose could efficiently target prediabetic individuals who are most likely to progress to diabetes is unclear. We aimed to evaluate the effect of introduction of the HbA_{1c} 5·7–6·4% criterion into diagnosis of prediabetes by

impaired fasting glucose, and to longitudinally assess differences in the progression rate to diabetes between individuals diagnosed with prediabetes on the basis of these two criteria in a large Japanese cohort. We tested whether the two tests used together could target people most likely to progress to diabetes, which would allow early intervention.

	Study participants (n=6241)
Age (years)	49·9 (8·7)
Men	4670 (75%)
Smoking habit	
Never	3342 (54%)
Former	1503 (24%)
Current	1396 (22%)
BMI ≥25·0 kg/m²	1215 (19%)
Hypertension	1320 (21%)
Dyslipidaemia	1962 (31%)
History of coronary heart disease	84 (1%)
History of stroke	16 (<1%)

Data are n (%) or mean (SD). Hypertension is defined as systolic blood pressure 140 mm Hg or higher, diastolic blood pressure 90 mm Hg or higher, or on treatment. Dyslipidaemia is defined as triglyceride concentration 1·7 mmol/L or higher, HDL cholesterol lower than 1·03 mmol/L, or on treatment. BMI=body-mass index.

Table 1: **Overall baseline characteristics**

Methods

Study population

The Toranomon Hospital Health Management Center Study (TOPICS) included a cohort consisting mainly of apparently healthy Japanese government employees who underwent annual examinations for health screening. The details of the study have been described previously.[9] The cohort consisted of 32 057 individuals who had a routine health check for the first time between 1997 and 2003 at the Health Management Center, Toranomon Hospital (Tokyo, Japan). Of these 32 057 individuals, our investigation included 6636 individuals who had annual examinations regularly for 4 years (n=1716) or 5 years (n=4920) after the initial examination. Registered nurses interviewed all participants at the time of each annual examination using standard questionnaires that gathered information about demographic characteristics, medical history, and health-related habits. We excluded 310 individuals who had diabetes at the baseline examination (192 individuals were previously diagnosed and 118 were undiagnosed) or who had missing data for baseline characteristics (n=89). Subsequently, 6241 individuals aged 24–82 years were eligible for our analysis.

The study protocol was consistent with the Japanese Government's ethics guidelines regarding epidemiological studies in accordance with the Declaration of Helsinki and was reviewed by the institutional review

		Prediabetes			p value		
	Normoglycaemia (group 1, n=4149)	IFG alone (group 2, n=1270)	HbA_{1c} 5·7–6·4% alone (group 3, n=412)	Both HbA_{1c} 5·7–6·4% and IFG (group 4, n=410)	1 *vs* 2	1 *vs* 3	1 *vs* 4
Age (years)	49·2 (48·9–49·4)	49·9 (49·5–50·4)	54·3 (53·5–55·1)	53·7 (52·8–54·5)	0·0048	<0·0001	<0·0001
Women	1252 (30%)	118 (9%)	130 (32%)	71 (17%)	<0·0001	0·56	<0·0001
Family history of diabetes	532 (13%)	194 (15%)	69 (17%)	90 (22%)	0·0247	0·0247	<0·0001
Current smoking	880 (21%)	312 (25%)	101 (25%)	103 (25%)	0·0115	0·12	0·0661
BMI (kg/m²)*	22·5 (22·4–22·6)	23·5 (23·3–23·6)	22·9 (22·7–23·2)	23·8 (23·6–24·1)	<0·0001	0·0034	<0·0001
Obesity (BMI ≥25·0 kg/m²)*	648 (16%)	370 (29%)	73 (18%)	124 (30%)	<0·0001	0·27	<0·0001
Systolic blood pressure (mm Hg)*	123 (123–124)	130 (129–131)	123 (121–124)	127 (126–129)	<0·0001	0·52	<0·0001
Diastolic blood pressure (mm Hg)*	75 (75–76)	80 (80–81)	75 (74–76)	78 (77–79)	<0·0001	0·37	<0·0001
Triglycerides (mmol/L)*	1·23 (1·21–1·26)	1·43 (1·38–1·47)	1·33 (1·25–1·41)	1·59 (1·51–1·67)	<0·0001	0·0179	<0·0001
Total cholesterol (mmol/L)*	5·22 (5·19–5·24)	5·37 (5·33–5·42)	5·35 (5·27–5·43)	5·46 (5·38–5·54)	<0·0001	0·0015	<0·0001
HDL cholesterol (mmol/L)*	1·41 (1·40–1·42)	1·42 (1·40–1·44)	1·33 (1·29–1·36)	1·31 (1·28–1·34)	0·49	<0·0001	<0·0001
γ-glutamyltransferase (units per L)*	46·1 (44·5–47·8)	63·8 (60·8–66·8)	50·3 (45·0–55·5)	65·0 (59·7–70·3)	<0·0001	0·14	<0·0001
Uric acid (μmol/L)*	333·7 (331·6–335·8)	349·1 (345·3–352·9)	334·5 (327·8–341·2)	354·9 (348·2–361·6)	<0·0001	0·83	<0·0001
eGFR (ml/min per 1·73m²)*	75·5 (75·1–75·8)	76·2 (75·6–76·9)	74·5 (73·3–75·6)	75·9 (74·7–77·1)	0·0559	0·11	0·50
White cell count (×10⁹/L)*	5·2 (5·2–5·3)	5·3 (5·2–5·4)	5·5 (5·4–5·7)	5·6 (5·5–5·8)	0·0654	0·0001	<0·0001
Haemoglobin (g/L)*	145 (144–145)	146 (146–147)	141 (140–142)	145 (144–146)	<0·0001	<0·0001	0·18
Fasting plasma glucose (mmol/L)*	5·1 (5·0–5·1)	5·8 (5·8–5·8)	5·1 (5·1–5·2)	6·0 (6·0–6·0)	<0·0001	<0·0001	<0·0001
HbA_{1c} (%)*	5·2% (5·2–5·2)	5·3% (5·3–5·3)	5·8% (5·8–5·8)	5·9% (5·8–5·9)	<0·0001	<0·0001	<0·0001

Data are n (%) or mean (95% CI). Categorical data were analysed with the χ^2 test. HbA_{1c} was estimated as the National Glycohemoglobin Standardization Program equivalent value (%). Normoglycaemia was defined as HbA_{1c} less than 5·7% and FPG lower than 5·6 mmol/L. Diagnosis of prediabetes was by IFG alone when HbA_{1c} less than 5·7% and FPG 5·6–6·9 mmol/L, by HbA_{1c} alone when HbA_{1c} 5·7–6·4% and FPG lower than 5·6 mmol/L, and by both HbA_{1c} and IFG when HbA_{1c} 5·7–6·4% and FPG 5·6–6·9 mmol/L. HbA_{1c}=glycated haemoglobin A_{1c}. IFG=impaired fasting glucose. FPG=fasting plasma glucose. BMI=body-mass index. eGFR=estimated glomerular filtration rate. *Adjusted for age and sex.

Table 2: **Baseline characteristics according to diagnosis of prediabetes by HbA_{1c} and IFG criteria**

board at Toranomon Hospital. Written informed consent was obtained from all participants.

Procedures

Blood samples were obtained after an overnight fast (12 h) and tested with an automatic clinical chemistry analyser (Hitachi, LABOSPECT 008, Tokyo, Japan). Blood glucose, serum triglyceride, total cholesterol, HDL cholesterol, and uric acid concentrations were measured by enzymatic methods. HbA_{1c} was assessed by high-performance liquid chromatography. The intra-assay coefficient of variation was 0·7% with a mean of 4·29%, and the interassay coefficient of variation was 0·7% with a mean of 4·29%. The value for HbA_{1c} was estimated as a National Glycohemoglobin Standardization Program equivalent value calculated with the formula:[10]

$$HbA_{1c}\,(\%) = HbA_{1c}\,(\text{Japan Diabetes Society})\,(\%) + 0{\cdot}4\%$$

Diabetes was defined in accordance with ADA guidelines[1] as a fasting plasma glucose (FPG) concentration of 7·0 mmol/L or higher, self-reported clinician-diagnosed diabetes, or HbA_{1c} 6·5% or higher. Baseline diagnosis of prediabetes was based on the new ADA criterion[1] of impaired fasting glucose (FPG 5·6–6·9 mmol/L) or HbA_{1c} 5·7–6·4%, or both. Participants were divided into four groups on the basis of baseline diagnosis of prediabetes: (1) normoglycaemia (HbA_{1c} <5·7% and FPG <5·6 mmol/L); (2) impaired fasting glucose alone (HbA_{1c} <5·7% and FPG 5·6–6·9 mmol/L); (3) HbA_{1c} 5·7–6·4% alone (HbA_{1c} 5·7–6·4% and FPG <5·6 mmol/L); and (4) both HbA_{1c} 5·7–6·4% and impaired fasting glucose (HbA_{1c} 5·7–6·4% and FPG 5·6–6·9 mmol/L). Additionally, to investigate whether similar associations between a baseline diagnosis of prediabetes and future risk of diabetes would be identified irrespective of the diagnostic criteria for incident diabetes, we did an analysis of incident cases of diabetes that were diagnosed with three other criteria: diabetes indicated by self-reported clinician-diagnosis; diabetes indicated by self-reported clinician-diagnosis or FPG 7·0 mmol/L or higher; or diabetes indicated by self-reported clinician-diagnosis or HbA_{1c} 6·5% or higher.

Statistical analysis

We used SPSS (version 16.0) for all analyses and regarded p values lower than 0·05 as significant. We compared baseline characteristics between the four prediabetic groups using a general linear model with adjustments for age and sex. The level of agreement of the diagnostic categories between FPG and HbA_{1c} criteria was examined with κ statistics.[11] The diagnostic property of HbA_{1c} for FPG 5·6–6·9 mmol/L was cross-sectionally evaluated by a receiver operating characteristic (ROC) curve. We also did an analysis when FPG concentrations of

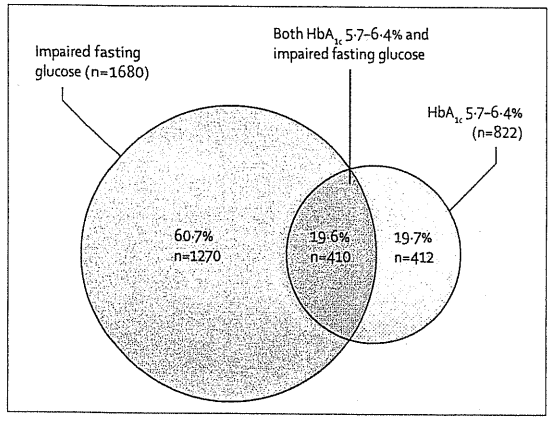

Figure 1: **Prevalence of individuals with prediabetes according to diagnosis by glycated haemoglobin A_{1c} (HbA_{1c}) 5·7–6·4% and impaired fasting glucose (fasting plasma glucose 5·6–6·9 mmol/L) criteria at a baseline examination (n=2092)**

	Proportion of total population (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
6·4%	<1%	1%	100%	100%	73%
6·3%	<1%	1%	100%	89%	73%
6·2%	1%	3%	100%	79%	74%
6·1%	2%	5%	99%	72%	74%
6·0%	3%	8%	99%	67%	74%
5·9%	5%	12%	97%	59%	75%
5·8%	8%	17%	95%	54%	76%
5·7%	13%	24%	91%	50%	77%
5·6%	21%	35%	85%	46%	78%
5·5%	30%	46%	76%	42%	79%
5·4%	41%	57%	65%	37%	80%
5·3%	53%	69%	52%	35%	82%
5·2%	66%	79%	40%	33%	84%

Impaired fasting glucose was defined as fasting plasma glucose 5·6–6·9 mmol/L. HbA_{1c}=glycated haemoglobin A_{1c}.

Table 3: **Sensitivity, specificity, and positive and negative predictive values for identification of individuals with impaired fasting glucose at different HbA_{1c} thresholds**

	Participants without diabetes (n=5903)	Participants with diabetes (n=338)
Normoglycaemia	4103 (70%)	46 (14%)
Baseline diagnosis of prediabetes		
IFG alone	1162 (20%)	108 (32%)
HbA_{1c} 5·7–6·4% alone	382 (6%)	30 (9%)
Both HbA_{1c} 5·7–6·4% and IFG	256 (4%)	154 (46%)

Data are n (%). Normoglycaemia was defined as HbA_{1c} lower than 5·7% and FPG lower than 5·6 mmol/L. Diagnosis of prediabetes was by IFG alone when HbA_{1c} lower than 5·7% and FPG 5·6–6·9 mmol/L; by HbA_{1c} alone when HbA_{1c} 5·7–6·4% and FPG lower than 5·6 mmol/L; and by both HbA_{1c} and IFG when HbA_{1c} 5·7–6·4% and FPG 5·6–6·9 mmol/L. IFG=impaired fasting glucose. HbA_{1c}=glycated haemoglobin A_{1c}. FPG=fasting plasma glucose.

Table 4: **Comparison of baseline diagnosis of prediabetes between individuals who did and did not develop type 2 diabetes**

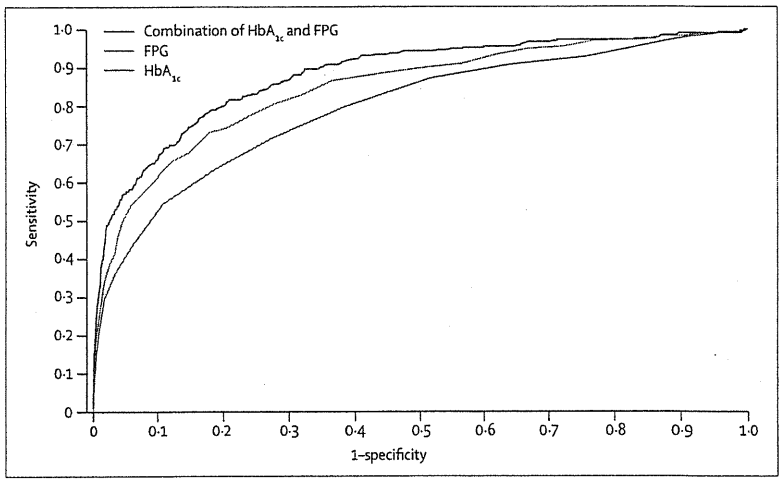

Figure 2: **ROC curve for prediction of future diabetes by HbA_{1c}, by FPG, and by the combination of HbA_{1c} and FPG**
ROC curve for HbA_{1c}, AUC 0·795 (95% CI 0·767–0·822); ROC curve for FPG, AUC 0·846 (0·821–0·870); ROC curve for the combination of HbA_{1c} and FPG, AUC 0·880 (0·859–0·901). ROC=receiver operating characteristic. HbA_{1c}=glycated haemoglobin A_{1c}. FPG=fasting plasma glucose. AUC=area under the curve.

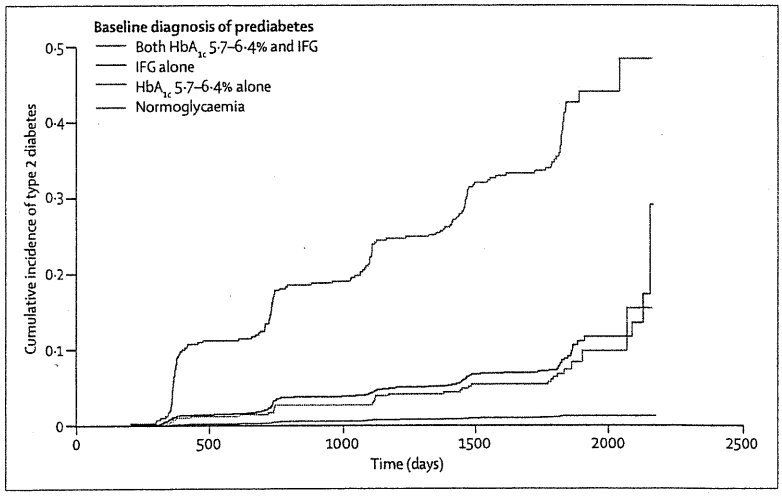

Figure 3: **Cumulative incidence of diabetes during follow-up according to baseline diagnosis of prediabetes**
Log-rank test, p=0·3317 between IFG alone and HbA_{1c} 5·7–6·4% alone. Normoglycaemia defined as HbA_{1c} lower than 5·7% and FPG lower than 5·6 mmol/L. Diagnosis of prediabetes by IFG alone defined as HbA_{1c} less than 5·7% and FPG 5·6–6·9 mmol/L, by HbA_{1c} alone defined as HbA_{1c} 5·7–6·4% and FPG lower than 5·6 mmol/L, and by both HbA_{1c} and IFG defined as HbA_{1c} 5·7–6·4% and FPG 5·6–6·9 mmol/L. HbA_{1c}=glycated haemoglobin A_{1c}. IFG=impaired fasting glucose. FPG=fasting plasma glucose.

6·1–6·9 mmol/L rather than 5·6–6·9 mmol/L were applied as the reference criterion.

In prospective analyses, we undertook an ROC analysis for prediction of risk of future diabetes on the basis of HbA_{1c}, FPG, and the combination of the two values (HbA_{1c} and FPG). Risk established for the combination of the two tests was calculated as: log hazard ratio = (β1×FPG) + (β2×HbA_{1c}). Unadjusted overall time to the development of diabetes was described by Kaplan-Meier analysis with log-rank testing. Cox regression was used to estimate the hazard ratios (HRs) and their 95% CIs for each baseline diagnosis of prediabetes with a normoglycaemic group as the reference. Follow-up for each participant was calculated from the date of the first examination to the date of confirmed diabetes or the date of the last follow-up examination.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Prevalence of diabetes in the entire study population was 5% (1684 of 32 057 people). Table 1 shows characteristics of study participants (n=6241). On the basis of the HbA_{1c} 5·7–6·4% criterion, 412 (7%) individuals in the study population had newly diagnosed prediabetes (table 2). Prediabetic individuals diagnosed by impaired fasting glucose but not by HbA_{1c} had significantly different characteristics at the baseline examination compared with those diagnosed by HbA_{1c} but not by fasting glucose. Those diagnosed on the basis of HbA_{1c} alone were more likely to be women, older, and less hypertensive, to have a lower body-mass index (BMI), lower triglyceride, uric acid, HDL cholesterol, and γ-glutamyltransferase concentrations, and higher leucocyte counts than those diagnosed by impaired fasting glucose alone (adjusted for age and sex).

Of 2092 prediabetic individuals at the baseline examination, only 20% (n=412) were classified as having prediabetes by the HbA_{1c} criterion without impaired fasting glucose (figure 1). Screening by HbA_{1c} alone missed 61% of the total number of prediabetic individuals diagnosed by a combination of impaired fasting glucose and HbA_{1c} 5·7–6·4%, and 1270 prediabetic individuals previously diagnosed by impaired fasting glucose (n=1680) were not classified as having prediabetes. The magnitude of overlap between the two criteria was low: 50% of prediabetic individuals diagnosed by HbA_{1c} also had impaired fasting glucose (n=410/822), and 24% of those diagnosed by impaired fasting glucose also had HbA_{1c} 5·7–6·4% (n=410/1680). We noted poor agreement between impaired fasting glucose and HbA_{1c} criteria (κ 0·18, 95% CI 0·16–0·21). HbA_{1c} ranging between 5·6% and 6·4% provided the highest agreement with impaired fasting glucose (κ 0·22, 0·19–0·24), although the improvement was small.

The area under the curve for the ROC analysis with HbA_{1c} for diagnosis of prediabetes by impaired fasting glucose was 0·656 (95% CI 0·641–0·672). For identification of individuals with impaired fasting glucose, a threshold of HbA_{1c} 5·7% showed high specificity of 91% and low sensitivity of 24%, wher eas HbA_{1c} 5·5% gave the highest combination of specificity (76%) and sensitivity (46%; table 3). When the more restrictive definition for impaired fasting glucose of FPG 6·1–6·9 mmol/L was

applied instead of 5·6–6·9 mmol/L, the prevalence of prediabetes by impaired fasting glucose decreased from 27% (n=1680) to 6% (n=380) among the total population, and 17% (n=1043) had prediabetes by either FPG 6·1–6·9 mmol/L or HbA_{1c} 5·7–6·4%. The area under the curve for the ROC analysis with HbA_{1c} for detection of FPG 6·1–6·9 mmol/L was 0·740 (95% CI 0·714–0·766), and the threshold of HbA_{1c} 5·7% showed a sensitivity of 42% and specificity of 89%.

After the baseline diagnosis of prediabetes, we documented 338 incident cases of diabetes during a mean 4·7 years' (SD 0·7) annual follow-up. A prediabetic state assessed by impaired fasting glucose alone, by HbA_{1c} 5·7–6·4% alone, or by both fasting glucose and HbA_{1c} preceded diabetes in 32% (n=108), 9% (n=30), and 46% (n=154) of incident cases of diabetes, respectively (table 4). Of the incident cases, 86% (n=292) were predicted by either impaired fasting glucose or HbA_{1c} 5·7–6·4%, whereas 14% (n=46) with normoglycaemia at baseline progressed straight to diabetes. Among normoglycaemic individuals (HbA_{1c} <5·7% and FPG <5·6 mmol/L), higher baseline levels of both HbA_{1c} and FPG, even though within a normal range, were associated with development of diabetes. As to age-adjusted and sex-adjusted HRs, for each 0·5% increase in HbA_{1c} there was a 2·57 (95% CI 1·32–5·00) increase in the HR and for each 0·55 mmol/L (10 mg/dl) increase in FPG values there was an increase of 2·33 (95% CI 1·19–4·57) in the HR.

The ROC curve plot for prediction of future diabetes by HbA_{1c}, by FPG, or by the combination of the two tests showed that the combination of FPG and HbA_{1c} slightly but significantly (p<0·0001) improved the area under the curve for prediction of future diabetes compared with use of only one test for screening (figure 2).

Figure 3 shows a Kaplan-Meier survival curve for prediction of diabetes after a baseline diagnosis of prediabetes. Incidence was 7% for HbA_{1c} alone (n=412 individuals and 30 incident cases) and 9% for impaired fasting glucose alone (n=1270, 108 incident cases). Overall cumulative probability did not differ significantly between the two (log-rank test, p=0·3317). Of prediabetic individuals who fulfilled both HbA_{1c} and fasting glucose criteria at baseline, 38% (n=154) progressed to diabetes within 5 years. If a definition for impaired fasting glucose of FPG 6·1–6·9 mmol/L was applied rather than FPG 5·6–6·9 mmol/L, incidence was 2% for HbA_{1c} lower than 5·7% and FPG lower than 6·1 mmol/L (n=5198 individuals and 100 incident cases). With HbA_{1c} alone, FPG alone, and both FPG and

	Total (n=6241)	Normoglycaemia (n=4149)	Prediabetes		
			IFG alone (n=1270)	HbA_{1c} 5·7–6·4% alone (n=412)	Both HbA_{1c} 5·7–6·4% and IFG (n=410)
Incidence of diabetes by self-reported clinician diagnosis					
Incident cases/person-years	157/29 856	34/19 982	43/6029	14/1985	66/1860
Incident rate (per 1000 person-years)	5·3	1·7	7·1	7·1	35·5
Age-adjusted and sex-adjusted hazard ratio (95% CI)	..	1·00	3·53 (2·24–5·55)	3·68 (1·96–6·91)	17·4 (11·4–26·6)
Multivariate hazard ratio (95% CI)	..	1·00	3·40 (2·14–5·39)	3·48 (1·85–6·54)	15·8 (10·2–24·6)
Incidence of diabetes by self-reported clinician diagnosis or HbA_{1c} ≥6·5%					
Incident cases/person-years	250/29 684	39/19 973	62/6006	26/1968	123/1737
Incident rate (per 1000 person-years)	8·4	2·0	10·3	13·2	70·8
Age-adjusted and sex-adjusted hazard ratio (95% CI)	..	1·00	4·54 (3·03–6·79)	6·63 (4·02–11·0)	33·8 (23·4–48·8)
Multivariate hazard ratio (95% CI)	..	1·00	4·34 (2·88–6·54)	6·24 (3·77–10·3)	30·5 (20·9–44·6)
Incidence of diabetes by self-reported clinician diagnosis or FPG ≥7·0 mmol/L					
Incident cases/person-years	298/29 558	43/19 965	101/5927	21/1978	133/1688
Incident rate (per 1000 person-years)	10·1	2·2	17·0	10·6	78·8
Age-adjusted and sex-adjusted hazard ratio (95% CI)	..	1·00	6·77 (4·73–9·7)	4·83 (2·85–8·17)	33·8 (23·8–47·9)
Multivariate hazard ratio (95% CI)	..	1·00	6·00 (4·16–8·6)	4·34 (2·56–7·35)	26·8 (18·7–38·4)
Incidence of diabetes by self-reported clinician diagnosis, HbA_{1c} ≥6·5% or FPG ≥7·0 mmol/L					
Incident cases/person-years	338/29 487	46/19 961	108/5920	30/1965	154/1641
Incident rate (per 1000 person-years)	11·5	2·3	18·2	15·3	93·8
Age-adjusted and sex-adjusted hazard ratio (95% CI)	..	1·00	6·86 (4·84–9·71)	6·53 (4·10–10·4)	38·6 (27·6–54·0)
Multivariate hazard ratio (95% CI)	..	1·00	6·16 (4·33–8·77)	6·00 (3·76–9·56)	31·9 (22·6–45·0)

Normoglycaemia was defined as HbA_{1c} lower than 5·7% and FPG less than 5·6 mmol/L. Diagnosis of prediabetes was by IFG alone when HbA_{1c} less than 5·7% and FPG 5·6–6·9 mmol/L, by HbA_{1c} alone when HbA_{1c} 5·7–6·4% and FPG less than 5·6 mmol/L, and by both HbA_{1c} and IFG when HbA_{1c} 5·7–6·4% and FPG 5·6–6·9 mmol/L. Multivariate model was adjusted for age, sex, smoking habit (never/former/current), parental history of diabetes, body-mass index, hypertension (systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or treatment), HDL cholesterol, log-transformed triglycerides, and γ-glutamyltransferase. IFG=impaired fasting glucose. HbA_{1c}=glycated haemoglobin A_{1c}. FPG=fasting plasma glucose.

Table 5: Hazard ratios for development of type 2 diabetes according to baseline diagnosis of prediabetes

HbA_{1c}, incidence rates were 13% (n=663 individuals and 83 incident cases), 24% (n=221, 54 cases), and 64% (n=159, 101 cases), respectively (log-rank test, p<0·0001).

Results of Cox analysis showed that the HR for incident diabetes was similarly increased for individuals with prediabetes discordantly diagnosed by either HbA_{1c} or fasting glucose criteria alone (table 5). The age-adjusted and sex-adjusted HR was 6·86 (95% CI 4·84–9·71) in those diagnosed with prediabetes by impaired fasting glucose alone and 6·53 (4·10–10·4) for those diagnosed with prediabetes on the basis of HbA_{1c} alone compared with normoglycaemic individuals. However, prediabetic individuals who fulfilled both criteria had a substantially increased risk, with an HR of 38·6 (95% CI 27·6–54·0). Although further adjustments for a parental history of diabetes, smoking habit (never, former, or current smokers), BMI, hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg, or both, or treatment), γ-glutamyltransferase, HDL cholesterol, and log-transformed triglycerides attenuated the HRs, the results were fundamentally the same.

Multivariate-adjusted HRs for impaired fasting glucose alone, HbA_{1c} 5·7–6·4% alone, and both HbA_{1c} and impaired fasting glucose were 6·16 (95% CI 4·33–8·77), 6·00 (3·76–9·56), and 31·9 (22·6–45·0), respectively. We noted similar associations irrespective of the diagnostic criteria for incident diabetes (table 5). The association of a diagnosis of prediabetes and risk of diabetes was fundamentally the same irrespective of the presence of anaemia, obesity, or becoming obese at follow-up (data not shown).

Discussion

This study showed that diagnosis of prediabetes by both new HbA_{1c} and fasting glucose criteria identified individuals in a Japanese population at a substantially increased risk for progression to diabetes within 5 years. Although the new HbA_{1c} criterion identified fewer participants at high risk than were identified by impaired fasting glucose, the predictive value for progression to diabetes assessed by HbA_{1c} 5·7–6·4% without impaired fasting glucose was similar to that assessed by fasting glucose alone. Our results suggest that introduction of the new HbA_{1c} criterion in addition to assessment of fasting plasma glucose could efficiently target patients who are most likely to progress to diabetes and allow for early intervention (panel).

A fifth of prediabetic individuals discordantly met the HbA_{1c} but not the impaired fasting glucose criterion for diagnosis in our study, which is in line with results of the National Health and Nutrition Examination Survey (NHANES) in US adults, which showed a rate of 17·2%.[2] Conversely, with respect to screening accuracy, the NHANES had a sensitivity of 27% and specificity of 93%, and showed that 61% of individuals with prediabetes diagnosed by HbA_{1c} also had impaired fasting glucose. Our results in a Japanese population showed lower sensitivity (21%) and specificity (91%), and only 50% of those with prediabetes diagnosed by HbA_{1c} also had impaired fasting glucose. Evidence has suggested that screening performance using HbA_{1c} values might differ according to ethnic origin.[4,5,15,16] Since only half of the prediabetic individuals in our study who met the new HbA_{1c} criterion also had impaired fasting glucose, a discrepancy in the degree of overlap might be more likely in Japanese than in US adults. A recent report of the New Hoorn Study[17] indicated that correlations between glucose and HbA_{1c} were moderate in the general population, although a strong correlation was seen in patients with known diabetes; therefore, the discrepancy in the degree of overlap might be higher for diagnosis of prediabetes than for diagnosis of type 2 diabetes. Other results of cross-sectional studies in US populations and Asian Indians[2–4,6] also reported a limited performance of the new HbA_{1c} criterion for identification of individuals at increased risk of diabetes compared with screening by impaired fasting glucose or impaired glucose tolerance, although none of these studies prospectively investigated the subsequent risk of progression to diabetes.

In our study, diagnosis of prediabetes on the basis of both criteria was strongly predictive of the risk of future diabetes. The increased HR shown by the overlap between the two prediabetic criteria might suggest that the predictive power achieved through use of the two criteria was multiplied rather than having an additive effect. Additionally, this association was noted irrespective of the diagnostic criteria for incident diabetes. Two cohort studies offered prospective data for new criteria for diagnosis of prediabetes including HbA_{1c} testing and the subsequent risk of diabetes.[7,8] The Atherosclerosis Risk in Communities (ARIC) study, which investigated the 10-year risk of diagnosed diabetes, reported that the 10-year cumulative incidence of diabetes was 10% for FPG lower than 5·6 mmol/L and HbA_{1c} 5·7–6·5%, 7% for FPG 5·6–7·0 mmol/L and HbA_{1c} less than 5·7%, and 23% for FPG 5·6–7·0 mmol/L and HbA_{1c} 5·7–6·5%, suggesting a dual role for HbA_{1c} and glucose in prediction of diabetes.[8] In Cederberg and colleagues' study,[7] which followed up 553 Finnish adults, 66% of incident cases of diabetes at 10 years were predicted by either HbA_{1c} 5·7–6·4%, impaired fasting glucose, or impaired glucose tolerance, and a raised HbA_{1c} 5·7–6·4% preceded diabetes in 33% of incident cases. Results of our study with a shorter follow-up showed that 86% of incident cases were predicted by either glucose tolerance or HbA_{1c} 5·7–6·4%, and that in 54% of incident cases a range of HbA_{1c} of 5·7–6·4% was seen at the baseline examination.

Two meta-analyses showed that the risk of development of diabetes established by HbA_{1c} was similar to that described for FPG and 2-h glucose.[12,13] Gerstein and colleagues[12] reported that dysglycaemic individuals were at about a five-to-ten times increased risk of diabetes compared with individuals without impaired fasting

glucose or impaired glucose tolerance. In a systematic review,[13] raised HbA_{1c} in the 5·0–6·5% range steeply increased the risk of diabetes and HbA_{1c} 5·5–6·5% was associated with a substantially increased risk of future diabetes. By contrast with FPG, which reflects acute dysglycaemia, HbA_{1c} reflects chronic hyperglycaemia, including postprandial glucose spikes.[1] The pathological disturbances of hepatic insulin resistance and reduced insulin secretion (first-phase and early-phase) led to isolated impaired fasting glucose.[18] In isolated impaired glucose tolerance, a severe deficit in late insulin secretion with muscle and hepatic insulin resistance leads to extended hyperglycaemia after a carbohydrate load.[18] Therefore, overlap with criteria for HbA_{1c} and impaired fasting glucose might allow for assessment of a substantially increased risk of diabetes.

Although we cannot establish the underlying mechanism in this observational study, we noted that prediabetic individuals discordantly diagnosed by either impaired fasting glucose or HbA_{1c} alone had significantly different cardiovascular risk profiles at the baseline diagnosis of prediabetes. The prevalence of obesity (BMI ≥25·0 kg/m²) was lower in individuals with prediabetes diagnosed by HbA_{1c} alone compared with those diagnosed by impaired fasting glucose alone in this study. These different factors[14] might affect progression to diabetes within each case of discordantly diagnosed prediabetes. However, the results were fundamentally the same after adjustments in multiple regression models. Data from an epidemiological study on the insulin resistance syndrome showed that HbA_{1c} predicted diabetes only in individuals with impaired fasting glucose.[19] On the other hand, the ARIC study reported that HbA_{1c} values were associated with a risk of diabetes irrespective of baseline FPG.[8,20] In this study, even without impaired fasting glucose, the new HbA_{1c} 5·7–6·4% criterion could be predictive of progression to diabetes, which was previously missed by screening for impaired fasting glucose.

In a more recent British prospective cohort study,[21] only 36% of incident cases of diabetes (defined as self-reported or HbA_{1c} ≥6·5%, or both) arose from the 6% of the total study population with baseline HbA_{1c} 6·0–6·4%, which suggests that most incident cases of diabetes occurred in those with baseline HbA_{1c} lower than 6·0%. Although we used a different threshold for HbA_{1c}, 46% of incident cases were without raised HbA_{1c} at baseline. Efficacy of the new HbA_{1c} 5·7–6·4% criterion will improve as a screening test and will be more predictive when used with impaired fasting glucose criterion for identification of individuals at substantially increased risk of developing diabetes. Nonetheless, the prediabetic state only indicates an individual's glycaemic condition at a single point in time of the natural history of development of diabetes. An analysis from the Whitehall II study[22] showed that individuals who developed diabetes had raised FPG as early as 13 years before diagnosis of diabetes compared with those who did not develop diabetes. Further studies will be needed to investigate the long-term trajectories of clinical markers, including HbA_{1c}, before the diagnosis of diabetes, especially in non-western populations.

Although the HbA_{1c} test has been standardised nationwide in Japan, there might be concerns about its cost and lack of standardisation in some countries. Investigations into how the predictive power would be improved by inclusion of simple risk factors for diabetes such as family history, obesity, or smoking habits and the derivation of algorithms would be of great importance. Also, whether introduction of HbA_{1c} into glucose testing is appropriate for detection of not only future diabetes but also cardiovascular disease events and mortality should be studied in various ethnic groups.

Strengths of our study include the assessment of a large sample size with annual data for HbA_{1c} and FPG and the investigation of a major topic in relation to the Asian population, in view of the increasing prevalence of prediabetes and diabetes.[23,24] Our findings through introduction of HbA_{1c} criteria in addition to impaired fasting glucose for screening of prediabetic individuals

Panel: **Research in context**

Systematic review

We searched PubMed for studies published in English from March 1, 1996, to Dec 31, 2010, that assessed the effect of introduction of the new glycated haemoglobin A_{1c} (HbA_{1c}) 5·7–6·4% criterion into screening of prediabetes and those that assessed the risk of future diabetes according to a baseline diagnosis by raised HbA_{1c}, impaired fasting glucose concentrations, or impaired glucose tolerance. Search terms used were "glycated hemoglobin", "HbA_{1c}", "A_{1c}", "impaired fasting glucose", "impaired glucose tolerance", "glucose intolerance", "prediabetes", or "pre-diabetes". A meta-analysis[12] reported that dysglycaemic individuals were at a roughly five-to-ten times increased risk of diabetes compared with individuals without impaired fasting glucose or impaired glucose tolerance. A recent systematic review[13] reported that HbA_{1c} 5·5–6·5% was associated with a substantially increased risk of future diabetes. Recent cross-sectional studies[2–5] reported that use of the HbA_{1c} 5·7–6·4% criterion for screening reclassified a large number of individuals previously diagnosed as having prediabetes on the basis of impaired fasting glucose, and that HbA_{1c} alone might miss prediabetes in many individuals. Two longitudinal studies[7,8] compared the risk of progression to diabetes according to a baseline diagnosis of prediabetes by the new HbA_{1c} 5·7–6·4% criterion and the impaired fasting glucose criterion.

Interpretation

Our study provides data for a cross-sectional comparison of the new HbA_{1c} criterion for prediabetes and impaired fasting glucose as predictors for progression to diabetes. We found that diagnosis of prediabetes by both the HbA_{1c} criterion and impaired fasting glucose identified individuals at a substantially increased risk for progression to diabetes more effectively than the use of only one of these indicators. Even though the new HbA_{1c} criterion identified fewer individuals at high risk than did impaired fasting glucose, the predictive value for progression to diabetes assessed by HbA_{1c} 5·7–6·4% without impaired fasting glucose was similar to that assessed by fasting glucose alone. Previously, there has been little clarification of the characteristics of people who progress to diabetes in a large population study,[14] especially related to the performance of screening with the proposed new HbA_{1c} criterion. Our results could contribute to targeting of people most likely to progress to diabetes and efficiently allow for early intervention.

contributed to identification of different groups of people who subsequently progressed to diabetes who would otherwise have been missed. These results have the potential to reduce the incidence of diabetes in the future by allowing such individuals to undertake strategies to prevent progression to diabetes. Our study also includes a systematic assessment of the incidence of diabetes with either FPG or HbA_{1c} alone and both FPG and HbA_{1c} diagnostic criteria for diabetes. Results suggested that use of both criteria could detect many individuals in need of early clinical care, in line with a recent study showing that use of either HbA_{1c} or FPG alone identified only 63% of incident cases of diabetes defined by a combination of FPG and HbA_{1c} criteria.[25]

Several limitations should be considered. First, data for the oral glucose tolerance test were not available for our study, and whether these data could have changed our results should be considered. Since we might have included individuals with postprandial hyperglycaemia in the category of HbA_{1c} 5·7–6·4% alone, the risk of development of diabetes for this group of patients might be overestimated by comparison with investigations using data for oral glucose tolerance. Second, we cannot deny the possibility of selection bias due to study participants being those who underwent routine medical checkups, and thus who paid more attention to health issues than those who did not. All participants might not have been properly fasting, although the prevalence of diabetes, obesity, and smoking were similar to those in general population-based studies in Japan.[26] Since we assessed the development of diabetes yearly, the incidence might be overestimated, although we noted that the incident rate of diabetes was similar to that of general Japanese populations.[27,28] We also need to consider that there could be many confounding factors such as differences in dietary habit of the participants for which we were not able to fully adjust. Additionally, the effect of factors that might change HbA_{1c} levels independently of glycaemia, such as anaemia or haemoglobinopathies, should be considered. Although the prevalence of haemoglobinopathies is reported to be very low in Japan (0·04%),[29] concern should be noted in some areas of southeast Asia that have high rates of haemoglobinopathies.[30]

In conclusion, diagnosis of prediabetes by introduction of the new HbA_{1c} criterion in addition to assessment of impaired fasting glucose could identify prediabetic individuals with a substantially increased risk of progression to diabetes. Although the new HbA_{1c} criterion identified fewer individuals at high risk than did impaired fasting glucose, the predictive value for progression to diabetes assessed by the HbA_{1c} criterion alone was similar to that assessed by impaired fasting glucose alone. The two tests used together could target individuals most likely to progress to diabetes and allow for early intervention.

Contributors

YH contributed to the study concept and design, acquisition of data, analysis and interpretation of data, drafting and critical revision of the manuscript, statistical analysis, and study supervision. SH, YA, SDH, HT, and YM contributed to the study concept and design, acquisition of data, drafting and critical revision of the manuscript, and technical or material support. KS, KF, and SK contributed to the study concept and design, acquisition of data, analysis and interpretation of data, drafting and critical revision of the manuscript, and statistical analysis. HSh contributed to study concept and design and critical revision of the manuscript. NY contributed to study concept and design, acquisition of data, and critical revision of the manuscript. KK contributed to the study concept and design and acquisition of data. HSo contributed to study concept and design, acquisition of data, analysis and interpretation of data, drafting and critical revision of the manuscript, statistical analysis, funding, and study supervision. All authors were involved in the writing of the manuscript and approved the final version of this article.

Conflicts of interest

We declare that we have no conflicts of interest.

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Establishment of chemiluminescence enzyme immunoassay for apolipoprotein B-48 and its clinical applications for evaluation of impaired chylomicron remnant metabolism

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ABSTRACT

Background: Apolipoprotein B-48 (apoB-48) is a constituent of chylomicron remnants synthesized in the small intestines. The serum concentration of apoB-48 at fasting has been reported to be a marker of postprandial hyperlipidemia, a presumed risk factor for atherosclerosis.
Methods: We evaluated the basal performance of a recently developed chemiluminescent enzyme immunoassay (CLEIA). We also examined the correlations between serum apoB-48 concentrations and other lipid concentrations or life style patterns, including smoking and drinking. We analyzed the data of 273 clinical samples by multiple regression analysis to examine the influence of other serum lipid values, age, sex, smoking, drinking status and BMI on serum apoB-48 values.
Results: Within-run and between-run precision was obtained with 1.7–2.7% and 1.2–7.3%, respectively. The correlativity of enzyme-linked immunosorbent assay was correlation coefficient $r = 0.953$, and regression $y = 1.02 \times -1.59$. Serum apoB-48 concentrations were higher in males than in females, and were correlated with the status of smoking as well as with remnant-like particle-cholesterol (RLP-C) concentrations. Patients with the metabolic syndrome showed higher values of serum apoB-48 compared with control subjects.
Conclusion: Serum apoB-48 measurement by CLEIA was satisfactory for clinical use to assess abnormalities in the chylomicron remnant metabolism.

1. Introduction

In the postprandial period, we observe an increase of serum triglycerides (TG), which are mainly transported by chylomicrons (CMs) and their remnants (CM remnants). Recently, postprandial hyperlipidemia has been considered as an independent determinant of cardiovascular diseases [1,2]. Dietary fats are absorbed by the small intestines and transported as chylomicrons (CMs), which are macromolecules synthesized exclusively by the small intestines. After the excretion of CMs into the intestinal lymph and their entrance into the systemic circulation, the TG moiety of CMs is promptly hydrolyzed by lipoprotein lipase (LPL), resulting in the production of CM remnants. Thereafter, CM remnants are promptly taken up by the liver via CM-remnant receptors.

Postprandial hyperlipidemia is a state characterized by the impaired catabolism of exogenous triglyceride-rich lipoproteins (TRL), in which the number of CMs and CM remnants is increased. However, no method has so far been developed to quantitatively and accurately measure the serum concentrations of CMs and CM remnants. CMs and CM remnants have a characteristic apolipoprotein B48 (apoB-48), each with one apoB-48 molecule per particle. In contrast, very-low-density lipoproteins (VLDL) and their remnants (intermediate-density-lipoproteins,

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IDL, or VLDL remnants) contain one apolipoprotein B-100 (apoB-100) molecule per particle. CM remnants contain apoB-48, but not apoB-100 [3]. CM remnants are taken up by monocyte-derived macrophages leading to the foam cell formation. Therefore, it is assumed that the measurement of serum apoB-48 concentration can help evaluate the synthesis and metabolism of CM remnants [4]. We have established an enzyme-linked immunosorbent assay (ELISA) to measure serum apoB-48 concentrations [5]. Thus, it has recently become possible to conveniently measure serum apoB-48 concentrations, thereby estimating the number of CMs and CM-remnant particles [4,5].

2. Materials and methods

2.1. Analysis equipment and reagents

We evaluated the basal performance of a recently developed CLEIA for apoB-48 measurement kit (Fujirebio Inc., Tokyo, Japan), carried out on the Lumipulse *f* fully automated immunoassay analyzer (Fujirebio). An in-house ELIS [4,5] provided by Fujirebio was used to measure serum apoB-48 concentrations and confirm the correlativity. Choletest CHO (Sekisui Medical Ltd., Tokyo, Japan) was used for the measurement of total cholesterol (T-CHO); Choletest TG (Sekisui) for triglycerides; Choletest LDL (Sekisui) for LDL-cholesterol; Choletest N HDL (Sekisui) for HDL-cholesterol; and Metabolead RemL-C (Kyowa Medex Co, Tokyo, Japan) for remnant lipoprotein cholesterol, respectively. TBA-200FRneo fully automated chemical analyzer (Toshiba Ltd., Tokyo, Japan) was used for automated measurements.

2.2. Principle of measurement of serum ApoB-48 concentrations

Serum samples were incubated with a treatment buffer solution supplemented with surfactants for separation of apoB-48 from CMs and CM remnants. The pre-treated samples were incubated with ferrite particles coupled with murine monoclonal antibody against apoB-48 in a solid phase. After incubation for 10 min at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase-conjugated anti-apoB-48 monoclonal antibody as a second antibody. After washing, AMPPD [3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) as a substrate was added to the test cartridge, and further incubation was performed for 5 min at 37 °C. Relative chemiluminescent intensity was measured and serum apoB-48 concentration was calculated by a standard curve.

2.3. Samples

We analyzed the data of 6 patients from Osaka University Hospital and 273 clinical samples from Health Care Center Osaka University and Minami-Osaka Total Health Screening Center by multiple regression analysis to examine the influence of other serum lipid values, as well as age, sex, smoking, drinking status and body mass index (BMI) on serum apoB-48 values. The experimental protocol was approved by the institutional ethics committee of the Osaka University Hospital and informed consent was obtained from all patients and healthy volunteers.

2.4. Storage of serum samples

Specimen preservation at room temperature, 4 °C, and −20 °C was investigated in sample sera from 6 control subjects. The preservation period for serum was for 1 week, 2 weeks, and 4 weeks, and collection day, respectively.

3. Methods

3.1. Precision

Within-run and between-run imprecision was evaluated with three concentrations of quality control (QC) material. Ten aliquots were analyzed in one analytical run. Briefly, three concentrations of QC material and three concentrations of human serum were prepared as individual pools. All samples were immediately stored at −80 °C. Duplicate apoB-48 analyses were performed on each pool in two separate runs per day for 10 days. Precision was evaluated as the coefficient of variation calculated from the date series mean and standard deviation.

3.2. Limit of blank and limit of detection

The limit of blank (LoB) and limit of detection (LoD) were determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) EP17-A requirement [6]. The LoD represents the 95th percentile value from measurements of analyte-free samples over several independent series.

3.3. Linearity

We assessed the dilution linearity by serial dilution of 3 human serum samples spiked with recombinant apoB-48 to concentrations covering the whole measuring range. Each sample was diluted 1:4 with analyte-free human serum as diluents in 5 consecutive steps. We measured all dilutions in duplicate with various assay applications and calculated linearity separately for each assay application and instrument.

3.4. Interference studies

The CLEIA for measuring serum apoB-48 concentration was evaluated for common interferences including those due to hyperlipidemia, hemolysis and bilirubinemia by using Interference Check A Plus (Sysmex Co, Hyogo, Japan). Each interference material was evaluated by supplementation of human serum with the indicated to create a high or low interference pool followed by serial dilution with the high to the low pool to create a dilution series.

3.5. Statistical analysis

Data were analyzed by using Stat Flex software (Ver.5.0, Artec Inc., Osaka, Japan), and Mann–Whitney test and two-way ANOVA were used to evaluate the between-group differences. A $p<0.05$ was considered statistically significant.

4. Results

4.1. Imprecision

Imprecision was evaluated as the coefficient of variation (CV%). Within-run and between-run variations were examined using 3 QC materials and 3 kinds of patient pool sera with different concentrations of serum apoB-48 (Low,Middle,High). The largest within-run CV% observed was 2.7% (apoB-48 = 29.0 μg/ml). The largest between-run CV% observed was 7.3% (apoB-48 = 3.2 μg/ml). ApoB-48 imprecision for all samples are summarized in Supplementary Table 1.

4.2. Limit of blank and limit of detection

LoB and LoD for the apoB-48 assay were determined to be 0.06 μg/ml and 0.125 μg/ml, respectively. Linearity was documented by dilution up

Table 1
Multiple regression analysis between serum ApoB-48 level and other lipids or life style (drinking, smoking and BMI).

Characteristics	Regression coefficient	SE	P value
Sex	0.787	0.248	<0.01
Age	−0.008	0.016	0.63
RLP-C	0.253	0.029	<0.01
TG	0.013	0.016	0.46
T-CHO	0.009	0.009	0.11
LDL-C	−0.013	0.009	0.11
HDL-C	−0.013	0.022	0.56
Drinking	0.014	0.013	0.92
Smoking	0.306	0.122	<0.01
BMI	-0.034	0.032	0.29

Male = 1, Female = 0; Yes = 1, No = 0 for drinking and smoking.

to 30.0 μg/ml (Fig. 1). Thus, the analytical measurement range was 0.125–30.0 μg/ml.

4.3. Recovery

ApoB-48 recovery was 96.3–103.5% after 1:9 dilution steps of 3 human serum samples spiked with recombinant apoB-48 to concentrations of approximately 15.1 and 39.9 μg/ml. Identical values were obtained for all assay applications and instruments.

4.4. Interferences

Interference was defined as a >10% change in apoB-48 concentration in the presence of supplemented interference compared to the apoB-48 concentration in the absence of supplemented interference. The acceptable interference concentrations were observed at 19.4 mg/dl (bilirubin F), 20.9 mg/dl (bilirubin C), 523 mg/dl (hemoglobin). These data are demonstrated in Supplementary Fig. 2a, b and c.

4.5. Correlation with ELISA

Fig. 2(a) shows the correlation of the apoB-48 data between CLEIA and ELISA. Correlation of the test results by the ELISA (x) with those by CLEIA (y) for serum samples gave the following regression equation for apoB-48: $y = 1.02 \times -1.59$, $r = 0.953$ ($p < 0.0001$). Fig. 2 (b) shows the Bland–Altman plot which demonstrates a slight overall bias of 0.3% for the CLEIA compared with the ELISA. The 95% confidence interval was from −0.2% to 0.8%.

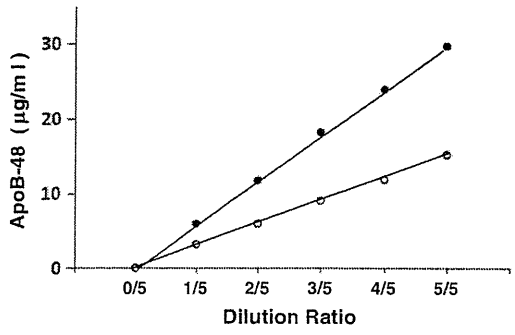

Fig. 1. Linearity of CLEIA for measuring serum ApoB-48. We used the dilution buffer as the blank. CLEIA was performed in duplicate measurements. Two serum samples with a high concentration of apoB-48 (30.0 μg/ml) and a low concentration of apoB-48 (20.6 μg/ml), respectively, were progressively diluted with a dilution buffer, and then assayed.

4.6. Storage of serum samples

Specimen preservation at room temperature, 4 °C, and −20 °C was investigated in sample sera from 6 control subjects. The preservation period for serum was for 1 week, 2 weeks, and 4 weeks, and collection day, respectively.

As shown in Fig. 3a, the apoB-48 concentrations of sample sera kept at room temperature for 2 weeks decreased gradually. The stability of samples was kept for at least 2 weeks at 4 °C (Fig. 3b). The apoB-48 concentration of one sample had decreased at 4 weeks from the beginning. The samples frozen at −20 °C remained stable for at least 4 weeks (Fig. 3c). Thus, it was preferable to keep the samples cold after blood drawing and to freeze them below −20 °C for prolonged storage.

4.7. Healthy reference value of serum ApoB-48

As shown in Fig. 4, the healthy reference value was confirmed for 273 clinical samples from Health Care Center Osaka University and Minami-Osaka Total Health Screening Center. Serum apoB48 concentration distributed log-normally, and the reference values were set at the 95th percentile of the distribution of serum apoB-48. The reference interval of apoB-48 was 0.8–9.7 μg/ml (median: 2.8 μg/ml) in the total subjects. In addition, the reference interval was 1.0–10.3 μg/ml (median: 3.3 μg/ml) for males and 0.7–5.6 μg/ml (median: 2.2 μg/ml) for females, respectively. Therefore, a significant gender difference was observed in the serum apoB-48 concentrations ($P < 0.01$).

4.8. Correlations of lipid values and lifestyle factor

We analyzed the data of 273 clinical samples from Health Care Center Osaka University and Minami-Osaka Total Health Screening Center by multiple regression analysis to examine the influence of other serum lipid values or Life Style (drinking, smoking and BMI). Serum apoB-48 concentrations were correlated with gender and RemL-C concentrations ($P < 0.01$). Moreover, the correlativity was admitted by smoking in the lifestyle as show in Table 1. We compared the serum apoB-48 concentrations according to smoking status and gender. In males, the concentrations of serum apoB-48 of smoker subjects was 1.0–10.8 μg/ml (median: 3.8 μg/ml), that of ex-smokers was 1.0–15.6 μg/ml (median: 2.8 μg/ml), and that of nonsmokers was 0.9–7.1 μg/ml (median: 2.8 μg/ml). The P value of difference by Mann–Whitney test between smokers and ex-smokers, smokers and nonsmokers, ex-smokers and nonsmokers was 0.006, 0.14, and 0.95, respectively (Fig. 5). In contrast, in female subjects, the concentrations of serum apoB-48 were 1.6–6.6 μg/ml (median: 2.3 μg/ml) for smokers, 0.8–4.6 μg/ml (median: 2.1 μg/ml) for ex-smokers, and 0.6–4.6 μg/ml (median: 2.1 μg/ml) for nonsmokers, respectively. The P value of difference by Mann–Whitney test between smokers and ex-smokers, smokers and nonsmokers, ex-smokers and nonsmokers was 0.11, 0.29, and 0.43, respectively. No significant differences in serum apoB-48 concentrations were observed in females according to the smoking status.

4.9. Serum ApoB-48 concentrations in patients with metabolic syndrome

In Japan, the patients with the metabolic syndrome (Mets) can be identified as increased waist circumference at the umbilicus concentration (≥85 cm in men and ≥90 cm in women, respectively), and the presence of 2 or more of the following components: 1) systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg, 2) fasting serum TG concentrations ≥150 mg/dl and/or serum HDL-C< 40 mg/dl, 3) fasting glucose ≥110 mg/dl [7]. Pre-Mets subjects are identified as having increased waist circumference and one Mets component. Among 211 patients who underwent medical examinations for Mets, 62 were classified as having Mets or pre-Mets. The apoB-48 concentration of these 62 Mets and pre-Mets subjects was higher than

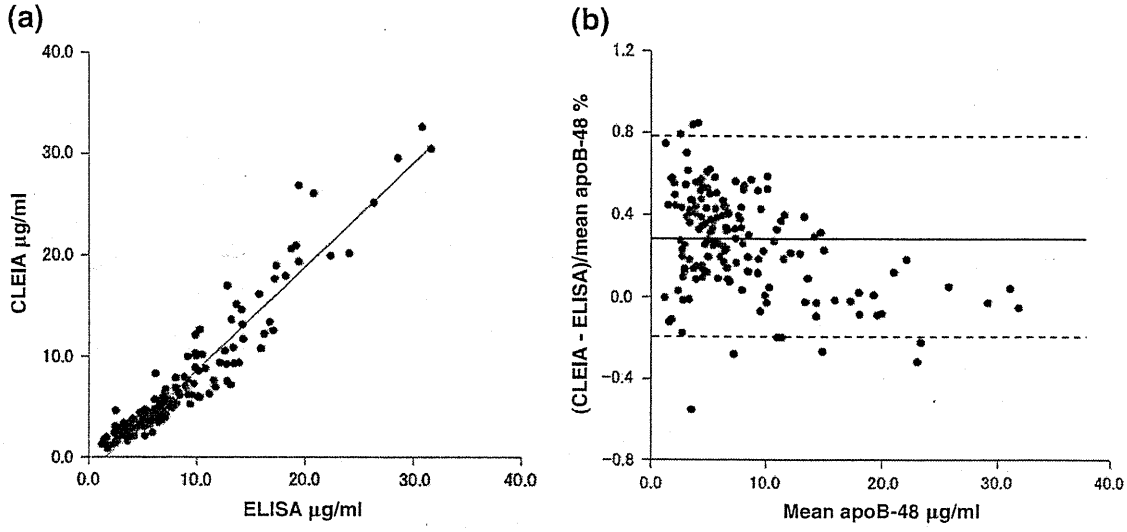

Fig. 2. Correlation of serum ApoB-48 levels determined by ELISA and CLEIA. (a) The serum apoB-48 levels determined by either ELISA or CLEIA in 159 serum samples were well correlated with a correlation coefficient r = 0.953 (y = 1.02× − 1.59). (b) Bland-Altman analysis showing overall bias of 0.3% for the CLEIA compared with the ELISA. The dashed lines represent 95% limit of agreement.

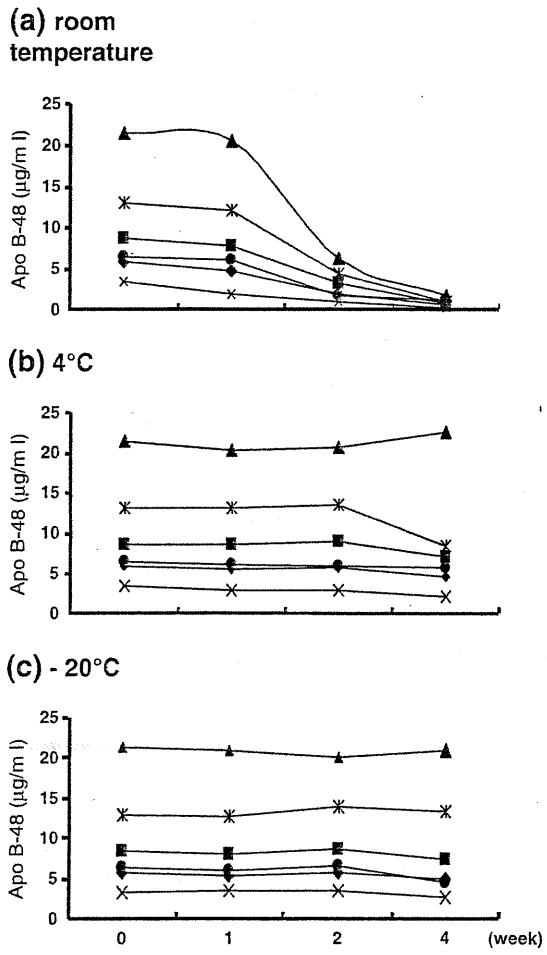

Fig. 3. Effects of storage conditions on serum ApoB-48 concentrations. (a) Samples were stored for indicated number of weeks at room temperature until analysis. (b) Samples were stored for indicated number of weeks in a refrigerator at 4 °C until analysis. (c) Samples were stored for indicated number of weeks in freezer at −20 °C until analysis.

that of non-Mets subjects. The apoB-48 values of Mets and pre-Mets subjects were 1.2–10.2 μg/ml (median: 3.1) while those of non-Mets subjects were 0.8–8.2 μg/ml (median: 2.8), thereby revealing a significant difference (P = 0.01) (Fig. 6).

5. Discussion

We previously established an ELISA to measure serum apoB-48 concentrations, using a microplate assay [4,5]. By using this ELISA, we have

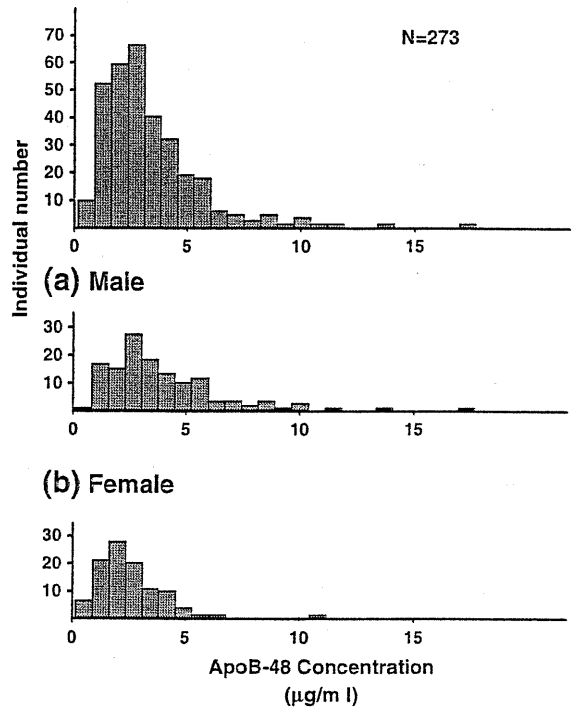

Fig. 4. Histogram of serum ApoB-48 concentration in males and females. (a) Histogram of serum apoB-48 concentration in males (n = 115). (b) Histogram of serum apoB-48 concentration in females (n = 96).