Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

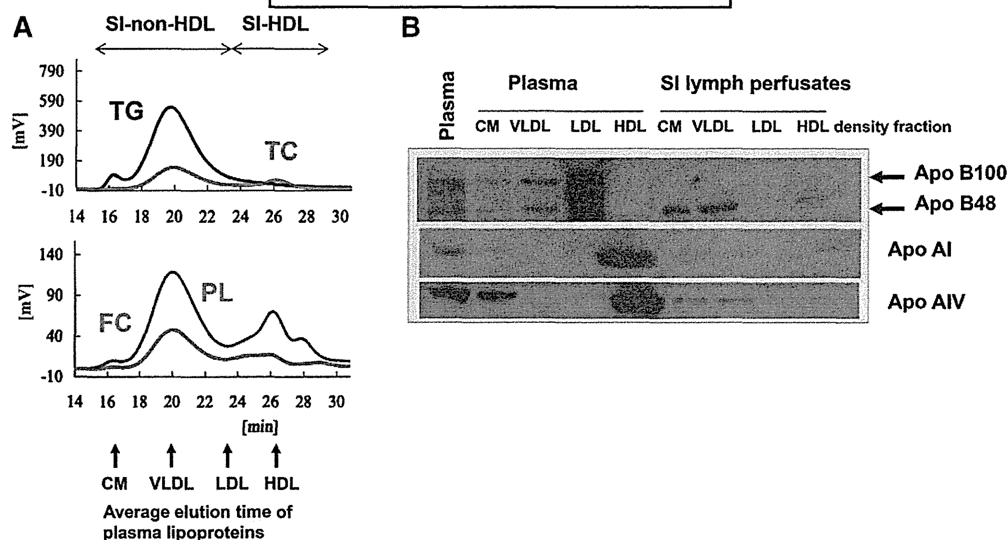


Fig. 3. HPLC analyses of lipids and preparative ultracentrifugation followed by Western blotting for apos in SI lymph perfusates. A: HPLC analyses of SI lymph perfusates from WT mice. Two hundred microliters of SI lymph perfusates was run on HPLC as described in the Methods. TC, FC, TG, and PL were measured enzymatically. Arrows denote the average elution time of indicated plasma lipoproteins in WT mice. B: Apo distribution among lipoproteins in SI lymph perfusates from WT mice separated by preparative ultracentrifugation. Lipoproteins in plasma and SI lymph perfusates were subjected to small-scale preparative ultracentrifugation to concentrate samples, and the concentrated samples were then run on SDS-PAGE followed by Western blot analysis using antibodies against the indicated apos. CM, VLDL, LDL, and HDL denote the density range of the indicated plasma lipoprotein fractions.

measurement of lipids to ensure that lipids in HDL are measured without possible interference from other lipoproteins. Supplementary Fig. II gives examples of the HPLC TC profile of HDL separated from plasma, lymph perfusates, and liver perfusates to show the purity of HDL. HPLC-separated HDL was measured for TC, TG, FC, and PL, and CE was calculated from TC and FC (47).

Table 1 and **Fig. 4A** show the contents of lipids and total protein in P-HDL, L-HDL, and SI-HDL. SI-HDL, similar to L-HDL, had a significantly higher protein content and lower lipid content than P-HDL (Table 1, Fig. 4A). CE and PL were the major lipids in SI-HDL and L-HDL, similar to P-HDL. However, the contents of CE and PL relative to protein in SI-HDL and L-HDL were significantly lower than those in P-LDL (Table 1).

Table 1 also shows the distribution of lipids in HDL. At ad libitum, SI-HDL tended to show less CE and more TG than P-HDL and L-HDL (Table 1). The distribution of CE and TG was significantly ($P < 0.05$) different between SI-HDL and L-HDL at ad libitum and between SI-HDL at ad libitum and at fasting, as assessed by a two-way ANOVA (data not shown). At fasting, TG was not detected in L-HDL but was detected in SI-HDL (Table 1).

Therefore, using our in situ perfusion model, we showed that SI-HDL was protein rich compared with HDL in plasma in WT mice and TG rich compared with L-HDL.

TABLE 1. Lipid and protein composition of HDL separated by ultracentrifugation from mouse plasma, liver perfusates, and SI lymph perfusates collected using an in situ perfusion model

	HDL in Plasma	HDL Produced from the Liver		HDL Produced from the SI	
	Ad libitum (n = 5)	Ad libitum (n = 5)	Fasting (n = 5)	Ad libitum (n = 5)	Fasting (n = 5)
HDL composition (% total mass)					
Protein	55.3 ± 2.1	83.8 ± 9.0[a]	83.3 ± 6.5[a]	86.0 ± 5.0[a]	88.8 ± 4.4[a]
Lipids	44.7 ± 2.1	16.2 ± 9.0[a]	16.7 ± 6.5[a]	14.0 ± 5.0[a]	11.2 ± 4.4[a]
FC	2.2 ± 0.5	1.0 ± 0.6[a]	1.3 ± 0.2[b]	1.3 ± 1.1	0.9 ± 0.7[a]
CE	17.9 ± 0.4	5.9 ± 2.9[a]	6.7 ± 2.9[a]	3.7 ± 2.2[a]	4.1 ± 1.8[a]
PL	24.1 ± 2.1	9.1 ± 5.5[a]	8.7 ± 3.7[a]	6.8 ± 2.2[a]	5.8 ± 3.6[a]
TG	0.5 ± 0.3	0.2 ± 0.4	0.0 ± 0.0[a]	2.3 ± 2.9	0.4 ± 0.9
HDL lipid composition (% of lipid mass)					
FC	5.0 ± 1.2	6.6 ± 6.1	8.5 ± 3.7	11.2 ± 13.4	7.0 ± 5.5
CE	40.1 ± 2.1	38.7 ± 9.8	39.3 ± 4.1	24.2 ± 14.0[a, c]	41.0 ± 18.4
PL	53.8 ± 2.3	54.2 ± 6.2	52.2 ± 2.5	50.2 ± 14.1	49.2 ± 17.9
TG	1.1 ± 0.6	0.6 ± 1.3	0.0 ± 0.0	14.3 ± 14.9[b, c]	2.8 ± 6.2

[a] $P < 0.05$, versus plasma, assessed by an ANOVA.
[b] $P < 0.1$, versus plasma, assessed by an ANOVA.
[c] $P < 0.1$, versus the liver, assessed by an ANOVA.

JOURNAL OF LIPID RESEARCH ASBMB

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

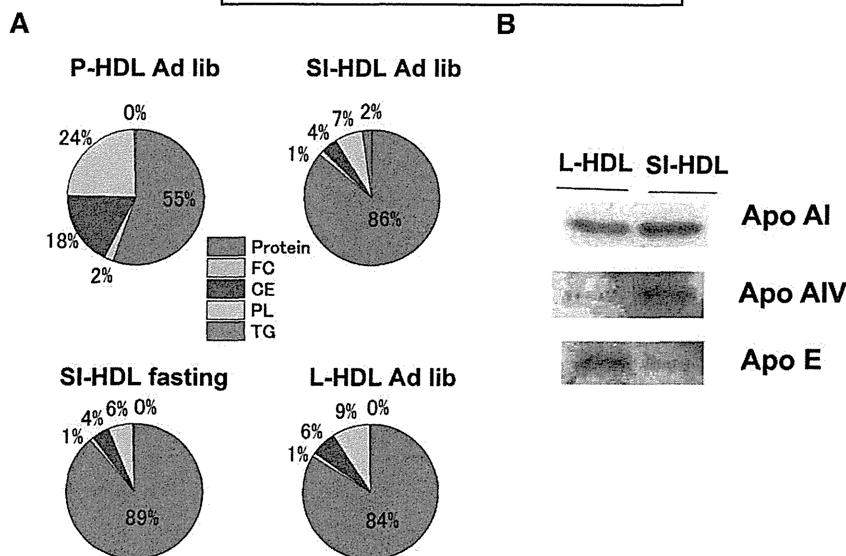


Fig. 4. Lipid and apo composition of HDL in SI lymph perfusates from WT mice. A: Lipid and protein composition of HDL in plasma (P-HDL), SI lymph perfusates (SI-HDL), and liver perfusates (L-HDL) separated by serial preparative ultracentrifugation. HDL was further separated by HPLC to determine its lipid composition. B: Comparison of apo compositions of SI-HDL and L-HDL. SI-HDL and L-HDL were obtained by in situ perfusion followed by serial preparative ultracentrifugation and subjected to SDS-PAGE followed by Western blot analysis using antibodies against the indicated apos.

Apo composition of SI-HDL

To characterize the apo composition of SI-HDL, L-HDL and SI-HDL separated by ultracentrifugation from liver perfusates and SI lymph perfusates, respectively, were run on SDS-PAGE and then subjected to Western blot analysis for apo AI, apo AIV, and apo E (Fig. 4B). As shown in Fig. 4B, L-HDL contained a very limited amount of apo AIV but a considerable amount of apo E, whereas an opposite trend was seen for SI-HDL. This result indicates that SI-HDL was rich in apo AIV compared with L-HDL, and SI-HDL is different from L-HDL with respect to the composition of apos.

Size distribution of SI-HDL

Because SI-HDL was protein rich compared with P-HDL (Table 1), we used EM to examine the size distribution of HDL separated using ultracentrifugation from SI lymph perfusates and plasma of WT mice (**Fig. 5**). Fig. 5A shows the representative negative-stain electron micrographs of SI-HDL and P-HDL. As shown, SI-HDL particles were a population of spheres with a very small number of discs. We measured the particle diameter of spherical particles in SI-HDL and P-HDL (Fig. 5B, C). Fig. 5B shows the size distribution of SI-HDL and P-HDL. As shown, SI-HDL particles, similar to P-HDL, were heterogeneous in size, but the distribution of SI-HDL particles was more diverse than that of P-HDL, and the size distributions of SI-HDL and P-HDL overlaid (Fig. 5B). However, SI-HDL apparently had a higher proportion of smaller particles as compared with P-HDL (Fig. 5B). Fig. 5C shows the individual data and the box plots of SI-HDL and P-HDL. As shown, although the size range of SI-HDL covered that of P-HDL, the peak diameter of SI-HDL particles shifted toward smaller particles as compared with that of P-HDL, and the average size of SI-HDL particles (mean ± SD: 11.06 ± 2.70 nm) was significantly ($P < 0.001$) smaller than that of P-HDL particles (12.94 ± 1.64 nm). This result indicates that the particle size of SI-HDL was smaller than that of P-HDL.

Inhibitors of ABCA1 and LCAT affect the formation of SI-HDL

Because we have shown that most of the HDL particles secreted from the SI are spherical using EM (Fig. 5A), to identify the mechanism for SI-HDL assembly, we examined the effects of inhibitors of ABCA1 and LCAT on the formation of SI-HDL. It is well known that ABCA1 lipidates apo AI to form HDL and LCAT converts lipid-poor pre-β-migrating HDL to mature α-migrating HDL. We used glyburide (26) and DTNB (27) as inhibitors of ABCA1 and LCAT, respectively (**Fig. 6A**). The effects of ABCA1 and LCAT inhibitors were examined by collecting SI lymph perfusates from WT mice that underwent in situ perfusion using buffers with and without the presence of the inhibitors (Fig. 6).

As shown in Fig. 6B, nondenaturing PAGE followed by Western blot analysis for apo AI showed that there was a marked increase in free apo AI and small HDL in SI lymph perfusates in the presence of glyburide compared with the absence of glyburide. This result suggests that ABCA1 is involved in the lipidation of apo AI to form SI-HDL.

As shown in Fig. 6C, two-dimensional electrophoresis of SI lymph perfusates followed by Western blotting for apo AI showed that the presence of premature HDL such as pre-β1- and pre-β2-HDL (41, 42) was observed in the presence of DTNB, but not in the absence of DTNB. A reduction in α-HDL particle size was also observed in the

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

ASBMB JOURNAL OF LIPID RESEARCH JLR

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

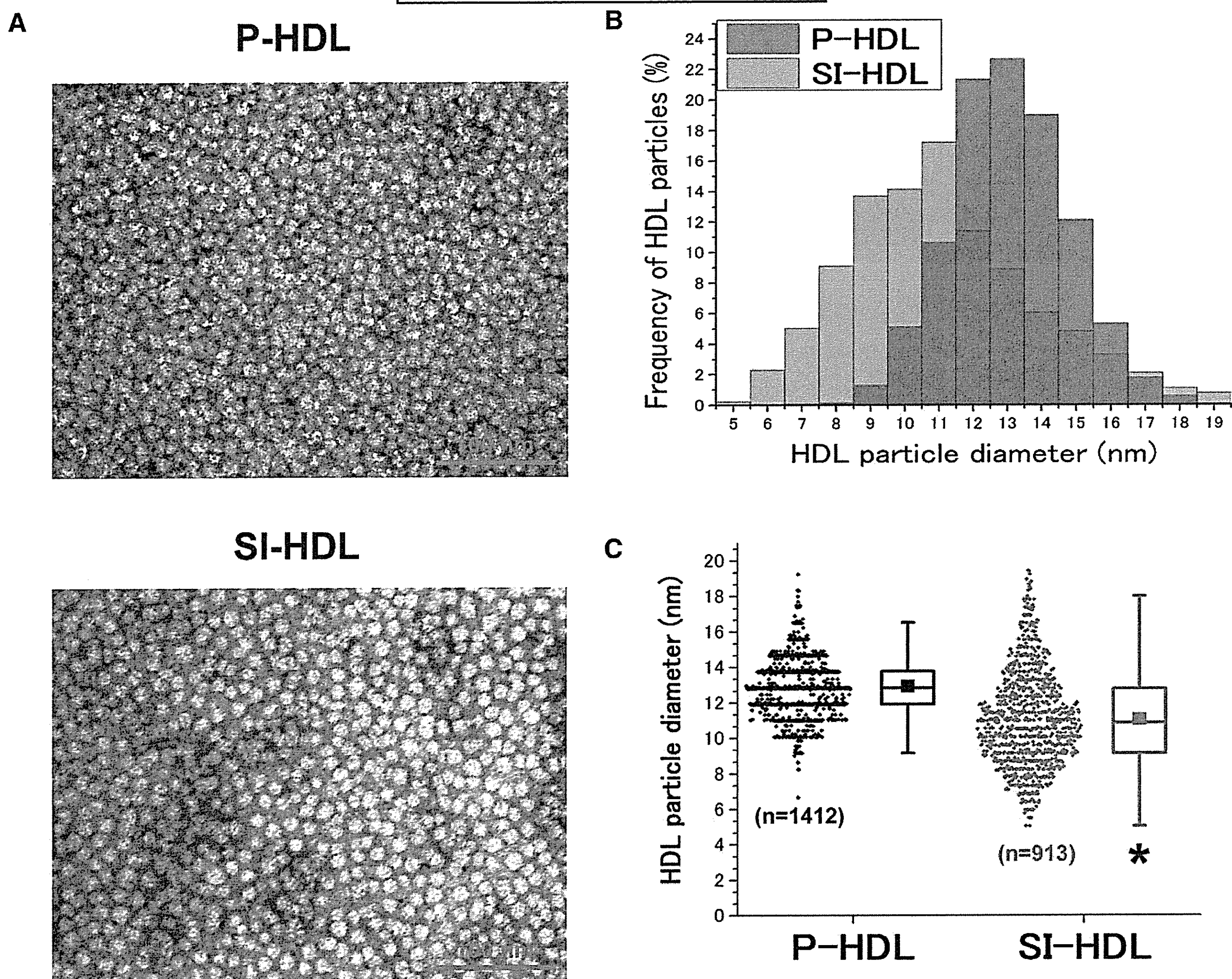


Fig. 5. Electron micrographs of negatively stained HDL from SI lymph perfusates. A: Representative negative-stain EM of HDL separated by serial ultracentrifugation from SI lymph perfusates (SI-HDL, lower panel) and plasma (P-HDL, upper panel). Magnification: 200,000×; scale bar: 100 nm. B: Size distribution of SI-HDL and P-HDL particles from negative-stain electron micrographs. The frequency distributions of the size of SI-HDL (pink bars; n = 913) and P-HDL (gray bars; n = 1,412) were plotted together, and the red bars represent the overlaid parts. Two measurements were made for the diameter of each HDL particle, and the mean diameter was used to calculate the size frequency. C: Box-and-whisker plots showing the mean (■), median (middle bar in the rectangle), and 10th (bottom bar), 25th (bottom of rectangle), 75th (top of rectangle), and 90th (top bar) percentiles of the sizes of SI-HDL (black) and P-HDL (red) particles. The individual data are shown on the left of the boxes. * $P < 0.001$, SI-HDL versus P-HDL, assessed by the Wilcoxon rank sum test.

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

presence of DTNB (Fig. 6C). This result suggests that LCAT may be involved in the maturation of SI-HDL.

Although our experiments were limited in that the inhibitory effects of ABCA1 and LCAT are unknown, our results suggest that ABCA1 and LCAT may play important parts in the formation of SI-HDL.

Nutritional regulation of the production of HDL from SI

A previous study showed that intestine significantly contributes to plasma HDL-C levels (44). We examined the effects of fasting and high-fat feeding on the production of HDL from SI in WT mice using our in situ perfusion model to clarify the nutritional regulation of SI-HDL. HDL in lymph perfusates collected under different nutritional conditions was analyzed by non-SDS-PAGE followed by Western blot analysis for apo AI. As shown in **Fig. 7A**, plasma HDL-C levels and the contents of HDL-apo AI in SI lymph perfusates from WT mice ad libitum were markedly reduced after 24 h fasting. In contrast, a 4-week high-fat diet markedly increased plasma HDL-C levels and the contents of HDL-apo AI in lymph perfusates from WT mice (Fig. 7B). These results indicate that fasting reduces and high-fat diet increases the production of HDL from the SI.

Apo E KO reduces the production of HDL from the SI

Apo E KO mice are characterized by a marked reduction of HDL-C levels in plasma. Because intestinal HDL has been shown to significantly contribute to plasma HDL (16), we used our in situ perfusion model to examine whether

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

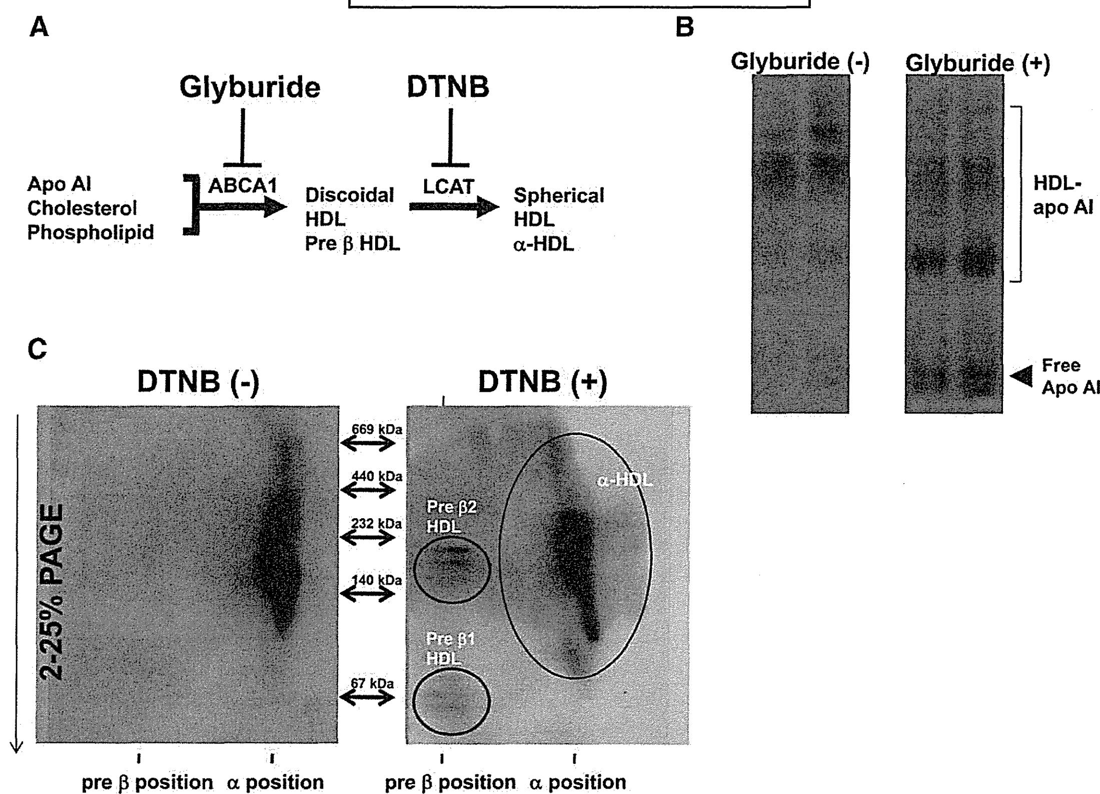


Fig. 6. Effects of glyburide and DTNB on the assembly of SI-HDL. A: Possible mechanism for the assembly of SI-HDL. Glyburide and DTNB are known inhibitors of ABCA1 and LCAT, respectively. B: Effect of glyburide on immunoblot patterns of HDL in SI lymph perfusates from WT mice. WT mice were subjected to in situ SI perfusion in the presence (right panel) and absence (left panel) of glyburide in the perfusion buffer. SI-HDL was run on non-SDS-PAGE followed by the detection of apo AI. Arrowhead represents free apo AI. C: Effect of DTNB on the formation of SI-HDL. SI lymph perfusates were obtained from WT mice that were perfused using a perfusion buffer in the presence (right panel) and absence (left panel) of DTNB in the perfusion buffer.

the production of HDL from the SI is reduced in apo E KO mice. As shown in Fig. 7C, apo E KO mice had markedly lower levels of HDL-C and reduced contents of HDL-apo AI in SI lymph perfusates as compared with WT mice. These results indicate that apo E may play a role in the biogenesis of SI-HDL.

DISCUSSION

To selectively evaluate HDL produced from the intestine, we developed an in situ perfusion model using surgically isolated mouse SI. Using our in situ perfusion model, we found that the SI produces HDL in mice and ABCA1 plays an important role in the production of SI-HDL, that SI-HDL is different from HDL produced by the liver, and that SI-HDL may be regulated by nutritional and genetic factors.

Our in situ perfusion model using surgically isolated mouse SI was developed for the selective evaluation of SI-HDL because HDL in mesenteric lymph collected from anesthetized mice originates either from the secretion by the SI or from the filtration from plasma through the blood capillary-lymph loop into the intestinal lymph duct (19–22).

Our novel in situ perfusion model achieves the selective evaluation of HDL by dissociating HDL production by the SI from the filtration of HDL from plasma. In this model, arterial blood supply for the SI is blocked by ligation of abdominal aorta and other arteries, leaving only the superior mesenteric artery open as the perfusion inlet (Fig. 1A). Perfusion buffer is pumped through a needle that is connected to a tube and inserted antegrade through the thoracic descending aorta into the abdominal aorta just before ligation of the abdominal aorta (Fig. 1A). Therefore, after perfusion starts, no additional systemic blood will enter the SI, and the possible filtration of plasma HDL from the systemic circulation into the SI lymph duct can be prevented. The SI lymph duct and portal vein are cannulated as outlets for perfusion buffer (Fig. 1A). Under these conditions, the HDL in the infusates collected from the SI lymph duct would originate only from the SI.

Using our in situ perfusion model, we found that HDL was detected in SI lymph perfusates from WT mice (Fig. 1B), indicating that the SI produces HDL. This finding supports the notion that the intestine, along with the liver, is an important site for the secretion of apo AI and the production of HDL (12–16). We did not detect HDL in SI lymph perfusates from ABCA1 mice (Fig. 1B), indicating that ABCA1 is essential for the production of HDL by the

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

JOURNAL OF LIPID RESEARCH ASBMB

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

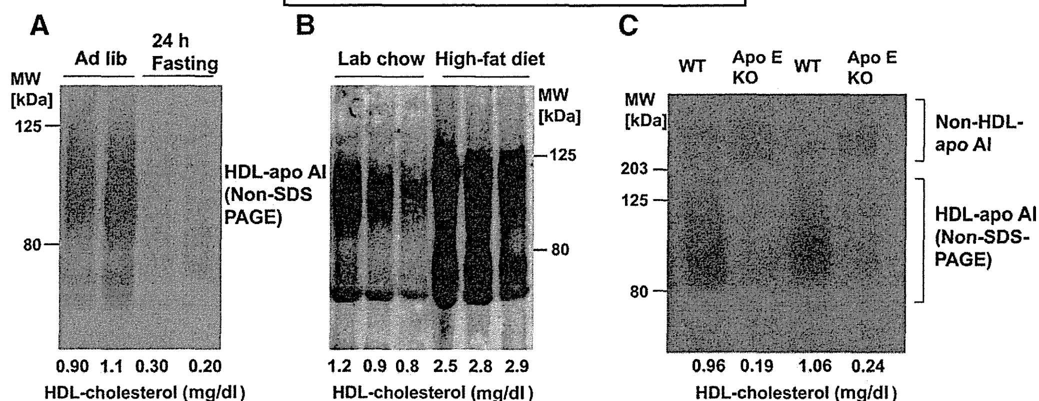


Fig. 7. Nutritional and genetic regulation of SI-HDL. The HDL-C concentration (mg/dl) in different perfusates was measured by HPLC as described in the Methods and is shown under each column. A: Effect of fasting on SI-HDL in WT mice. Mice were subjected to in situ perfusion at ad libitum and after 24 h of fasting. SI lymph perfusates were analyzed by using non-SDS-PAGE followed by Western blot analysis for apo AI. B: Effect of a high-fat diet on SI-HDL in WT mice. Mice were subjected to in situ SI perfusion after being fed a high-fat diet for 4 weeks. C: Comparison of SI-HDL production in 14-week-old WT and apo E KO mice.

SI. This finding supports those of Brunham et al. (16) that mice that specifically lack ABCA1 in the intestine had ~30% lower plasma HDL-C levels. However, we detected free apo AI in SI lymph perfusates from ABCA1 mice (Fig. 1B), indicating that cellular lipids are not available for the lipidation of apo AI to form SI-HDL in the absence of ABCA1 (45).

Because SI-HDL is not formed in the absence of ABCA1, we used ABCA1 KO mice to clarify whether plasma HDL can filtrate from the abdominal aorta through the SI into a lymph duct. We perfused the SI of ABCA1 KO mice using perfusion buffer to which had been added serum from WT mice and found that the collected SI lymph perfusates contained a substantial amount of HDL (Fig. 1C). It is possible that lipids in lipoproteins in the aortic perfusate may be delivered to the SI through transintestinal transport, that is, via the transintestinal cholesterol efflux pathway (48), and lipidate apo AI from intestinal secretion to form HDL. However, in the absence of ABCA1, which is located at the basolateral membrane of enterocytes (49), cellular lipids will not be available to free apo AI to form HDL. Therefore, HDL that was detected in SI lymph perfusates of ABCA1 mice perfused with serum from WT mice (Fig. 1C) can originate only from filtration of plasma HDL. Using our in situ perfusion model, we provided direct evidence that systemic plasma HDL can filtrate into the SI lymph duct.

One of the most likely mechanisms responsible for this perfusion is the "blood capillary-lymph loop" (50). Our finding that no apo B was present in SI lymph perfusates from ABCA1 KO mice that were perfused with buffer containing serum from WT mice indicated that HDL from the systemic circulation can, whereas apo-B-containing lipoproteins cannot, filter into the SI lymph duct (Fig. 1C), suggesting that bigger molecules cannot pass through the blood-capillary wall. Consistent with this finding, apo B48, but not apo B100, was detected in our analyses of apos in ultracentrifugation density fractions of lipoproteins in SI lymph perfusates from WT mice (Fig. 3B). Therefore, we consider the conventional cannulation experiment to be useful for the analysis of apo-B-containing lipoproteins because we used it to demonstrate an increase in the production of CMs in CD36 KO mice (51). However, for the selective evaluation of SI-HDL, the newly developed perfusion technique may be the best method available for eliminating the interference of plasma HDL.

Our finding that HDL was produced by the SI (Fig. 1A) but plasma HDL can also filtrate into the SI lymph duct (Fig. 1B) resolves controversies regarding intestinal-derived HDL (19–22). A previous study showed that HDL from the intestinal lymph duct obtained in vivo from anesthetized mice is likely to contain HDL from the systemic circulation, the majority of which is derived from the liver (16). Therefore, peptide mapping of HDL using LC/MS was performed to compare SI-HDL in SI lymph perfusates collected using our in situ perfusion model, C-HDL collected from the SI lymph duct in anesthetized mice, and L-HDL collected from liver perfusates (Fig. 2). C-HDL and L-HDL were very similar in that they had the same number of major peptides and relative peptide-ion intensities (Fig. 2C, D). This result agrees with the finding of Brunham et al. (16) that intestinal HDL in mice that lacked intestinal ABCA1 predominantly originates from the liver.

However, we found that SI-HDL in SI lymph perfusates was different from C-HDL and L-HDL in that SI-HDL contained additional major peptides (*m/z* 542 and *m/z* 524) that were not detected in L-HDL or C-HDL (Fig. 2). It is possible that the SI may secrete some known or unknown apos that are not secreted by the liver, and this proposition will need to be examined in future studies.

Our result that the two peptides (*m/z* 542 and *m/z* 524), which were detected as major peaks in SI-HDL obtained using our in situ perfusion model, were not detected in C-HDL obtained from the SI lymph duct in anesthetized mice (Fig. 2), suggests that rate of the production of HDL by the SI is slow as compared with that of filtration of preexisting liver-originated HDL from the abdominal aorta into the SI lymph duct, and thus liver-originated HDL is

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

predominant in the SI lymph duct in anesthetized mice. This finding explains the result of Brunham et al. (16) that lymph from mice that specifically lack ABCA1 in the liver had no detectable HDL-C.

Our finding, obtained with peptide mapping of HDL using LC/MS, that C-HDL was very similar to L-HDL but different from SI-HDL (Fig. 2) further confirms our finding, obtained by perfusion of ABCA1 KO mice with serum from WT mice, that plasma HDL can filtrate from the abdominal aorta into the SI lymph duct (Fig. 1C). Therefore, our novel findings indicate that our in situ perfusion model can selectively evaluate HDL produced from the SI without possible interference from plasma HDL or HDL derived from the liver.

Using the novel in situ perfusion model, we characterized SI-HDL in comparison with plasma HDL and L-HDL. We found that SI-HDL had a much higher protein content and a lower lipid content than plasma HDL and that CE and PL were the major lipids (Table 1, Fig. 4A). Consistent with these findings, by examining SI-HDL using EM, we found that most SI-HDL was spherical and HDL was smaller than plasma HDL (Fig. 5). Our finding that SI-HDL is small and dense compared with plasma HDL suggests that SI-HDL may have higher antiatherogenic activity than plasma HDL (52).

When mice were fed ad libitum, SI-HDL separated from lymph perfusates by ultracentrifugation contained more TG and less CE than that from liver perfusates (Table 1), suggesting that the composition of core lipids of intestinal HDL is different from that of hepatic HDL. It is possible that HDL becomes TG rich due to fusion between nascent HDL and TG-rich lipoprotein (TRL). We have previously shown that HDL reconstituted from apo AI and PLs remodels plasma apo-B-containing lipoprotein from a patient with Tangier disease, which was TG rich (53), and from a patient with hypercholesterolemia (35). TG in HDL is known to be hydrolyzed by hepatic TG lipase. A lack of hepatic TG lipase in SI lymph perfusates may also lead to TG-rich SI-HDL.

Our analyses of apos in ultracentrifugation density fractions of lipoproteins in SI lymph perfusates showed that apo AIV and apo AI were distributed in both the HDL and non-HDL fractions (Fig. 3B). We compared the compositions of apos of intestinal and hepatic HDL in HDL density fractions separated from SI lymph perfusates and liver perfusates from WT mice using ultracentrifugation (Fig. 4B). We found that L-HDL contained a very limited amount of apo AIV but a considerable amount of apo E, whereas an opposite trend was seen with SI-HDL (Fig. 4B).

Ohta et al. (54) showed that apo AIV exists as a complex with apo AI. They separated apo-AIV-containing HDL using an anti-apo AIV immunoabsorbance column from a human lymph TRL fraction, lymph lipoprotein-deficient fraction (LDF), plasma HDL, and plasma LDF and analyzed apos after separation by SDS-PAGE. Also, Böttcher et al. (55), who separated plasma HDL into charge-based subfractions using preparative isotachophoresis, showed that slow-migrating HDL contained both apo AIV and apo AI, whereas fast-migrating HDL contained only apo AI. However, Duka et al. (56) showed that apo-AIV-containing HDL is formed in the absence of apo AI by using apo $AI^{-/-}$ mice that had been transfected with the apo AIV gene. Therefore, apo AIV coexists with apo AI but can form HDL independent of apo AI if apo AI is absent. It would be interesting to know whether apo-AIV-containing HDL is formed in patients with a genetic apo AI deficiency.

Apo AIV, which is mainly expressed in the SI, is a 46 kDa plasma protein associated with CM and HDL (54, 57) and reportedly can inhibit lipid oxidation and enhance cholesterol efflux. In addition, the overexpression of apo AIV was found to reduce atherosclerosis in mice models (58–60). Therefore, it would be of considerable interest to determine the function and relevance of SI-HDL, particularly with respect to atherosclerosis.

Because the examination of SI-HDL by EM showed spherical particles (Fig. 5), we examined the involvement of ABCA1 and LCAT in the formation of SI-HDL by using inhibitors of ABCA1 and LCAT (Fig. 6). We found that in SI lymph perfusates from WT mice markedly increased levels of free apo AI were detected in the presence of an ABCA1 inhibitor in the perfusion buffer, and pre-β-HDL appeared in the presence of an LCAT inhibitor in the perfusion buffer (Fig. 6). This finding indicates that both ABCA1 and LCAT may be involved in the formation of SI-HDL (2).

Complete inhibition of ABCA1 or LCAT was not achieved in our experiments, and this may have been due to technical reasons; that is, the selection and dosage of inhibitors was limited because of the sensitivity of the SI to the organic solvents (methanol and ethanol) used for solving the inhibitors. However, because we have shown that SI-HDL is rich in apo AIV, our finding is consistent with that of Duka et al. (56), who showed that ABCA1 and LCAT participate in the biogenesis of apo-AIV-containing particles by using $ABCA1^{-/-}$ and $LCAT^{-/-}$ mice that had been transfected with the apo AIV gene.

Using this novel in situ perfusion model, we also found that fasting drastically reduced, and high-fat feeding drastically increased, HDL-apo AI and HDL-C levels in SI lymph perfusates from WT mice (Fig. 7A, B). Our findings indicate that the production of SI-HDL can be dynamically regulated by nutritional factors. It would be interesting to determine whether the ratio of apo AI in HDL to lipid-poor apo AI is similar under different dietary conditions. Our in situ perfusion model should be useful for further investigating the regulation of the production of SI-HDL by various diet components such as saturated and unsaturated fatty acids.

We found that the production of SI-HDL was markedly reduced in the major experimental murine model for atherosclerosis, apo E KO mice (Fig. 7C). Reduced HDL-apo AI and HDL-C in SI lymph perfusates from apo E KO mice may be caused by a redistribution of apos from HDL to non-HDL due to substantial hyperlipidemia and abnormal lipoprotein metabolism. Our results showed that apo AI in SI lymph perfusates was distributed in HDL in WT mice but was distributed in non-HDL in apo E KO mice (Fig. 7C). Duka et al. (56) showed that apo AIV was contained

JOURNAL OF LIPID RESEARCH ASBMB

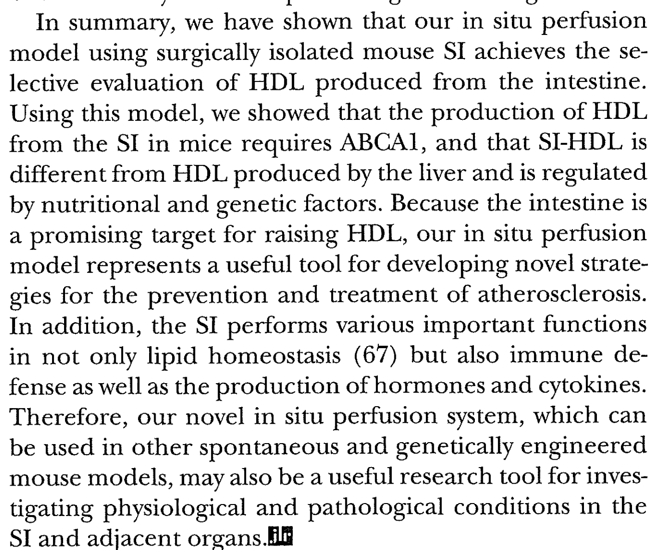
Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

in HDL in apo $AI^{-/-}$ mice but was redistributed to non-HDL in apo $AI^{-/-}$ × apo $E^{-/-}$ mice.

Using our in situ perfusion model, we obtained novel information regarding the production of HDL by the SI, the characteristics of SI-HDL, and the regulation of HDL. However, this model is limited because the effects of anesthesia on gut motility and intestinal lipid trafficking are not clear.

The inhibition of cholesteryl ester transfer protein (CETP) may be a strategy for raising HDL. However, it has been demonstrated that such a strategy needs to be reconsidered because some clinical trials with CETP inhibitors have failed and been terminated (61–63). We reported in the 1990s that genetic human CETP deficiency was atherogenic rather than beneficial (64–66). Based on both these previous and current studies, an increase in the production of SI-HDL may be a therapeutic target for raising HDL.



In summary, we have shown that our in situ perfusion model using surgically isolated mouse SI achieves the selective evaluation of HDL produced from the intestine. Using this model, we showed that the production of HDL from the SI in mice requires ABCA1, and that SI-HDL is different from HDL produced by the liver and is regulated by nutritional and genetic factors. Because the intestine is a promising target for raising HDL, our in situ perfusion model represents a useful tool for developing novel strategies for the prevention and treatment of atherosclerosis. In addition, the SI performs various important functions in not only lipid homeostasis (67) but also immune defense as well as the production of hormones and cytokines. Therefore, our novel in situ perfusion system, which can be used in other spontaneous and genetically engineered mouse models, may also be a useful research tool for investigating physiological and pathological conditions in the SI and adjacent organs.

The authors thank Drs. Seiichiro Tarui and Masao Kawasaki for their helpful comments and discussion, Dr. Kazumitsu Ueda for providing antibody against ABCA1, and Mr. Jan K. Visscher for editing and proofreading the manuscript.

REFERENCES

1. Rader, D. J. 2006. Molecular regulation of HDL metabolism and function: implications for novel therapies. *J. Clin. Invest.* **116:** 3090–3100.
2. Nicholls, S. J., K. A. Rye, and P. J. Barter. 2005. High-density lipoproteins as therapeutic targets. *Curr. Opin. Lipidol.* **16:** 345–349.
3. Miller, N. E. 1990. Raising high density lipoprotein cholesterol. The biochemical pharmacology of reverse cholesterol transport. *Biochem. Pharmacol.* **40:** 403–410.
4. Miller, G. J., and N. E. Miller. 1975. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet.* **1:** 16–19.
5. Castelli, W. P., J. T. Doyle, T. Gordon, C. G. Hames, M. C. Hjortland, S. B. Hulley, A. Kagan, and W. J. Zukel. 1977. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation.* **55:** 767–772.
6. Brooks-Wilson, A., M. Marcil, S. M. Clee, L. H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J. A. Collins, H. O. Molhuizen, et al. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22:** 336–345.
7. Bodzioch, M., E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, et al. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22:** 347–351.
8. Rust, S., M. Rosier, H. Funke, J. Real, Z. Amoura, J. C. Piette, J. F. Deleuze, H. B. Brewer, N. Duverger, P. Denefle, et al. 1999. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* **22:** 352–355.
9. Murthy, S., E. Born, S. N. Mathur, and F. J. Field. 2002. LXR/RXR activation enhances basolateral efflux of cholesterol in CaCo-2 cells. *J. Lipid Res.* **43:** 1054–1064.
10. Ohama, T., K. Hirano, Z. Zhang, R. Aoki, K. Tsujii, Y. Nakagawa-Toyama, K. Tsukamoto, C. Ikegami, A. Matsuyama, M. Ishigami, et al. 2002. Dominant expression of ATP-binding cassette transporter-1 on basolateral surface of Caco-2 cells stimulated by LXR/RXR ligands. *Biochem. Biophys. Res. Commun.* **296:** 625–630.
11. Iqbal, J., K. Anwar, and M. M. Hussain. 2003. Multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells. *J. Biol. Chem.* **278:** 31610–31620.
12. Johansson, C., S. Rossner, and G. Walldius. 1978. H.D.L. secretion from human intestinal tract. *Lancet.* **1:** 324–325.
13. Green, P. H., R. M. Glickman, C. D. Saudek, C. B. Blum, and A. R. Tall. 1979. Human intestinal lipoproteins. Studies in chyluric subjects. *J. Clin. Invest.* **64:** 233–242.
14. Green, P. H., A. R. Tall, and R. M. Glickman. 1978. Rat intestine secretes discoid high density lipoprotein. *J. Clin. Invest.* **61:** 528–534.
15. Timmins, J. M., J. Y. Lee, E. Boudyguina, K. D. Kluckman, L. R. Brunham, A. Mulya, A. K. Gebre, J. M. Coutinho, P. L. Colvin, T. L. Smith, et al. 2005. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J. Clin. Invest.* **115:** 1333–1342.
16. Brunham, L. R., J. K. Kruit, J. Iqbal, C. Fievet, J. M. Timmins, T. D. Pape, B. A. Coburn, N. Bissada, B. Staels, A. K. Groen, et al. 2006. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J. Clin. Invest.* **116:** 1052–1062.
17. Joyce, C. W., E. M. Wagner, F. Basso, M. J. Amar, L. A. Freeman, R. D. Shamburek, C. L. Knapper, J. Syed, J. Wu, B. L. Vaisman, et al. 2006. ABCA1 overexpression in the liver of LDLr-KO mice leads to accumulation of pro-atherogenic lipoproteins and enhanced atherosclerosis. *J. Biol. Chem.* **281:** 33053–33065.
18. Lo Sasso, G., S. Murzilli, L. Salvatore, I. D'Errico, M. Petruzzelli, P. Conca, Z. Y. Jiang, L. Calabresi, P. Parini, and A. Moschetta. 2010. Intestinal specific LXR activation stimulates reverse cholesterol transport and protects from atherosclerosis. *Cell Metab.* **12:** 187–193.
19. Forester, G. P., A. R. Tall, C. L. Bisgaier, and R. M. Glickman. 1983. Rat intestine secretes spherical high density lipoproteins. *J. Biol. Chem.* **258:** 5938–5943.
20. Bearnot, H. R., R. M. Glickman, L. Weinberg, P. H. Green, and A. R. Tall. 1982. Effect of biliary diversion on rat mesenteric lymph apolipoprotein-I and high density lipoprotein. *J. Clin. Invest.* **69:** 210–217.
21. Sipahi, A. M., H. C. Oliveira, K. S. Vasconcelos, L. N. Castilho, A. Bettarello, and E. C. Quintao. 1989. Contribution of plasma protein and lipoproteins to intestinal lymph: comparison of long-chain with medium-chain triglyceride duodenal infusion. *Lymphology.* **22:** 13–19.
22. Vasconcelos, K. S., A. M. Sipahi, H. C. Oliveira, L. N. Castilho, N. De Luccia, and E. C. Quintao. 1989. Origin of intestinal lymph cholesterol in rats: contribution from luminal absorption, mucosal synthesis and filtration from plasma. *Lymphology.* **22:** 4–12.
23. Nutescu, E. A., N. L. Shapiro, and A. Chevalier. 2008. New anticoagulant agents: direct thrombin inhibitors. *Cardiol. Clin.* **26:** 169–187.
24. Kavin, H., N. W. Levin, and M. M. Stanley. 1967. Isolated perfused rat small bowel–technic, studies of viability, glucose absorption. *J. Appl. Physiol.* **22:** 604–611.
25. Windmueller, H. G., and A. E. Spaeth. 1972. Fat transport and lymph and plasma lipoprotein biosynthesis by isolated intestine. *J. Lipid Res.* **13:** 92–105.
26. Wang, N., D. L. Silver, C. Thiele, and A. R. Tall. 2001. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J. Biol. Chem.* **276:** 23742–23747.
27. Dory, L., C. H. Sloop, L. M. Boquet, R. L. Hamilton, and P. S. Roheim. 1983. Lecithin:cholesterol acyltransferase-mediated modification of discoidal peripheral lymph high density lipoproteins: possible mechanism of formation of cholesterol-induced high density lipoproteins (HDLc) in cholesterol-fed dogs. *Proc. Natl. Acad. Sci. USA.* **80:** 3489–3493.
28. Sugano, T., K. Suda, M. Shimada, and N. Oshino. 1978. Biochemical and ultrastructural evaluation of isolated rat liver systems perfused with a hemoglobin-free medium. *J. Biochem.* **83:** 995–1007.

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/02/25/jlr.M047761.DC1
.html

29. Rappsilber, J., M. Mann, and Y. Ishihama. 2007. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* **2:** 1896–1906.
30. Sano, S., S. Tagami, Y. Hashimoto, K. Yoshizawa-Kumagaye, M. Tsunemi, M. Okochi, and T. Tomonaga. 2014. Absolute quantitation of low abundance plasma APL1beta peptides at sub-fmol/mL level by SRM/MRM without immunoaffinity enrichment. *J. Proteome Res.* **13:** 1012–1020.
31. Usui, S., Y. Hara, S. Hosaki, and M. Okazaki. 2002. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J. Lipid Res.* **43:** 805–814.
32. Okazaki, M., S. Usui, M. Ishigami, N. Sakai, T. Nakamura, Y. Matsuzawa, and S. Yamashita. 2005. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography. *Arterioscler. Thromb. Vasc. Biol.* **25:** 578–584.
33. Toshima, G., Y. Iwama, F. Kimura, Y. Matsumoto, M. Miura, J. Takahashi, H. Yasuda, N. Arai, H. Mizutani, K. Hata, et al. 2013. LipoSEARCH®; analytical GP-HPLC method for lipoprotein profiling and its applications. *J Biol Macromol.* **13:** 21–32.
34. Furusyo, N., M. Ai, M. Okazaki, H. Ikezaki, T. Ihara, T. Hayashi, S. Hiramine, K. Ura, T. Kohzuma, E. J. Schaefer, et al. 2013. Serum cholesterol and triglyceride reference ranges of twenty lipoprotein subclasses for healthy Japanese men and women. *Atherosclerosis.* **231:** 238–245.
35. Zhang, B., Y. Uehara, S. Hida, S. Miura, D. L. Rainwater, M. Segawa, K. Kumagai, K. A. Rye, and K. Saku. 2007. Effects of reconstituted HDL on charge-based LDL subfractions as characterized by capillary isotachophoresis. *J. Lipid Res.* **48:** 1175–1189.
36. Zhang, B., E. Kawachi, A. Matsunaga, S. Imaizumi, K. Noda, Y. Uehara, S. Miura, K. Yoshinaga, M. Kuroki, and K. Saku. 2012. Reactivity of direct assays for low-density lipoprotein (LDL) cholesterol toward charge-modified LDL in hypercholesterolemia. *Circ. J.* **76:** 2241–2248.
37. Zhang, B., A. Matsunaga, D. L. Rainwater, S. Miura, K. Noda, H. Nishikawa, Y. Uehara, K. Shirai, M. Ogawa, and K. Saku. 2009. Effects of rosuvastatin on electronegative LDL as characterized by capillary isotachophoresis: the ROSARY Study. *J. Lipid Res.* **50:** 1832–1841.
38. Zhang, B., A. Böttcher, S. Imaizumi, K. Noda, G. Schmitz, and K. Saku. 2007. Relation between charge-based apolipoprotein B-containing lipoprotein subfractions and remnant-like particle cholesterol levels. *Atherosclerosis.* **191:** 153–161.
39. Saku, K., B. Zhang, and K. Noda, and Patrol Trial Investigators. 2011. Randomized head-to-head comparison of pitavastatin, atorvastatin, and rosuvastatin for safety and efficacy (quantity and quality of LDL): the PATROL trial. *Circ. J.* **75:** 1493–1505.
40. Mills, G. L., P. A. Lane, and P. K. Weech. 1984. A guidebook to lipoprotein technique. *In* Laboratory Techniques in Biochemistry and Molecular Biology. R. H. Burdon and P. H. van Knippenberg, editors. Elsevier, New York, NY. 18–78.
41. Miida, T., K. Inano, T. Yamaguchi, T. Tsuda, and M. Okada. 1997. LpA-I levels do not reflect pre beta1-HDL levels in human plasma. *Atherosclerosis.* **133:** 221–226.
42. Miida, T., T. Yamada, U. Seino, M. Ito, Y. Fueki, A. Takahashi, K. Kosuge, S. Soda, O. Hanyu, K. Obayashi, et al. 2006. Serum amyloid A (SAA)-induced remodeling of CSF-HDL. *Biochim. Biophys. Acta.* **1761:** 424–433.
43. SAS Institute Inc. 2009. SAS Online Doc 9.2. SAS Institute Inc., Cary, NC.
44. Brunham, L. R., J. K. Kruit, T. D. Pape, J. S. Parks, F. Kuipers, and M. R. Hayden. 2006. Tissue-specific induction of intestinal ABCA1 expression with a liver X receptor agonist raises plasma HDL cholesterol levels. *Circ. Res.* **99:** 672–674.
45. McNeish, J., R. J. Aiello, D. Guyot, T. Turi, C. Gabel, C. Aldinger, K. L. Hoppe, M. L. Roach, L. J. Royer, J. de Wet, et al. 2000. High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc. Natl. Acad. Sci. USA.* **97:** 4245–4250.
46. Hirano, K., S. G. Young, R. V. Farese, Jr., J. Ng, E. Sande, C. Warburton, L. M. Powell-Braxton, and N. O. Davidson. 1996. Targeted disruption of the mouse apobec-1 gene abolishes apolipoprotein B mRNA editing and eliminates apolipoprotein B48. *J. Biol. Chem.* **271:** 9887–9890.
47. Shen, B. W., A. M. Scanu, and F. J. Kezdy. 1977. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA.* **74:** 837–841.
48. Temel, R. E., and J. M. Brown. 2012. Biliary and nonbiliary contributions to reverse cholesterol transport. *Curr. Opin. Lipidol.* **23:** 85–90.
49. Bonamassa, B., and A. Moschetta. 2013. Atherosclerosis: lessons from LXR and the intestine. *Trends Endocrinol. Metab.* **24:** 120–128.
50. Granger, D. N., J. D. Valleau, R. E. Parker, R. S. Lane, and A. E. Taylor. 1978. Effects of adenosine on intestinal hemodynamics, oxygen delivery, and capillary fluid exchange. *Am. J. Physiol.* **235:** H707–H719.
51. Masuda, D., K. Hirano, H. Oku, J. C. Sandoval, R. Kawase, M. Yuasa-Kawase, Y. Yamashita, M. Takada, K. Tsubakio-Yamamoto, Y. Tochino, et al. 2009. Chylomicron remnants are increased in the postprandial state in CD36 deficiency. *J. Lipid Res.* **50:** 999–1011.
52. Kontush, A., P. Therond, A. Zerrad, M. Couturier, A. Negre-Salvayre, J. A. de Souza, S. Chantepie, and M. J. Chapman. 2007. Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small dense HDL3 particles: relevance to antiapoptotic and antioxidative activities. *Arterioscler. Thromb. Vasc. Biol.* **27:** 1843–1849.
53. Uehara, Y., Y. Tsuboi, B. Zhang, S. Miura, Y. Baba, M. A. Higuchi, T. Yamada, K. A. Rye, and K. Saku. 2008. POPC/apoA-I discs as a potent lipoprotein modulator in Tangier disease. *Atherosclerosis.* **197:** 283–289.
54. Ohta, T., N. H. Fidge, and P. J. Nestel. 1984. Characterization of apolipoprotein A-IV complexes and A-IV isoforms in human lymph and plasma lipoproteins. *J. Biol. Chem.* **259:** 14888–14893.
55. Böttcher, A., J. Schlosser, F. Kronenberg, H. Dieplinger, G. Knipping, K. J. Lackner, and G. Schmitz. 2000. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. *J. Lipid Res.* **41:** 905–915.
56. Duka, A., P. Fotakis, D. Georgiadou, A. Kateifides, K. Tzavlaki, L. von Eckardstein, E. Stratikos, D. Kardassis, and V. I. Zannis. 2013. ApoA-IV promotes the biogenesis of apoA-IV-containing HDL particles with the participation of ABCA1 and LCAT. *J. Lipid Res.* **54:** 107–115.
57. Bisgaier, C. L., O. P. Sachdev, L. Megna, and R. M. Glickman. 1985. Distribution of apolipoprotein A-IV in human plasma. *J. Lipid Res.* **26:** 11–25.
58. Duverger, N., N. Ghalim, G. Ailhaud, A. Steinmetz, J. C. Fruchart, and G. Castro. 1993. Characterization of apoA-IV-containing lipoprotein particles isolated from human plasma and interstitial fluid. *Arterioscler. Thromb.* **13:** 126–132.
59. Qin, X., D. K. Swertfeger, S. Zheng, D. Y. Hui, and P. Tso. 1998. Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation. *Am. J. Physiol.* **274:** H1836–H1840.
60. Duverger, N., G. Tremp, J. M. Caillaud, F. Emmanuel, G. Castro, J. C. Fruchart, A. Steinmetz, and P. Denefle. 1996. Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science.* **273:** 966–968.
61. Nissen, S. E., J. C. Tardif, S. J. Nicholls, J. H. Revkin, C. L. Shear, W. T. Duggan, W. Ruzyllo, W. B. Bachinsky, G. P. Lasala, E. M. Tuzcu, et al. 2007. Effect of torcetrapib on the progression of coronary atherosclerosis. *N. Engl. J. Med.* **356:** 1304–1316. [Erratum. 2007. *N. Engl. J. Med.* 357: 835.]
62. Barter, P. J., M. Caulfield, M. Eriksson, S. M. Grundy, J. J. Kastelein, M. Komajda, J. Lopez-Sendon, L. Mosca, J. C. Tardif, D. D. Waters, et al. 2007. Effects of torcetrapib in patients at high risk for coronary events. *N. Engl. J. Med.* **357:** 2109–2122.
63. Schwartz, G. G., A. G. Olsson, M. Abt, C. M. Ballantyne, P. J. Barter, J. Brumm, B. R. Chaitman, I. M. Holme, D. Kallend, L. A. Leiter, et al. 2012. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N. Engl. J. Med.* **367:** 2089–2099.
64. Hirano, K., S. Yamashita, Y. Kuga, N. Sakai, S. Nozaki, S. Kihara, T. Arai, K. Yanagi, S. Takami, M. Menju, et al. 1995. Atherosclerotic disease in marked hyperalphalipoproteinemia. Combined reduction of cholesteryl ester transfer protein and hepatic triglyceride lipase. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1849–1856.
65. Hirano, K., S. Yamashita, N. Nakajima, T. Arai, T. Maruyama, Y. Yoshida, M. Ishigami, N. Sakai, K. Kameda-Takemura, and Y. Matsuzawa. 1997. Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the Omagari area of Japan. Marked hyperalphalipoproteinemia caused by CETP gene mutation is not associated with longevity. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1053–1059.
66. Hirano, K., S. Yamashita, and Y. Matsuzawa. 2000. Pros and cons of inhibiting cholesteryl ester transfer protein. *Curr. Opin. Lipidol.* **11:** 589–596.
67. Abumrad, N. A., and N. O. Davidson. 2012. Role of the gut in lipid homeostasis. *Physiol. Rev.* **92:** 1061–1085.

Downloaded from www.jlr.org at OSAKA U SEIMEIKAGAKU, on April 29, 2014

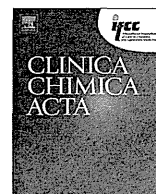
ASBMB JOURNAL OF LIPID RESEARCH JLR

Clinica Chimica Acta 430 (2014) 134–139

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim



Mean postprandial triglyceride concentration is an independent risk factor for carotid atherosclerosis in patients with type 2 diabetes

Mayumi Idei [a], Satoshi Hirayama [a,*], Noriko Miyake [a], Mika Kon [a], Yuki Horiuchi [a], Tsuyoshi Ueno [a], Kazunori Miyake [a], Naotake Satoh [b], Hidenori Yoshii [c], Keiko Yamashiro [c], Tomio Onuma [c], Takashi Miida [a]

[a] *Department of Clinical Laboratory Medicine, Juntendo University Graduate School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan*
[b] *Department of Clinical Laboratory, Juntendo Tokyo Koto Geriatric Medical Center, Shinsuna 3-3-20, koto-ku, Tokyo 136-0075, Japan*
[c] *Department of Internal Medicine, Diabetes and Endocrinology, Juntendo Tokyo Koto Geriatric Medical Center, Shinsuna 3-3-20, koto-ku, Tokyo 136-0075, Japan*

ARTICLE INFO

Article history:
Received 30 August 2013
Received in revised form 14 December 2013
Accepted 17 December 2013
Available online 27 December 2013

Keywords:
Postprandial hypertriglyceridemia
Diabetic dyslipidemia
Carotid atherosclerosis

ABSTRACT

Background: Postprandial hypertriglyceridemia is a risk factor for atherosclerotic disease. However, the postprandial triglyceride (PTG) concentration fluctuates markedly and is poorly reproducible. The aim of this study was to determine whether the mean-PTG concentration is a risk factor for carotid atherosclerosis in patients with type 2 diabetes.
Methods: We measured the fasting and postprandial lipid concentrations and the maximum intima-media thickness (max IMT) of carotid arteries by ultrasound in 115 diabetic patients. A carotid plaque was defined as max IMT of >1.0 mm. The mean-PTG concentration was calculated from several PTG concentrations measured on different days during a 1-year follow-up period.
Results: PTG concentrations showed marked intra-individual variability and ranged from 0.29 to 6.03 mmol/l. Patients with carotid plaques had higher mean-PTG concentrations than those without carotid plaques (1.51 ± 0.57 vs. 1.29 ± 0.47 mmol/l, $p = 0.025$). Neither fasting triglycerides nor one-point PTG concentrations differed between the two groups. Multivariate stepwise logistic regression analysis revealed that the mean-PTG concentration was significantly associated with carotid plaques [OR 1.20 (95% CI, 1.05–1.37), $p = 0.009$], even after adjusting for traditional risk factors, including HDL-cholesterol, LDL-cholesterol, age, hypertension, and duration of diabetes.
Conclusions: The mean-PTG concentration is an independent risk factor for carotid atherosclerosis in patients with type 2 diabetes.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Accumulating evidence suggests that postprandial hypertriglyceridemia is an important risk factor for cardiovascular disease (CVD) in patients with type 2 diabetes. Recent epidemiological studies have shown that the postprandial triglyceride (PTG) concentration is more closely associated with CVD than is the fasting triglyceride (FTG) concentration, which is independent of traditional CVD risk factors [1–4]. Triglyceride-rich lipoproteins (TRLs) are composed of chylomicrons (CM), very low-density lipoproteins (VLDL), and their remnants. Remnant lipoproteins increase in the postprandial state and have greater atherogenicity than their precursors [5–8]. Because hypertriglyceridemia is a common feature in patients with type 2 diabetes, the PTG concentration is likely to be a better predictor of CVD than the FTG concentration in these patients.

Despite its potential usefulness as a diagnostic marker, no clinical guidelines provide a definitive cutoff value for the PTG concentration. The PTG concentration is poorly reproducible and is affected considerably by the meal content and fasting interval [2,9,10]. Most studies evaluated the PTG concentration only once (one-point PTG) or after oral loading with a high-fat diet, which has a much greater fat content than regular meals. Therefore, repeated measurements of PTG might yield a more reliable marker for postprandial dyslipidemia. The aim of this study was to elucidate whether the mean-PTG concentration is an independent risk factor for carotid atherosclerosis in patients with type 2 diabetes. To assess carotid atherosclerosis, we used carotid ultrasonography because it is a noninvasive and quantitative method and because the extent of carotid atherosclerosis is positively correlated with an increased risk of CVD [11,12].

2. Methods

2.1. Recruitment of study subjects

A total of 177 patients with type 2 diabetes were recruited from those who underwent carotid ultrasonography at Juntendo Tokyo Koto Geriatric Medical Center between April 2007 and March 2009. Type 2 diabetes was defined by the criteria of the Japan Diabetes Society [13]. Patients who received hypoglycemic medications or insulin

* Corresponding author. Tel.: +81 3 3813 3111x3373; fax: +81 3 5684 1609.
E-mail address: sthiraya@juntendo.ac.jp (S. Hirayama).

0009-8981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2013.12.022

therapy and/or lipid-lowering medications including statins and fibrates were eligible. We excluded patients with the following disorders: acute or chronic infections, cancer, liver cirrhosis, biliary tract disease, pancreatitis, chronic kidney disease, endocrine disease, and steroid-induced diabetes. Finally, data for 115 patients were collected and analyzed. The study protocol was approved by the Juntendo Tokyo Koto Geriatric Medical Center Research Ethics Committee.

2.2. Study protocol

In all patients, lipoprotein profiles were determined at 1- to 3-month intervals at the outpatient clinic. As a rule, blood samples were obtained 2 to 4 h after breakfast. However, fasting blood samples were obtained once during the 1-year follow-up period within 4 weeks before/after the ultrasound examination. Fasting and postprandial blood samples were taken on different days. In each patient, all PTG concentrations were used to calculate the mean-PTG concentration, while the PTG concentration measured nearest to the time of ultrasonography was defined as the one-point PTG concentration. The number of measurements of postprandial blood samples for each patient is shown in Supplementary Fig. 1. For subgroup analysis, we calculated the average and standard deviation (SD) of the mean-PTG of 115 patients. The patients were then classified into three groups: low group [(< average mean-PTG minus 1SD); <0.88 mmol/l (77.5 mg/dl)], middle group [(average mean-PTG minus 1SD) to (average mean-PTG plus 1SD); 0.88–1.95 mmol/l (77.5–172.5 mg/dl)], and high group [(≥ average mean-PTG plus 1SD); ≥1.95 mmol/l (172.5 mg/dl)].

Anthropometric data and medical history were collected from the medical records. Diabetic retinopathy, nephropathy, and neuropathy were categorized as microvascular complications, while coronary heart disease (CHD), cerebrovascular disease, and peripheral arterial disease were categorized as macrovascular complications. Smoking status was assessed by a questionnaire. Hypertension was defined as a systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg or current use of antihypertensive medications. Dyslipidemia was defined as an FTG of ≥1.69 mmol/l (150 mg/dl), HDL-C of <1.04 mmol/l (40 mg/dl), or LDL-C of ≥3.63 mmol/l (140 mg/dl) according to the guideline of the Japan Atherosclerosis Society [14] or current use of lipid-lowering medications.

All patients received diet therapy based on treatment guidelines for diabetes recommended by the Japan Diabetes Society and underwent nutritional guidance by a registered dietician before

Table 1
Patients' characteristics and laboratory data according to mean-PTG level.

	Low	Middle	High	*p*-value
N	17	76	22	
Age (years)	67.0 ± 7.9	64.2 ± 9.6	60.5 ± 10.9	0.10
Men	7 (41)	43 (57)	10 (45)	0.42
BMI (kg/m^2)	23.7 ± 3.1	25.0 ± 3.7	24.6 ± 3.0	0.35
Waist circumference (cm)	86.3 ± 7.0	88.0 ± 9.9	87.3 ± 9.4	0.79
Hypertension	11 (65)	47 (62)	14 (64)	1.0
Systolic BP (mmHg)	133 ± 15	133 ± 13	131 ± 12	0.88
Diastolic BP (mmHg)	81 ± 10	79 ± 9	79 ± 9	0.67
Dyslipidemia	11 (65)	63 (83)	22 (100)**†	0.007
Duration of diabetes (years)	8.5 ± 7.3	9.7 ± 6.3	11.7 ± 7.0	0.30
Micro-/macrovascular complications	6 (35)/2 (12)	37 (49)/23 (30)	11 (50)/10 (45)	0.64/0.07
Current smoker[a]	5 (33)	27 (42)	12 (55)	0.44
Medications				
ACE inhibitors or ARBs	3 (18)	22 (29)	5 (23)	0.67
Calcium channel blockers	3 (18)	15 (20)	8 (36)	0.27
β-Blockers/diuretics	0 (0)/0 (0)	1 (1)/2 (3)	1 (5)/2 (9)	0.57
Statins/fibrates	4 (24)/1 (6)	22 (29)/3 (4)	10 (45)/1 (5)	0.27/0.82
Oral hypoglycemic agents/insulin	14 (82)/1 (6)	61 (80)/4 (5)	20 (91)/4 (18)	0.57/0.14
Fasting data				
FPG (mmol/L)	7.6 ± 2.2	7.7 ± 2.5	8.3 ± 2.7	0.60
A1C (%)	7.8 ± 1.7	7.4 ± 1.2	8.2 ± 2.0	0.31
Insulin (pmol/L)[b]	25 ± 13	49 ± 39**	68 ± 86**	<0.001
HOMA-IR[b]	1.2 ± 0.7	2.6 ± 3.0**	4.4 ± 7.7**	0.003
FTG (mmol/L)	0.75 ± 0.22	1.20 ± 0.37**	2.01 ± 0.54**‡	<0.001
TC (mmol/L)	4.86 ± 0.91	5.28 ± 0.85	5.66 ± 1.13*	0.028
HDL-C (mmol/L)	1.62 ± 0.31	1.35 ± 0.33**	1.22 ± 0.24**	<0.001
LDL-C (mmol/L)[c]	2.89 ± 0.80	3.39 ± 0.69	3.51 ± 1.05	0.07
ApoB (g/L)	0.82 ± 0.20	1.00 ± 0.18**	1.14 ± 0.25**†	<0.001
ApoCIII (g/L)	0.07 ± 0.02	0.09 ± 0.02	0.12 ± 0.03**‡	<0.001
ApoE (g/L)	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01*†	0.013
RLP-C (mmol/L)	0.08 ± 0.03	0.11 ± 0.04*	0.16 ± 0.07**‡	<0.001
FFA (mmol/L)	0.66 ± 0.20	0.49 ± 0.20**	0.54 ± 0.20	0.001
hs-CRP (mg/L)	0.27 (0.20–3.00)	0.71 (0.33–1.74)	1.31 (0.61–3.62)	0.05
Postprandial data				
One-point PTG (mmol/L)	0.77 ± 0.20	1.26 ± 0.47**	2.40 ± 0.87**‡	<0.001
Mean-PTG (mmol/L)	0.75 ± 0.12	1.31 ± 0.29**	2.27 ± 0.29**‡	<0.001

Data are expressed as *n* (%), mean ± SD, or median (interquartile).
The low group was defined as a mean-PTG of <0.88 (mean − 1SD) mmol/L, the middle group as a mean-PTG of 0.88–1.95 (mean ± 1 SD) mmol/L, and the high group as a mean-PTG of >1.95 (mean + 1SD) mmol/L.
BP, blood pressure; FPG, fasting plasma glucose; FTG, fasting triglyceride; PTG, postprandial triglyceride.
*$p < 0.05$, **$p < 0.01$ vs. low; †$p < 0.05$, ‡$p < 0.01$ vs. middle.
[a] Smoking status data were missing for two subjects in the low group and for eleven subjects in the middle group.
[b] We excluded nine patients from the analysis (one from the low group, four from the middle group, and four from the high group) because they were treated with insulin. HOMA-IR, as a marker of insulin resistance, was calculated using the formula: FPG (mg/dl) × fasting insulin (μU/ml) / 405.
[c] LDL-C was calculated by the Friedewald formula. In all samples, FTG concentrations were <4.52 mmol/L (400 mg/dl).

the 1-year follow-up period. The content of diet therapy was as follows: a total energy was calculated by standard body weight ([body height (m)]^2 × 22) × 25–30 kcal/kg, determined by the amount of individual physical activity. The ratio of protein, fat, and carbohydrate (PFC ratio) were 15%–20%, 20%–25%, and 50%–60%, respectively. Patients were instructed to take one third of the total calorie at breakfast and to not alter the PFC ratio for each meal. Consuming alcohol was avoided the day before the blood test.

2.3. Laboratory tests

Laboratory tests were performed on a Hitachi 7180 analyzer (Hitachi) unless otherwise indicated. Plasma glucose was measured enzymatically, while plasma insulin was measured by electro-chemiluminescence immunoassay (ECLusys Insulin; Roche Diagnostics) using a Cobas 6000 (Roche). A1C was determined by high-performance liquid chromatography (HLC-723G7; Tosoh) and expressed as National Glycohemoglobin Standardization Program (NGSP) values [15]. Total cholesterol (TC), TG, and free fatty acid (FFA) were measured enzymatically (NEFA-SS; Eiken Chemical). LDL-C was calculated by the Friedewald formula [14] using overnight fasting samples. In all samples, FTG concentrations were <4.52 mmol/l (400 mg/dl). HDL-C was determined by a homogeneous method (Cholestest N HDL; Sekisui Medical). Remnant-like particle-cholesterol (RLP-C) was measured by an immunoseparation method (JIMRO II; Otsuka). Apolipoproteins were measured by an immunoturbidimetric method (B, CIII, E Auto N [Daiichi]; Sekisui Medical). High-sensitivity C-reactive protein (hs-CRP) was determined by a latex-enhanced immunonephelometric method (Bering Nepherometer II; Siemens Healthcare Diagnostics).

2.4. Assessment of carotid plaque

In the supine position, the bilateral walls of the common carotid arteries, carotid bulbs, and internal carotid arteries were visualized using a linear array 11-MHz transducer with a Philips SONOS 5500 (Philips Electronics). The maximum intima-media thickness (max IMT) was measured in the observable areas. A carotid plaque was defined as max IMT of > 1.0 mm with a point of inflection on the surface of the intima-media complex [16]. All ultrasound examinations were performed by two experienced laboratory technicians, and the recorded images were stored as digital data. The IMT was measured by one physician (M.I) using recorded images of the carotid artery. The personnel performing these procedures were blinded to the patient's clinical characteristics and laboratory test results.

2.5. Statistical analysis

Statistical analyses were performed using Stat Flex ver.6 for Windows (Artech Co., Ltd.,). Results are expressed as numbers (%), mean ± SD, or median (interquartile). For categorical variables, 2- and 3-group comparisons were performed with the χ^2 test or Fisher's exact probability test. For continuous variables, 2-group comparisons were performed with Student's t-test or the Wilcoxon rank-sum test, while 3-group comparisons were performed with one-way ANOVA followed by Tukey test or Kruskal–Wallis test followed by Dunn test. Univariate and multivariate stepwise logistic regression analyses yielding odds ratios (ORs) and 95% confidence intervals (CIs) were used to identify risk factors for carotid plaques. A 2-tailed p value of <0.05 was considered statistically significance.

3. Results

3.1. Patients' characteristics and laboratory test results

The mean age was 63.9 ± 9.8 years, and the mean duration of diabetes was 9.9 ± 6.6 years in all subjects. Patients' characteristics and laboratory test results among the low, middle, and high groups classified by mean-PTG level are shown in Table 1. The prevalence of dyslipidemia was significantly higher in the high group than the low and middle groups. Other characteristics including age, hypertension, duration of diabetes, and proportion of current smokers did not differ among the groups. Although > 60% of the patients had dyslipidemia in each of the three groups, most of them did not receive fibrates, and there was no significant difference in the number of patients who received fibrates among the groups. Insulin, HOMA-IR, FTG, apolipoproteins, and RLP-C were significantly higher, and HDL-C was significantly lower in the high group compared to the low group. On the other hand, LDL-C did not differ among the three groups.

More than half of the study subjects had carotid plaques; thus, we compared the patient's background according to carotid plaques (Table 2). The plaque (+) group was 7.5 years older and had a 3.6-year longer duration of diabetes than the plaque (−) group. Furthermore, the prevalence of hypertension and macrovascular complications was 20% to 30% higher in the plaque (+) group than in the plaque (−) group. On the other hand, sex, BMI, waist circumference, and the proportion of current smokers were not significantly different between

Table 2
Patients' characteristics and laboratory data according to the presence or absence of carotid plaques.

	Plaque (−)	Plaque (+)	p-value
N	51	64	
Age (years)	59.7 ± 10.8	67.2 ± 7.4	<0.001
Men	22 (43)	38 (59)	NS
BMI (kg/m^2)	24.9 ± 3.8	24.6 ± 3.3	NS
Waist circumference (cm)	88.6 ± 10.3	86.9 ± 8.6	NS
Hypertension	26 (51)	46 (72)	0.021
Systolic BP (mmHg)	132 ± 14	133 ± 12	NS
Diastolic BP (mmHg)	81 ± 9	77 ± 9	0.032
Dyslipidemia	41 (80)	55 (86)	NS
Duration of diabetes (years)	7.9 ± 5.3	11.5 ± 7.1	0.002
Micro-/macrovascular complications	19 (37)/6 (12)	35 (55)/29 (45)	0.06/<0.001
Current smoker[a]	16 (36)	28 (49)	NS
Medications			
ACE inhibitors or ARBs	11 (22)	19 (30)	NS
Calcium channel blockers	5 (10)	21 (33)	0.003
β-Blockers/diuretics	0 (0)/1 (2)	2 (3)/3 (5)	NS/NS
Statins/fibrates	15 (29)/3 (6)	21 (33)/2 (3)	NS/NS
Oral hypoglycemic agents/insulin	41 (80)/4 (8)	53 (83)/5 (8)	NS/NS
Fasting data			
FPG (mmol/l)	7.4 ± 2.2	8.2 ± 2.6	NS
A1C (%)	7.5 ± 1.6	7.7 ± 1.4	NS
Insulin (pmol/l)[b]	44 ± 30	52 ± 60	NS
HOMA-IR[b]	2.2 ± 1.9	3.2 ± 5.2	NS
FTG (mmol/l)	1.23 ± 0.44	1.34 ± 0.62	NS
TC (mmol/l)	5.34 ± 0.86	5.26 ± 0.99	NS
HDL-C (mmol/l)	1.45 ± 0.33	1.30 ± 0.32	0.019
LDL-C (mmol/l)[c]	3.33 ± 0.75	3.34 ± 0.85	NS
ApoB (g/l)	0.99 ± 0.20	1.01 ± 0.23	NS
ApoCIII (g/l)	0.09 ± 0.02	0.09 ± 0.03	NS
ApoE (g/l)	0.04 ± 0.01	0.04 ± 0.01	NS
RLP-C (mmol/l)	0.11 ± 0.04	0.12 ± 0.06	NS
FFA (mmol/l)	0.53 ± 0.20	0.51 ± 0.21	NS
hs-CRP (mg/l)	0.69 (0.25–1.77)	0.89 (0.32–2.15)	NS
Postprandial data			
One-point PTG (mmol/l)	1.29 ± 0.60	1.49 ± 0.84	NS
Mean-PTG (mmol/l)	1.29 ± 0.47	1.51 ± 0.57	0.025

Data are expressed as n (%), mean ± SD, or median (interquartile).
Abbreviations as in Table 1.
[a] Smoking status data were missing for six subjects in the plaque (−) group and for 7 subjects in the plaque (+) group.
[b] We excluded nine patients from the analysis (four from the plaque (−) group and 5 from the plaque (+) group) because they were treated with insulin. HOMA-IR was calculated as in Table 1.
[c] LDL-C was calculated as in Table 1.

the groups. Among many laboratory test results related to lipid and glucose metabolism, only HDL-C and mean-PTG showed a significant difference between the two groups. In the plaque (+) group, mean-PTG was higher and HDL-C was lower than in the plaque (−) group. It should be noted that neither FTG nor one-point PTG was significantly different between the groups.

3.2. Variability in PTG concentration

From the 115 patients, we took a total of 827 blood samples for PTG measurements (Fig. 1). The mean number of measurements was 7.2 ± 1.9 per patient (Supplementary Fig. 1). PTG concentration exhibit marked intra-individual variability, ranging from 0.29 to 1.32 mmol/l, 0.41 to 3.60 mmol/l, and 0.37 to 6.03 mmol/l in the low, middle, and high groups, respectively (Fig. 1). Even in the high group, 20% of the PTG values were < 1.69 mmol/l. Individual CVs of the PTG values ranged from 13.4% to 95.5% ($n = 115$, 31.6% ± 13.7%). There were no significant differences among the groups.

To determine the number of times that PTG measurement should be repeated to obtain stable mean-PTG values, we calculated the average PTG concentration at fewer sampling points (average PTG_x, where $x = 1, 2, 3...$) in each patient. The percentage difference between the mean-PTG and the average PTG_x [Δmean-PTG (%)] was obtained by the following equation: (average PTG_x − mean-PTG) × 100 / mean-PTG. The Δmean-PTG (%) steeply converged as the sampling number increased, and the 95% CI of the Δmean-PTG (%) of 5 measurements became approximately half as narrow as that of one measurement (95% CI: one measurement, −50% to 47%; 5 measurements, −25% to 16%) (Supplementary Fig. 2).

3.3. Relationship between mean-PTG concentration and carotid plaque

As the mean-PTG concentration increased, the prevalence of carotid plaques increased significantly. The χ^2 test revealed that the mean-PTG concentration had a significant effect on the incidence of carotid plaques (Fig. 2). The high group had a significantly higher prevalence of carotid plaques than did the middle and low groups. Univariate analysis also showed that the mean-PTG concentration was significantly associated with carotid plaques, as were other conventional risk factors such as age, hypertension, duration of diabetes, and HDL-C (Table 3). On the other hand, neither FTG nor one-point PTG had a significant association with carotid plaques.

Finally, we carried out multivariate stepwise logistic analysis with two models. A carotid plaque was used as the dependent variable in both models. Along with the other risk factors, either mean-PTG (Model A) or one-point PTG (Model B) was used as the independent variable. The mean-PTG, but not the one-point PTG, was selected as an independent risk factor for carotid plaques after adjustment for multiple risk factors including age, sex, BMI, hypertension, current smoker, A1C, HDL-C, and LDL-C [OR 1.20 (95% CI, 1.05–1.37), $p = 0.009$] (Table 4).

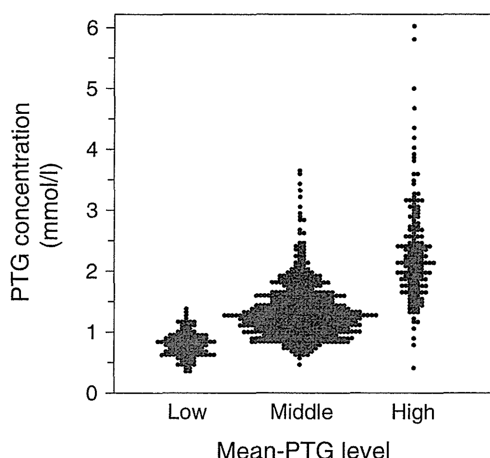


Fig. 1. Distribution of PTG concentration according to mean-PTG level. All PTG concentrations in each patient were plotted in the Low ($n = 17$, no. of samples = 120), Middle ($n = 76$, no. of samples = 535), and High ($n = 22$, no. of samples = 172) groups. The Low, Middle, and High groups were defined as in Table 1. PTG, postprandial triglycerides.

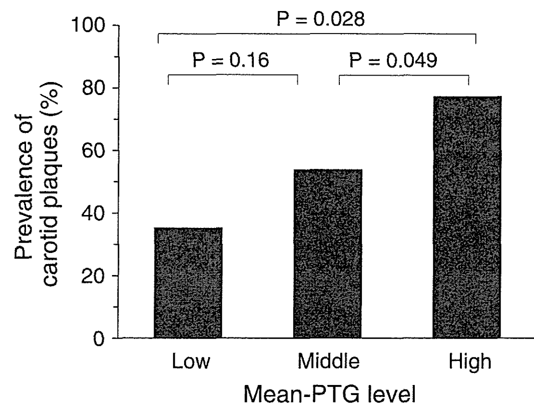


Fig. 2. Prevalence of carotid plaques according to mean-PTG level. The prevalence of carotid plaques was compared. The p value from χ^2 test among the 3 groups was 0.029. The results of χ^2 tests between each group are shown in the figure. The low, middle, and high groups were defined as in Table 1. PTG, postprandial triglycerides.

4. Discussion

Our results indicate that the mean-PTG concentration is an independent risk factor for carotid atherosclerosis and is superior to either the FTG or one-point PTG concentration in patients with type 2 diabetes. Multivariate stepwise logistic regression analysis revealed that the mean-PTG concentration, but not one-point PTG concentration, was associated with carotid atherosclerosis even after adjusting for other conventional risk factors such as HDL-C, LDL-C, age, hypertension, and duration of diabetes.

Dyslipidemia is common in patients with type 2 diabetes and is considered to promote atherosclerosis. Even in diabetic patients, LDL-C is a major risk factor for atherosclerotic disease. The lowering of LDL-C is the

Table 3
Univariate logistic regression analysis for carotid plaques.

Variable	Unadjusted OR (95% CI)	p-value
Age (years)	2.52 (1.56–4.08)	<0.001
Men	1.93 (0.91–4.06)	NS
BMI (kg/m^2)	0.98 (0.88–1.09)	NS
Hypertension	2.46 (1.13–5.33)	0.023
Current smoker	1.75 (0.79–3.90)	NS
Duration of diabetes (years)	1.11 (1.03–1.19)	0.005
Fasting data		
A1C (%)	1.14 (0.87–1.47)	NS
Insulin (pmol/l)	1.04 (0.95–1.14)	NS
FTG (mmol/l)	1.04 (0.97–1.13)	NS
HDL-C (mmol/l)	0.69 (0.51–0.95)	0.021
LDL-C (mmol/l)	1.01 (0.89–1.13)	NS
ApoB (g/l)	1.05 (0.88–1.25)	NS
ApoCIII (g/l)	1.05 (0.90–1.22)	NS
ApoE (g/l)	0.88 (0.62–1.24)	NS
RLP-C (mmol/l)	1.02 (0.81–1.28)	NS
hs-CRP (mg/l)	1.06 (0.90–1.24)	NS
Postprandial data		
One-point PTG (mmol/l)	1.04 (0.98–1.11)	NS
Mean-PTG (mmol/l)	1.10 (1.01–1.19)	0.031

ORs are expressed for 10 unit increases in age and insulin, 0.11 unit (10 mg/dl) increases in TG, 0.26 unit (10 mg/dl) increases in HDL-C and LDL-C, 0.03 unit (1 mg/dl) increases in RLPC, 0.1 unit increases in apoB, 0.01 unit increases in apoCIII and apoE, and 1 unit increases in other continuous variables. Abbreviations as in Table 1.

Table 4
Multivariate stepwise logistic regression analysis for carotid plaques, adjusted for conventional risk factors.

Variable	Adjusted OR (95% CI)	p-value
Model A[a]		
Age (years)	3.45 (1.80–6.61)	0.020
Hypertension	3.26 (1.15–9.62)	0.013
Duration of diabetes (years)	1.14 (1.04–1.26)	0.008
Mean-PTG (mmol/l)	1.20 (1.05–1.37)	0.009
Model B[b]		
Age (years)	2.74 (1.51–4.98)	<0.001
Hypertension	3.93 (1.22–12.7)	0.022
Duration of diabetes (years)	1.14 (1.04–1.25)	0.006

ORs are expressed for 10 unit increases in age, 1 unit increases in duration of diabetes, and 0.11 unit (10 mg/dl) increases in mean-PTG.
Models A and B were adjusted for age, sex, BMI, current smoker, hypertension, A1C, HDL-C, and LDL-C.
Abbreviations as in Table 1.
[a] Model A included age, sex, BMI, hypertension, current smoker, duration of diabetes, A1C, insulin, FTG, HDL-C, LDL-C, apoB, apoCIII, apoE, RLP-C, hs-CRP, and mean-PTG.
[b] Model B included all variables of Model A, except that mean-PTG was replaced by one-point PTG.

primary therapeutic target for diabetic dyslipidemia; this therapeutic goal is equivalent to that in patients with coronary artery disease. The guidelines of the American Diabetic Association recommend that the LDL-C concentration in patients with diabetes should be maintained at <2.59 mmol/l (100 mg/dl) [17]. However, many clinical trials have shown that statins, the most potent LDL-C-lowering drugs, reduce CVD events by only 25% to 50% [18,19].

High TG and low HDL-C concentrations are additional risk factors for macrovascular complications in patients with diabetes [20]. In a subanalysis of the Japan Diabetic Complication Study (JDCS), the TG concentration was the strongest predictor of CHD in Japanese females with diabetes [21]. Moreover, fibrates, which mainly reduce TG and raise HDL-C, significantly decreased CVD events in the patients with diabetes who had high TG concentrations [22,23]. Thus, both LDL-C and TG concentrations should be maintained within appropriate ranges.

Although PTG is more closely related to the development of atherosclerosis than is FTG [1–4], the marked intra-individual variability in the PTG concentration hinders the development of a consensus regarding the PTG concentration cutoff value [2,9,10]. Even in the high mean-PTG group in our study, the PTG concentration was within the normal range in 1 of every 5 blood samples (Fig. 1). To overcome these drawbacks of PTG, we adopted the mean values of several PTG concentrations measured at different times within 1 year. Our data suggest that the mean-PTG concentration calculated using more than five PTG values could be a stable marker of postprandial hypertriglyceridemia (Supplementary Fig. 2). The mean-PTG concentration can be calculated at any clinical institute and is readily applicable to daily medical practice.

The impact of the mean-PTG concentration on carotid atherosclerosis can be explained by the following mechanisms. In the postprandial state, CM is secreted from the intestine. Therefore, TRLs and their remnants accumulate in the circulation [5,6]. Remnants can enter the subendothelial space and be taken up without oxidation by macrophages. In diabetic patients with hypertriglyceridemia, TG-rich VLDL secretion is promoted in the liver due to high TG availability, while lipoprotein lipase (LPL) activity, a key enzyme for the catabolism of TRLs, is reduced. In the circulation, LPL hydrolyzes VLDL particles and forms LDL particles. Under the condition of abundant TRLs, cholesterol ester transfer protein (CETP) significantly transfers TRL triglyceride to these LDL particles, which are hydrolyzed by hepatic lipase (HL). As a result, both LDL size and density further decrease, turning into atherogenic small dense LDLs (sd-LDLs), which are more atherogenic than large buoyant LDLs. Insulin resistance induces and exaggerates these abnormalities and promotes the formation of sd-LDLs [6,24–26]. In addition, high TRLs in the postprandial state may also contribute to proatherosclerotic and prothrombotic processes, including inflammation, oxidative stress, and endothelial dysfunction [20,24,25].

The mean-PTG concentration has some advantages over other markers related to TRLs, such as sd-LDL, apo CIII, and RLP-C. For example, the sd-LDL concentration is higher in patients with diabetes than in healthy subjects. As we reported previously, however, sd-LDL did not increase but rather decreased in the postprandial state [27]. Apo CIII resides mainly on TRLs and partly on HDL particles and inhibits LPL activity [28]. The apo CIII concentration is constant during the day [29]. Therefore, sd-LDL and apo CIII do not directly reflect postprandial hypertriglyceridemia. The RLP-C level could be another candidate marker of postprandial hyperlipidemia because it is elevated in the postprandial state [6,10,24,25]. Unfortunately, few studies have examined the association of postprandial RLP-C and atherosclerotic disease. On the other hand, several studies have demonstrated an association of the one-point PTG concentration or non-fasting TG concentration with CVD risk. Our results using the mean-PTG concentration are consistent with these observations [1–4,30].

In summary, the mean-PTG concentration is an independent risk factor for carotid atherosclerosis and is superior to either the FTG or one-point PTG concentration in patients with type 2 diabetes. Because the mean-PTG concentration is readily measured at outpatient clinics, the cutoff value should be determined in future studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2013.12.022.

Acknowledgements

The authors thank Munechika Segawa (Juntendo Tokyo Koto Geriatric Medical Center, Tokyo, Japan) for his technical assistance.

References

[1] Iso H, Naito Y, Sato S, et al. Serum triglycerides and risk of coronary heart disease among Japanese men and women. Am J Epidemiol 2001;153:490–9.
[2] Nordestgaard BG, Benn M, Schnohr P, et al. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. JAMA 2007;298:299–308.
[3] Bansal S, Buring JE, Rifai N, et al. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. JAMA 2007;298:309–16.
[4] Mora S, Rifai N, Buring JE, et al. Fasting compared with nonfasting lipids and apolipoproteins for predicting incident cardiovascular events. Circulation 2008;118:993–1001.
[5] Zilversmit DB. Atherogenesis: a postprandial phenomenon. Circulation 1979;60:473–85.
[6] Karpe F. Postprandial lipoprotein metabolism and atherosclerosis. J Intern Med 1999;246:341–55.
[7] McNamara JR, Shah PK, Nakajima K, et al. Remnant-like particle (RLP) cholesterol is an independent cardiovascular disease risk factor in women: results from the Framingham Heart Study. Atherosclerosis 2001;154:229–36.
[8] National Cholesterol Education Program (NCEP) Expert Panel on Detection. Ea, and treatment of high blood cholesterol in adults (Adult Treatment Panel III), Third Report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. Circulation 2002;106:3143–421.
[9] Cohn JS, McNamara JR, Cohn SD, et al. Plasma apolipoprotein changes in the triglyceride-rich lipoprotein fraction of human subjects fed a fat-rich meal. J Lipid Res 1988;29:925–36.
[10] Ai M, Tanaka A, Ogita K, et al. Relationship between plasma insulin concentration and plasma remnant lipoprotein response to an oral fat load in patients with type 2 diabetes. J Am Coll Cardiol 2001;38:1628–32.
[11] O'Leary, DH Polak JF, Kronmal RA, et al. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999;340:14–22.
[12] Jashari F, Ibrahimi P, Nicoll R, et al. Coronary and carotid atherosclerosis: similarities and differences. Atherosclerosis 2013;227:193–200.
[13] The Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetol Int 2010;1:2–20. http://dx.doi.org/10.1007/s13340-010-0006-7.
[14] Teramoto T, Sasaki J, Ueshima H, et al. Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. J Atheroscler Thromb 2007;14:155–8.

[15] Kashiwagi A, Kasuga M, Araki E, et al. International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values. Diabetol Int 2012;3:8–10. http://dx.doi.org/10.1007/s13340-012-0069-8.
[16] Terminology and Diagnostic Criteria Committee. Japan Society of Ultrasonics in Medicine. Standard method for ultrasound evaluation of carotid artery lesions. Jpn J Med Ultrasonics 2009;36:510–8. http://dx.doi.org/10.1007/s10396-009-0238-y.
[17] American Diabetes Association. Standards of medical care in diabetes-2013. Daibetes Care 2013;36(Suppl. 1):S11–66.
[18] Collins R, Armitage J, Parish S, et al. MRC/BHF heart protection study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. Lancet 2003;361:2005–16.
[19] Colhoun HM, Betteridge DJ, Durrington PN, et al. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentrerandomised placebo-controlled trial. Lancet 2004;364:685–96.
[20] Mazzone T, Chait A, Plutzky J. Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. Lancet 2008;371:1800–9.
[21] Sone H, Tanaka S, Iimuro S, et al. Comparison of various lipid variables as predictors of coronary heart disease in Japanese men and women with type 2 diabetes: subanalysis of the Japan Diabetes Complications Study. Diabetes Care 2012;35:1150–7.
[22] Keech A, Simes RJ, Barter P, et al. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. Lancet 2005;366:1849–61.
[23] Ginsberg HN, Elam MB, Lovato LC, et al. Effects of combination lipid therapy in type 2 diabetes mellitus. N Engl J Med 2010;362:1563–74.
[24] Ginsberg HN, Illingworth DR. Postprandial dyslipidemia: an atherogenic disorder common in patients with diabetes mellitus. Am J Cardiol 2001;88:9H–15H.
[25] Taskinen MR. Diabetic dyslipidaemia: from basic research to clinical practice. Diabetologia 2003;46:733–49.
[26] Austin MA, Breslow JL, Hennekens CH, et al. Low-density lipoprotein subclass patterns and risk of myocardial infarction. JAMA 1988;260:1917–21.
[27] Hirayama S, Soda S, Ito Y, et al. Circadian change of serum concentration of small dense LDL-cholesterol in type 2 diabetic patients. Clin Chim Acta 2010;411:253–7.
[28] Bobik A. Apolipoprotein CIII and atherosclerosis: beyond effects on lipid metabolism. Circulation 2008;118:702–4.
[29] Kosuge K, Miida T, Takahashi A, et al. Estimating the fasting triglyceride concentration from the postprandial HDL-cholesterol and apolipoprotein CIII concentrations. Atherosclerosis 2006;184:413–9.
[30] Teno S, Uto Y, Nagashima H, et al. Association of postprandial hypertriglyceridemia and carotid intima-media thickness in patients with type 2 diabetes. Diabetes Care 2000;23:1401–6.

Original Article

High-Density Lipoprotein Levels Have Markedly Increased Over the Past Twenty Years in Japan

Shinji Yokoyama[1], Hirotsugu Ueshima[2], Takashi Miida[3], Masakazu Nakamura[4], Koki Takata[5], Tatsuyuki Fukukawa[6], Takaaki Goto[7], Mariko Harada-Shiba[8], Michitaka Sano[9], Kiminori Kato[10] and Kazuhiro Matsuda[10]

Shinji Yokoyama and Hirotsugu Ueshima contributed equally to this work.

[1]Nutritional Health Science Research Center, Chubu University, Kasugai, Japan
[2]Health Science, Shiga University of Medical Science, Otsu, Japan
[3]Clinical Laboratory Medicine, Juntendo University Graduate school of Medicine, Tokyo, Japan
[4]Lipid Reference Laboratory at National Cerebral and Cardiovascular Center under The Cholesterol Reference Method Laboratory Network of CDC, Suita, Japan
[5]Preventive Medicine Center, Chugoku Rosai Hospital, Hiroshima, Japan
[6]Quality Assurance Department, SRL Inc., Hachioji, Japan
[7]Clinical Chemistry Laboratory, Nagoya City University Hospital, Nagoya, Japan
[8]Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, Suita, Japan
[9]Clinical Chemistry of National Cerebral and Cardiovascular Center Hospital, Suita, Japan
[10]Niigata Association of Occupational Health, Niigata, Japan

Aim: The high-density lipoprotein cholesterol (HDL-C) level is a major negative risk factor for atherosclerotic diseases dependent on various lifestyle parameters. Changes in the lifestyle of Japanese individuals over the past several decades is believed to have increased their total cholesterol levels and the incidence of cardiovascular disease in Japan. It is therefore important to assess the long-term trends in the HDL-C levels with respect to public health in the community.
Methods: In this study, accumulated data for the serum/plasma HDL-C levels published in cohort studies and obtained during health checkup programs in Japan were analyzed with respect to time-dependent changes.
Results: The levels of HDL-C have continuously and significantly increased over the past 20 years by 12-15% according to the National Health and Nutrition Study, other cohort studies and commercially available data. On the other hand, the non-HDL-cholesterol levels demonstrated no changes or only a slight decrease during the same period. This finding is consistent with several sets of data obtained from health checkup programs. The commercially measured levels of serum apoA-I, an independent parameter of serum HDL, also showed a similar long-term increase, supporting the above findings.
Conclusion: We concluded that the serum/plasma HDL concentrations in Japanese individuals, selectively, have increased continuously and significantly over the past 20 years or more. The reasons for this phenomenon and the consequent public health outcomes have yet to be investigated.

J Atheroscler Thromb, 2014; 21:151-160.

Key words: HDL, HDL cholesterol, ApoA-I, Non-HDL cholesterol, Japanese

Introduction

The serum/plasma high-density lipoprotein cholesterol (HDL-C) level is a strong negative risk factor for atherosclerotic vascular diseases, such as coronary heart disease (CHD), as demonstrated by most epidemiological studies historically worldwide. It is therefore important to assess and monitor the HDL-C lev-

els in the community in order to improve public health. A recent report by Huxley *et al.*[1] found that isolated low HDL levels are more common in Asians with a high risk of CHD. This finding appears to be somewhat controversial, as the prevalence of CHD is traditionally low in Japan, while the HDL-C levels are generally considered to be high. The data used in this meta-analysis indeed showed relatively high levels among other Asian ethnic groups. We therefore evaluated the historic trend of the Japanese HDL-C levels.

Methods

Sources of Data for the Analysis

The data used in this analysis were available for public use or obtained in previous cohort studies. The National Health and Nutrition Survey (NHNS) conducted by the Ministry of Health, Labour and Welfare of Japan (MHLWJ) maintains files of serum HDL-C data collected since 1989[2, 3]. The data were obtained in the laboratory of SRL, Inc., a commercial clinical chemistry laboratory in Tokyo, based on the standardization and validation protocols issued for nearly 20 years by The Lipid Reference Laboratory in Osaka under the Cholesterol Reference Method Laboratory Network of the CDC (CDC/CRMLN). The SRL has also maintained their own data obtained using commercially ordered laboratory tests, including the serum HDL-C levels, since 1984. In addition, lipid surveys were conducted by the Research Groups on Serum Lipid Level Survey under the MHLWJ in 1990[4] and 2000[5] (measured by SRL and BML, Inc., respectively). Cohort study data have also been accumulated in the Circulatory Risk in Communities Study (CIRCS) at the Kyowa site in Ibaraki[6] and in the Occupational Health Check Program conducted by the Niigata Association of Occupational Health; in both of these studies, the HDL-C assays were standardized according to the criteria of the CDC/CRMLN. Follow-up data obtained in the health checkup program are available at Chugoku Rosai (Labor Welfare) Hospital in Hiroshima, without standardization by the CDC/CRMLN. The HDL-C data obtained in the beta quantification procedure conducted in the CDC/CRMLN Lipid Reference Laboratory in 2011 were also used[7]. Historic data are also available in the clinical laboratories of a few other

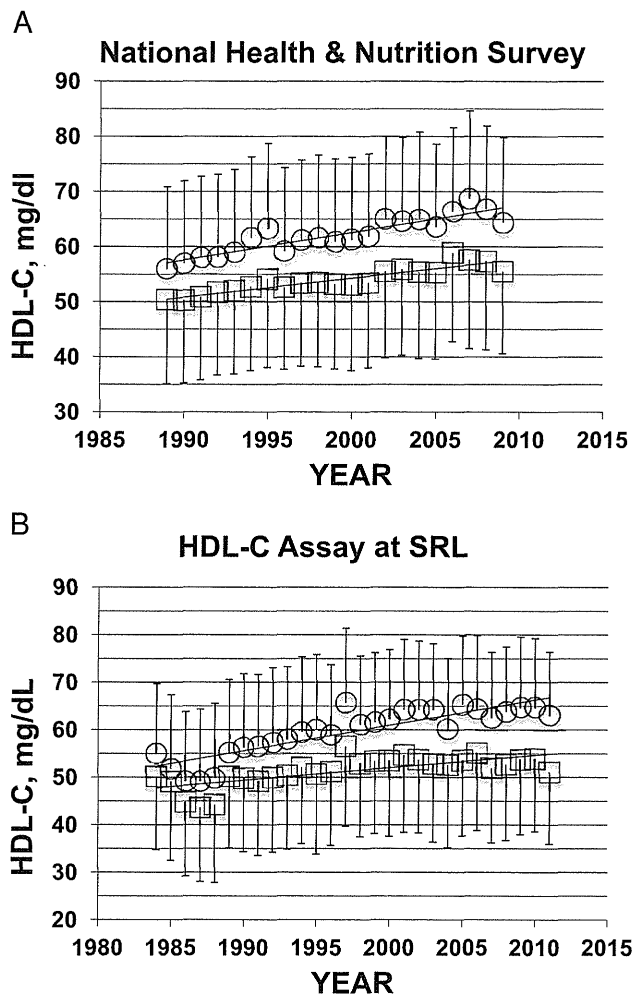


Fig. 1. A. HDL-C data obtained in the National Health and Nutrition Study, mean ± SD, for men (squares) and women (circles). The slopes are 0.35 mg/dL/year and 0.5 mg/dL/year for men and women, respectively, with $p < 0.001$ for both. B. HDL-C data commercially measured in the SRL laboratory, mean ± SD, for men (squares) and women (circles). The slopes are 0.27 mg/dL/year and 0.5 mg/dL/year for men and women, respectively, with $p < 0.001$ for both.

institutions. In addition, the SRL maintains assay data for the levels of serum apolipoprotein A-I (apoA-I), an independent parameter of HDL-C, for determining the HDL concentrations. The assay system was based on standardization by the International Federation of Clinical Chemistry Standardization Project for Measurement of Apolipoproteins A-I and B[8]. The numbers of case samples in each study are listed in **Supplementary Tables 1** and **2**. The assay reagents and systems are listed in **Supplementary Table 3**, with as much detail as possible. The NHNS, CIRCS and

Address for correspondence: Shinji Yokoyama, Nutritional Health Research Center, Chubu University, Matsumoto-cho 1200, Kasugai 487-8501, Japan
E-mail: syokoyam@med.nagoya-cu.ac.jp
Received: August 5, 2013
Accepted for publication: August 30, 2013

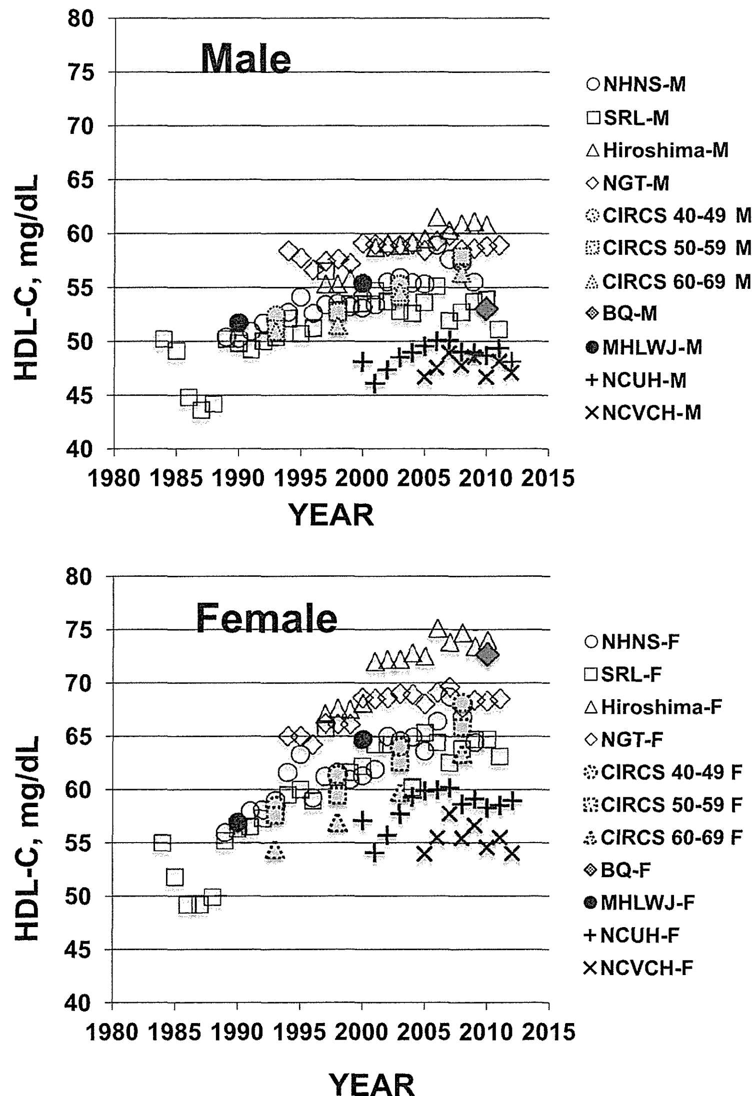


Fig. 2. HDL-C data for men (M) and women (F) obtained from cohort studies, health checkup programs and other sources in Japan. NHNS, National Health and Nutrition Study; SRL, data commercially measured in the SRL laboratory; Hiroshima, health checkup data program at Chugoku Rosai Hospital; NGT, Niigata, health checkup program data for the Niigata Association of Occupational Health; CIRCS, data obtained from CIRCS at the Kyowa site in each age group indicated for every 5-year average represented as each center year time point; BQ, beta-quantification data obtained at the CDC/CRMLN Lipid Laboratory at the National Cerebral and Cardiovascular Research Center for the evaluation of homogeneous LDL measurement[7]; MHLWJ, data obtained from the Serum Lipid Survey conducted by the Ministry of Health, Labour and Welfare of Japan in 1990 and 2000[4, 5]; NCUH and NCVCH, patient data obtained from the Clinical Laboratories of Nagoya City University Hospital and National Cerebral and Cardiovascular Center Hospital.

MHLWJ are cohort studies of the general population. The Hiroshima and Niigata studies are occupational health surveys that include actively working groups. The data obtained from the NCUH and NCVCH studies represent patients who visited the respective hospitals. The SRL samples include those submitted from clinics and hospitals nationwide.

Statistical Analysis

A multivariate regression analysis and the t-test were applied to the serially collected data in order to determine the historical trend.

Results

The HDL-C data obtained in the NHNS study

are shown in **Fig. 1**. The HDL-C levels steadily increased in both men and women over 20 years from 50 to 58 mg/dL and 55 to 67 mg/dL, respectively, (0.35 and 0.50 mg/dL/year with $p<0.001$ for both) (**Fig. 1A**). The commercially measured HDL-C SRL data were very similar to those of the NHNS, also showing an increase with statistical significance (0.27 and 0.50 mg/dL/year, for men and women, both with $p<0.001$) (**Fig. 1B**). This trend did not change among the SRL data samples for the age range of 30-59 years (0.40 and 0.69 mg/dL/year, for men and women). The cohort data obtained from the CIRCS Kyowa study in every age group and those obtained from the Niigata health checkup program both coincided with the NHNS data (**Fig. 2**). The increase in the CIRCS data was statistically significant[6]. The follow-up study conducted at Chugoku Rosai Hospital also showed a statistically significant increase in the levels of HDL-C since 1997, with apparently higher values than those discussed above in both men and women (**Fig. 2**). The HDL-C data obtained using beta-quantification in 2011[7] yielded "elevated values" in the blood samples of patients without lipid disorders (serum total cholesterol <250 mg/dL and triglycerides <150 mg/dL) consistent with a long-term increasing trend (**Fig. 2**).

It is notable that the non-HDL-C ([total cholesterol] − [HDL-C]) levels showed no or only marginally significant decreases in the NHNS data (−0.33 mg/dL/year with $p=0.01$ and −0.29 mg/dL/year with $p=0.07$ for men and women) (**Fig. 3**).

Supporting these trends, the data obtained by MHLWJ Research Group showed a comparable increase in the levels of HDL-C from 1990 to 2000 in every age group (**Fig. 4**). The non-HDL-C levels calculated using the MHLWJ Research Group data were very similar between 1990 and 2000 for both men and women with respect to age distribution (**Fig. 4**).

The increase in the levels of HDL-C reaches a plateau in the most recent several years. This is also indicated by the data obtained from the Clinical Laboratories at Nagoya City University Hospital (NCUH) and National Cerebral and Cardiovascular Center Hospital (NCVCH), which showed almost no tendency toward an increase over a relatively short period comprising the immediate past few years for which reliable data are available (**Fig. 2**). The levels of HDL-C in these institutions are much lower than those observed in the general population, perhaps representing high-risk patients, especially in the NCVCH study.

The levels of serum apoA-I, an independent parameter of serum HDL, have been commercially measured at SRL for years. The accumulated data showed a similar increasing trend in the levels of HDL-C (0.49 and 1.06 mg/dL/year for men and women, both with $p<0.001$) (**Fig. 5**).

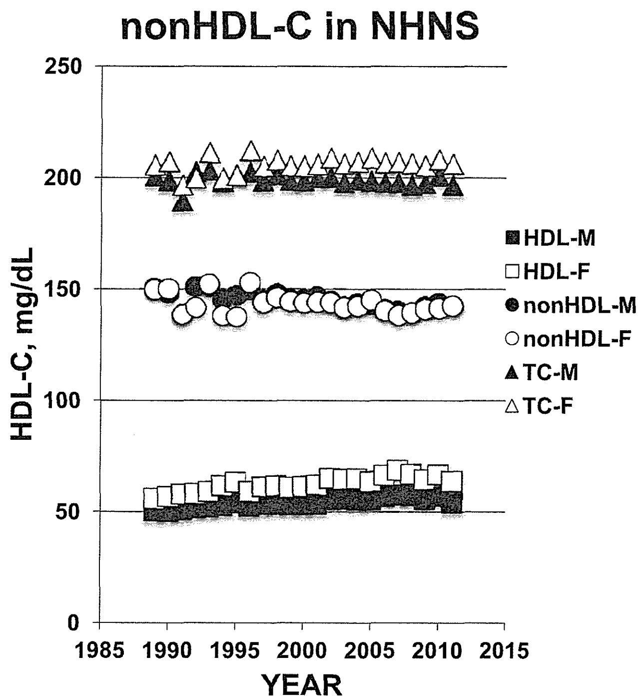


Fig. 3. Non-HDL-C data obtained in the NHNS study presented as [total cholesterol] − [HDL-C] for men (M) and women (F). The slopes and p values are: −0.330 mg/dL/year and 0.010 for men and −0.294 mg/dL/year and 0.070 for women, respectively.

From these data, we may conclude that the serum/plasma HDL concentrations of Japanese patients have increased by 12-15% in both men and women over the past two to three decades.

Discussion

The data analyzed in this study were obtained from mixed sources, collected in epidemiological studies in a somewhat controlled manner and including arbitrarily collected data from patients seen at ordinary regular clinics. It should also be noted that the procedures for measuring the HDL-C levels have varied over the past two decades included in the study period. Routine statistical approaches, such as standardization and adjustment, may therefore not be appropriate or valid for analyzing these sets of data.

The most troublesome concern we have is whether this trend is the result of methodological bias. A variety of methods for measuring the HDL-C levels have been historically applied, including ultracentrifugation, precipitation with polysaccharide sulfate with

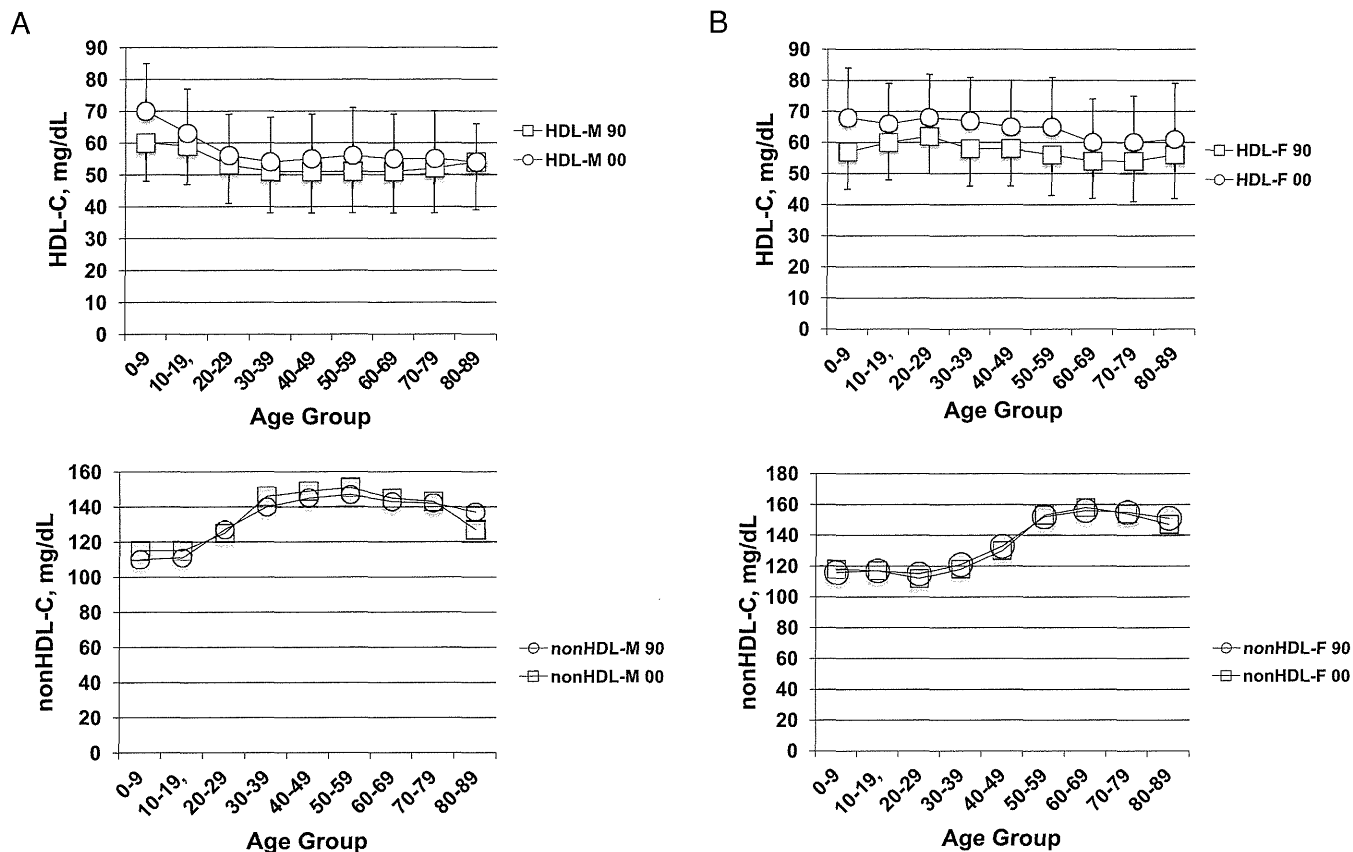


Fig. 4. Analysis of the data obtained in the Serum Lipid Survey conducted by the Ministry of Health, Labour and Welfare of Japan in 1990 and 2000[4, 5] for men (A) and women (B). The levels of HDL-C and non-HDL-C are plotted against age. The HDL-C levels increased significantly in both men and women, with $p<0.001$ according to the t-test, while no changes were observed in the non-HDL levels.

divalent cations and, more recently, homogeneous assay systems[9, 10] developed based on the principles of precipitation methods. The SRL used the heparin-calcium precipitation method until 1995, after which the homogeneous method was employed. Many other clinical laboratories rapidly switched to this method around that time. However, the HDL-C data appear to have continuously increased over this transition time; therefore the change in method is unlikely a major cause of the increase. Nevertheless, continuous efforts by industries to standardize and adjust the calibration of assay systems may ironically have caused a gradual shift in values over the years, inadvertently showing a great and continuous change.

Three lines of evidence may exclude this possibility. First, the results of the apoA-I measurements also showed an increasing trend very similar to that of the HDL-C levels (**Fig. 4**). This assay employs the enzyme immunoassay system[11], which has been based on international standardization since it was established in the mid 1980's; therefore, there have been no changes in the assay environment during this historical data collection period. This finding supports the view that the HDL concentrations in fact increased. Second, all methods of HDL-C measurement used by the SRL were validated using reference methods based on strictly standardized beta-quantification by the CDC/CRMLN Lipid Reference Laboratory. The beta-quantification HDL-C data obtained in the CDC/CRMLN Lipid Reference Laboratory in 2011[7] are consistent with the "elevated values" observed in the long-term increasing trend (**Fig. 2**); therefore, the HDL-C levels appear to be currently high in Japan. Third, the analysis of the data performed by the MHLWJ Research Group revealed interesting results. While the HDL-C levels measured in 1990 and 2000 were consistent with the increasing trend that observed in other sets of data, as a whole and with respect to age distribution, the non-HDL-C data were remarkably similar in the age distribution profiles of both

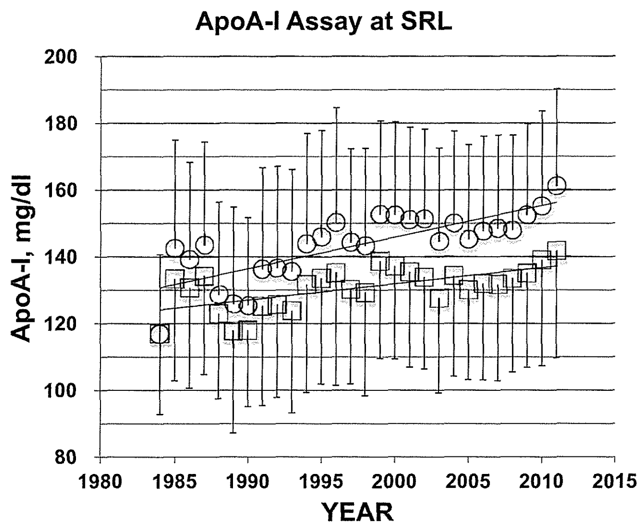


Fig. 5. The apoA-I levels commercially measured in the SRL laboratory, mean ± SD, for men (squares) and women (circles). The slopes are 0.485 mg/dL/year and 1.064 mg/dL/year for men and women, respectively, with $p < 0.001$ for both.

men and women between the two measurements (**Fig. 4**). This finding is consistent with the trend observed in the non-HDL-C levels obtained in the NHNS study (**Fig. 3**). These findings indicate that the long-term increasing trend in the HDL-C levels is less likely to be an artifact and is instead a real phenomenon. It is remarkable that the magnitude of increase (12-15%) is greater than that achieved in most interventional trials using statins or fibrates. The increasing trend in the HDL levels was apparent regardless of the type of data background, such as cohort studies, commercially measured samples or health checkup programs, or regional factors, such as nationwide or local data. Little information is available in the literature regarding the long-term trends in serum lipoprotein profiles. Carroll *et al.* reported that the HDL-C levels showed no changes in the period of 1966-2002, while a slight and significant increase was observed during the time period of 1988-2010 (45.6 to 47 mg/dL by 3% and 55.4 to 57.6 mg/dL by 4% in men and women) in the United States National Health and Nutritional Examination Survey[12, 13]. The current findings indicate that the levels and magnitude of the increase are both much higher among Japanese individuals.

Various factors are known to influence the serum/plasma HDL levels. Nutritional changes may influence the HDL-C level[14], as an increase in calories or fat or cholesterol intake generally increases both the LDL and HDL levels, unless accompanied by elevation of the serum/plasma triglyceride (TG) levels. An increase in physical activity also increases the HDL level by decreasing the TG level. A decrease in the smoking rate should also increase the HDL levels. Moderate habitual alcohol intake is known to result in increased HDL levels. Hypolipidemic drugs, such as fibrates and statins increase the HDL levels, while bile acid sequestering resins and probucol decrease this parameter.

Drastic changes in the eating habits of Japanese individuals took place in the post-WWII period[15]. A marked increase in fat and protein intake was observed until the mid-1970's and stabilized thereafter, while a decrease in carbohydrate intake has continued throughout the postwar period. Interestingly, the total energy intake increased until the mid-1970's then began to gradually decrease thereafter. Therefore, it is not apparent whether there are any specific nutritional causes of the increase in HDL-C observed over the past two to three decades. There may be some prolonged effects of the drastic nutritional changes that occurred in the early postwar period.

More people may currently make an effort to engage in physical exercise; however, overall, the physical activity of Japanese individuals decreased during the period of this survey. The plasma/serum TG levels showed no change, not to account for the increase in HDL-C observed in the NHNS data. Statins were introduced into the market in 1989. However, the proportion of NHNS subjects prescribed these drugs has remain between 10% and 12% over the past 10 years, and the average HDL-C levels observed after excluding these people showed very little changes. The rate of smoking among Japanese men was high and decreased during this period; however, the rate of smoking among women was low and instead increased. The rate of alcohol consumption in Japan has not changed enough to account for such a large increase in the levels of HDL-C. The body mass index has decreased over the past few decades among young Japanese women according to the NHNS data, which may partly account for the increase in the HDL levels observed in women.

From these facts, it can be concluded that the serum/plasma HDL concentrations have increased for at least two decades, from the late 1980's to the mid 2000's. The magnitude in the increase is as large as 10% to 15%. At this point, no apparent reasons accounting for this change can be identified. On the other hand, there were no significant changes in the levels of the atherogenic lipoprotein indicator, non-HDL-C, during this period. Both the mortality and incidence of heart disease and myocardial infarction