Title:

Synergistic association of elevated serum free fatty acid and glucose levels with large arterial stiffness in a general population: The Nagahama Study

Short title:

Free fatty acids, insulin resistance and arterial stiffness

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ABSTRACT

Background: Previous studies have reported that artificial increases in circulating free fatty acid (FFA) levels might have adverse effects on the vasculature. However, whether or not this effect can be extrapolated to physiological variations in FFA levels has not been clarified. Given that FFAs exert a lipotoxic effect on pancreatic β-cells and might directly damage the arterial endothelium, we hypothesized that these adverse effects might synergize with hyperglycemia.

Methods: A total of 9,396 Japanese subjects were included in the study. Serum FFA levels were measured at baseline examination. Brachial-to-ankle pulse wave velocity (baPWV) was measured as an index of arterial stiffness.

Results: As serum levels of FFA were markedly lower in subjects with higher insulin level, a significant association between FFA levels and baPWV was observed only in subjects with blood samples taken under fasting (≥12 h, $P<0.001$) or near-fasting (5–11 h, $P<0.001$) conditions, and not in those taken under non-fasting (<5 h, $P=0.307$) conditions. Although type 2 diabetes and HbA1c showed a strong association with baPWV, the association between FFA level and baPWV remained significant ($\beta=0.052$, $P<0.001$) after adjustment for glycemic levels. In addition to their direct relationship, FFA and glucose levels were synergistically associated with baPWV (FFA*glucose; $\beta=0.036$, $P<0.001$). Differences in baPWV between

the lowest and highest subgroups divided by a combination of FFA and glucose reached approximately 300 cm/sec.

Conclusions: Physiological variations in FFA concentrations might be a risk factor for large arterial stiffness. FFA and hyperglycemia exert a synergistic adverse effect on the vasculature.

HIGHLIGHTS

- Serum free fatty acid levels are associated with large arterial stiffness.
- The effect of serum free fatty acid levels on large arterial stiffness was increased with increasing glucose levels in a synergistic manner.
- Serum free fatty acid may be a marker of cardiovascular risk.

KEYWORDS

Free fatty acid, insulin, hyperglycemia, arterial stiffness

ABBREVIATIONS

baPWV, brachial-to-ankle pulse wave velocity; DBP, diastolic blood pressure; FFA, free fatty acid; hsCRP, high sensitive C-reactive protein; SBP, systolic blood pressure

INTRODUCTION

Circulating free fatty acid (FFA) is released mainly by adipose tissue and is used as a major energy source by cardiac and skeletal muscles [1]. However, excessive FFA exposure induces a lipotoxic effect on pancreatic β-cells, which might reduce insulin secretion [2] and increase β-cell apoptosis [3]. These adverse effects of FFA were first observed *in vitro* and subsequently confirmed *in vivo* in both animal models [4] and human studies [5, 6], which reported an association of chronically high FFA levels with reduced insulin sensitivity and impaired compensatory increases in insulin secretion.

In addition to lipotoxic effects on the insulin pathway that might increase the risk of type 2 diabetes [7], elevated circulating levels of FFA might exert a direct adverse effect on large arteries via impaired insulin-mediated vasodilation [8]. FFA might also exert adverse effects via impaired endothelium-dependent vasodilation [9]. However, nearly all human studies investigating the adverse effects of FFA on insulin signaling [5] and vascular function [8, 9] have used lipid infusion to increase circulating FFA levels. Although one small-scale study (n=105) [10] reported an inverse association between serum FFA levels and abdominal aortic distensibility, we are unaware of any data from large-scale populations on the potential effects of physiological variations in FFA levels on arterial properties. Longitudinal studies in

a general population [11] and in patients undergoing coronary angiography [12] reported positive associations between the elevation of FFA levels and incidence of ischemic heart disease, as well as cardiovascular mortality. Therefore, even physiological variations in FFA might result in adverse effects on arteriosclerotic vasculature change. Further, as FFA might exert adverse effects on arteries by bidirectional pathways [13], namely direct effects on vascular endothelium [14] and indirect effects via lipotoxicity, we hypothesized that higher circulating FFA and glucose levels have a synergistic association with arteriosclerosis.

Here, to further clarify the direct and synergistic adverse effects of FFA on arterial stiffness, we conducted a cross-sectional study by analyzing a dataset of the Nagahama Prospective Cohort for Comprehensive Human Bioscience (the Nagahama Study), which is a large-scale population-based cohort study in Japan. We also investigated factors that are potentially associated with circulating FFA levels to elucidate the descriptive and epidemiological characteristics of FFA in a general population.

METHODS

Study participants

Study participants consisted of 9,396 apparently healthy middle-aged to elderly citizens who

were participants of the Nagahama Study. The study cohort was recruited from 2008 to 2010 from the general population living in Nagahama City, a largely rural city of 125,000 inhabitants located in central Japan. Residents aged 30 to 74 years living independently in the community and with no physical impairment or dysfunction were recruited for the Nagahama cohort. Of a total of 9,804 participants, those meeting any of the following conditions were excluded: history of cardiovascular diseases (n=266), presently taking insulin therapy (n=22), pregnant (n=43), or no data for or outlying clinical parameters required for this study (n=77).

All study procedures were approved by the ethics committee of Kyoto University Graduate School of Medicine and the Nagahama Municipal Review Board. Written informed consent was obtained from all participants.

Clinical characteristics of study subjects

Basic clinical parameters, including plasma markers, were measured at baseline examination. Each participant was asked the time of their last meal, and fasting conditions were defined as follows: fasting, 12 h or more; near-fasting, 5 to 11 h; and non-fasting, less than 5 h. Serum FFA levels were quantified using an enzymatic assay (NEFA-HR; Wako Pure Chemical Industries, Ltd., Osaka, Japan). In FFA measurements, the intra-assay coefficient of variation

was 1.42% and inter-assay coefficient of variation was 1.79%. Smoking status and medication use were evaluated using a structured, self-administered questionnaire.

Evaluation of arterial stiffness

Brachial-to-ankle pulse wave velocity (baPWV) was used as an index of arterial stiffness. baPWV was measured by applying cuffs to both brachia and ankles, and blood pressure was simultaneously measured using a cuff-oscillometric device (Vasera-1500; Fukuda Denshi, Tokyo, Japan). Pulse volume waveforms were also simultaneously recorded using a plethysmographic sensor connected to the cuffs. baPWV was calculated from the time interval between the wave fronts of the brachial and ankle waveforms and the path length from the brachia to ankle (0.597 × height + 14.4014) [15]. Co-linearity of baPWV with a carotid-to-femoral PWV, a standard measure of arterial stiffness, has been reported [16].

Assessment of other risk factors

Hypertension was defined as any or all of brachial systolic blood pressure (SBP) ≥140 mmHg, diastolic BP (DBP) ≥90 mmHg, or taking antihypertensive medication. Type 2 diabetes was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200

mg/dl, HbA1c ≥6.5%, and use of hypoglycemic treatment.

Statistical analysis

Group differences in numeric and categorical variables were assessed by analysis of variance (ANOVA) or a chi-squared test. Quartiles of numeric variables were calculated within each sub-group divided by fasting condition. Factors independently associated with FFA and baPWV were analyzed by multiple linear regression analysis. Statistical analysis was performed using JMP 9.0.3 software (SAS Institute, Cary, NC, USA). *P*-values less than 0.05 were considered significant.

RESULTS

The structure of the Results section is schematically shown in Supplementary Figure 1.

Determinants of serum FFA levels

Clinical characteristics of subjects are summarized in Table 1. A marked sex difference in serum FFA level was observed, with a 20% increase in females (female=0.78±0.28, male=0.65±0.26 mEq/l, $P<0.001$). As a large proportion of serum FFA is bound to albumin, a

strong association was noted between serum FFA and albumin levels (r=0.188, $P<0.001$), but not with age (r=0.082) or body mass index (BMI; r=0.070).

Blood specimens of a substantial number of subjects were drawn under non-fasting conditions (Table 1). Subjects were therefore sub-divided according to fasting duration, and clinical characteristics were then summarized separately (Supplementary Table 1). Marked inter-group differences were observed in insulin and FFA levels, with the former being considerably higher and the latter considerably lower under non-fasting conditions. FFA and insulin levels showed dramatic variation depending on fasting condition (Figure 1A), and the pattern of variation in FFA levels was antithetical to that of insulin. Further, an inverse association between FFA and insulin was also observed on detailed analysis within each time slot (Figure 1B, Supplementary Table 2), but prolonged fasting durations diminished the differences in insulin levels. In contrast, no clear relationship was observed with other glycemic parameters, namely diabetes status or HbA1c quartile (Supplementary Table 3).

Smoking status was also strongly associated with serum FFA level (Supplementary Figure 2). Current smokers exhibited significantly lower FFA levels in both sexes, particularly under near-fasting and fasting conditions.

Multivariate analysis was then performed to identify factors independently

associated with serum FFA levels (Supplementary Table 4). Results indicated that factors strongly associated with FFA level were female sex (β=0.232, P<0.001), albumin (β=0.226, P<0.001), and near-fasting conditions (β=0.340, P<0.001), while those inversely associated with FFA level were insulin (β=-0.142, P<0.001) and non-fasting conditions (β=-0.200, P<0.001). When multivariate analysis was performed by fasting condition, the association of insulin with FFA was significant only under non-fasting conditions (non-fasting, β=-0.481, P<0.001; near-fasting, β=-0.006, P=0.749; fasting, β=-0.036, P=0.046) (Supplementary Table 5).

FFA and arterial stiffness

Differences in baPWV by serum FFA quartile are shown in Figure 2. In contrast to the relationship with insulin, FFA levels were positively associated with baPWV under near-fasting and fasting conditions. Consistent with previous reports, type 2 diabetes and impaired glycemic control were also strongly associated with baPWV in our datasets (Supplementary Figure 2). However, the association of FFA level with baPWV under near-fasting and fasting conditions remained significant (β=0.052, P<0.001) even after adjustment for plasma insulin or glucose level, as well as possible covariates (Models 1 and 2,

Table 2). Further, in addition to their direct relationship, FFA and glucose levels were synergistically associated with baPWV (FFA*glucose; β=0.036, P<0.001) (Model 3, Table 2). Subjects with higher plasma glucose levels, as well as patients with type 2 diabetes, exhibited larger increases in baPWV by FFA quartile than those with relatively low plasma glucose levels (Figure 3, Supplementary Table 6). In contrast, under non-fasting conditions, FFA and insulin were not identified as independent determinants for baPWV (insulin: β=0.013, P=0.585; FFA: β=0.030, P=0.137).

DISCUSSION

In this cross-sectional study of a large general population, we confirmed our hypothesis of an association between elevated serum FFA levels and large arterial stiffness [8-10] in an epidemiological setting. Further, the adverse effect of high FFA levels was prominent under poor glycemic control, particularly in patients with type 2 diabetes. Although a previous experimental study [17] reported a synergistic association of glucose and free fatty acid with lipid accumulation in macrophages, to our knowledge, this is the first large-scale report that physiological variation in serum FFA level might be a risk factor for large arterial stiffness, the effect of which increased with glucose levels in a synergistic manner.

Previous experimental studies demonstrated the lipotoxic effect of chronically high FFA levels on insulin secretion and pancreatic β-cell function [2, 3], which worsens glycemic control and results in insulin resistance and type 2 diabetes. In the present study, although both HbA1c and type 2 diabetes were factors that increased baPWV (Supplementary Figure 2), the positive relationship between FFA and baPWV was independent of plasma levels of insulin and glucose (Table 2). These results support previous findings that suggest an insulin-independent pathophysiological pathway between FFA and large arteries, namely direct impairment of endothelium function [13], and might explain the mechanism by which elevated FFA levels synergize with glucose levels. This notion is supported by findings from a previous study in healthy volunteers of a concomitant decrease in systemic glucose disposal rate and insulin-dependent vasodilation after FFA infusion [8].

As the adverse effects of FFA were synergistic with those of glucose in subjects under fasting or near-fasting conditions (Table 2), differences in baPWV between the lowest and highest subgroups divided by a combination of physiological variation of FFA and glucose reached approximately 300 cm/sec (Figure 2). Further, results of multiple regression analysis indicate that the correlation coefficient of FFA for baPWV is equivalent to that of glucose (Table 2). More attention should therefore be paid to FFA as a cardiovascular risk

factor in epidemiological studies among general populations, as well as in clinical settings.

Serum FFA levels were lowest under non-fasting conditions, whereas insulin levels were highest under these conditions (Figure 1). The relatively low levels of FFA under fasting conditions might therefore be explained by decreases in insulin-mediated FFA secretion from adipose tissue and increases in FFA uptake in the liver and muscle. Similar patterns of change in FFA and insulin levels were observed after intravenous infusion of glucose [18, 19]. As delayed exogenous insulin infusion did not affect these patterns [19], the initial endogenous secretion of insulin might be an important determinant of FFA level after glucose load. FFA levels are thus primarily influenced by insulin levels and as such, the use of FFA as a cardiovascular risk factor should be done with careful consideration to the involvement of confounding factors that may alter insulin levels, such as dietary intake and insulin therapy. Given that FFA levels were lower in current smokers (Supplementary Figure 2), smoking status should be considered as another confounding factor. A previous interventional study in heavy smokers [20] reported acute increases in FFA levels 3 h after smoking. The chronic and acute effects of smoking on serum FFA levels might differ. In addition, marked circadian variation in FFA levels (approximately 20% reduction during sleep) [21] should also be considered.

In a sub-analysis of the 10 time slots shown in Figure 1B, insulin levels were lower in subjects with higher FFA quartiles. This inverse association remained significant after adjustment for glucose level, possibly demonstrating the lipotoxic effect of FFA. Given that the inverse association between FFA and insulin disappeared under fasting conditions, the lipotoxic effect of FFA against pancreatic β-cells mainly impaired additional insulin secretion after glycemic load.

Several limitations to the present study warrant mention. First, repeated measure of FFA levels was not available. Although some day-to-day variation in FFA levels may exist, we did not consider intra-individual variation in the present study. Second, as this was an observational study, the fasting duration-related changes in FFA and insulin levels were compared by sub-population and not by time-dependent analysis. However, as our study had a large sample size, individual differences between each subgroup might be negligible when evaluating the pattern of changes in FFA and insulin. Third, we did not measure the composition of FFA. Eguchi et al. reported that palmitic acid might play a key role in β cell dysfunction and islet inflammation [22]. Detailed analysis based on fatty acid composition might provide further insight into the adverse effects of FFA. Fourth, our study was conducted under a cross-sectional design, and longitudinal investigations are required to clarify the

prognostic significance of FFA on cardiovascular outcomes. A limited number of longitudinal studies in patients with myocardial infarction [23] and those scheduled for coronary angiography [24] have suggested a positive relationship between elevated FFA and cardiovascular and all-cause mortality.

In conclusion, physiological variation in FFA levels might increase the risk of large arterial stiffness in a general population, and the pathophysiological pathway might be independent of FFA lipotoxicity. As insulin affects serum FFA levels, factors that potentially confound circulating insulin levels should be carefully considered when evaluating the adverse effects of FFA.

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DISCLOSURE SUMMARY

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Ya.T., study design, data analysis, data interpretation, and manuscript writing; Yo.T., K.S., T.K., N.G., C.T., data collection; R.Y., S.K., A.S., T.N., cohort design, and data collection; F.M., supervised cohort study.

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FIGURE LEGENDS

Figure 1. Association between serum FFA and insulin levels by fasting status

A: Fasting time-related changes in free fatty acid (FFA) and insulin levels. **B**: Differences in insulin levels by quartile of FFA and fasting time. Subjects were divided into quartiles within each subgroup. Significance was assessed by analysis of covariance. Detailed data are shown in Supplementary Table 2.

Figure 2. Association of FFA quartile with arterial stiffness

Fasting condition at blood sampling was defined as follows: ≥12 h, fasting; 5–11 h, near-fasting; and <5 h, non-fasting. Quartiles of free fatty acid (FFA) levels were calculated within subgroups. Differences in brachial-to-ankle pulse wave velocity (baPWV) were assessed by analysis of variance. Numbers of subjects in each subgroup are shown in columns.

Figure 3. Synergistic association of FFA and glycemic level with arterial stiffness in subjects under fasting or near-fasting conditions (n=8,059)

Quartiles of free fatty acid (FFA) were calculated within subgroups stratified by fasting condition (fasting and near-fasting) and then combined. Type 2 diabetes (T2DM) was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, glycosylated hemoglobin A1c ≥6.5%, and use of hypoglycemic treatment. Remaining subjects who did not receive hypoglycemic medication were subdivided by plasma glucose level. Detailed data are shown in Supplementary Table 6.

Table 1. Clinical characteristics of subjects (n=9,396)

Age (years)	53±13
Sex (male %)	32.3
BMI (kg/m^2)	22.3±3.3
Current smoking (%)	14.5
Fasting condition (fasting/near-fasting/non-fasting)	4,324/3,735/1,337
Blood pressure	
SBP (mmHg)	123±18
DBP (mmHg)	76±11
Antihypertensive medication (%)	15.9
Hypertension (%)	29.7
Metabolic parameters	
Glucose (mg/dl)	90±14
Insulin (μU/ml)	5.37±4.95
HbA1c (%)	5.5±0.5
Hypoglycemic medication (%)	2.4
Type 2 diabetes (%)	3.7
Total cholesterol (mg/dl)	207±35
HDL cholesterol (mg/dl)	65±17
LDL cholesterol (mg/dl)	123±31
Triglyceride (mg/dl)	98±66
Albumin (g/dl)	4.48±0.23
FFA (mEq/l)	0.74±0.28
hsCRP (μg/ml)	0.88±3.40
baPWV (cm/sec)	1,261±228

BMI, body mass index; HbA1c, glycosylated hemoglobin A1c; FFA, free fatty acid; hsCRP, high sensitive C-reactive protein; baPWV, brachial-to-ankle pulse wave velocity

Fasting condition at blood sampling was defined as follows: fasting, ≥12 h; near-fasting, 5–11 h; and non-fasting, <5 h. Hypertension was defined as any or all of brachial systolic blood pressure (SBP) ≥140 mmHg, diastolic BP (DBP) ≥90 mmHg, or use of antihypertensive medication. Type 2 diabetes was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, HbA1c ≥6.5%, and use of hypoglycemic treatment.

Table 2. Multiple linear regression analysis for arterial stiffness in subjects under fasting or near-fasting conditions (n=8,059)

	baPWV (cm/sec)					
	Model 1		Model 2		Model 3	
	β	*P*	*β*	*P*	*β*	*P*
Age (years)	0.426	<0.001	0.414	<0.001	0.413	<0.001
Sex (male)	0.019	0.028	0.012	0.154	0.014	0.101
BMI (kg/m^2)	−0.093	<0.001	−0.088	<0.001	−0.088	<0.001
Current smoking	<0.001	0.986	0.001	0.912	<0.001	0.963
SBP (mmHg)	0.389	<0.001	0.384	<0.001	0.383	<0.001
Antihypertensive medication	0.108	<0.001	0.108	<0.001	0.107	<0.001
Albumin (g/dl)	0.037	<0.001	0.035	<0.001	0.034	<0.001
HDL cholesterol (mg/dl)	−0.024	0.006	−0.026	0.003	−0.027	0.002
LDL cholesterol (mg/dl)	0.008	0.309	0.007	0.353	0.007	0.378
Triglyceride (mg/dl)	0.011	0.183	0.009	0.275	0.009	0.313
Glucose (mg/dl)			0.063	<0.001	0.066	<0.001
Antihyperglycemic treatment	0.047	<0.001	0.030	<0.001	0.032	<0.001
Insulin (log-normalized)	0.059	<0.001	0.043	<0.001	0.043	<0.001
hsCRP (log-normalized)	0.071	<0.001	0.067	<0.001	0.067	<0.001
FFA (mEq/l)	0.051	<0.001	0.052	<0.001	0.054	<0.001
Glucose*FFA interaction					0.036	<0.001

baPWV, brachial-to-ankle pulse wave velocity; BMI, body mass index; SBP, systolic blood pressure; hsCRP, high-sensitive C-reactive protein; FFA, free fatty acid

FIGURE 1

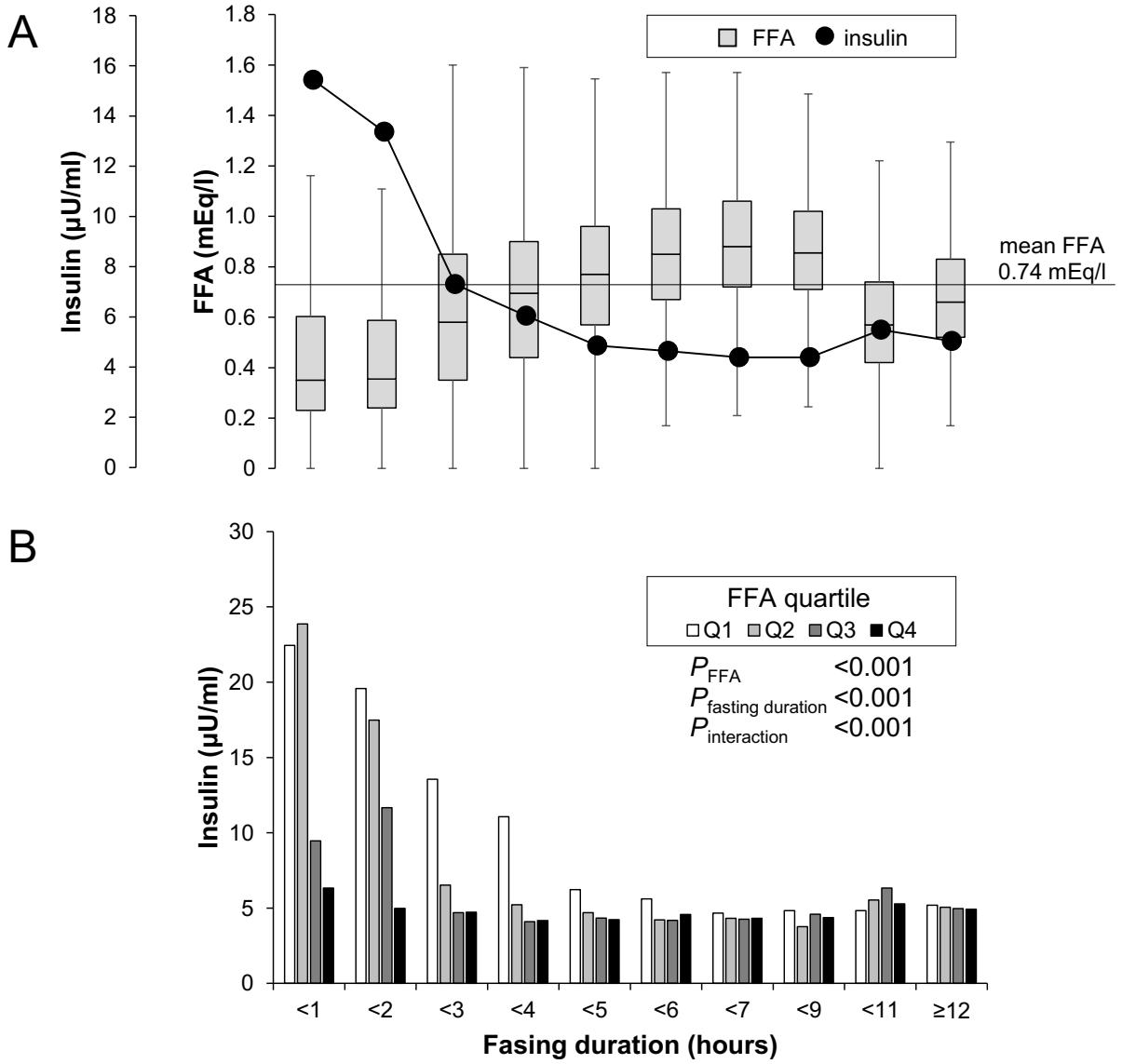

A
Insulin (µU/ml)
FFA (mEq/l)
FFA
insulin
mean FFA
0.74 mEq/l
B
Insulin (µU/ml)
FFA quartile
Q1 Q2 Q3 Q4
P_{FFA} <0.001
$P_{fasting\ duration}$ <0.001
$P_{interaction}$ <0.001
<1 <2 <3 <4 <5 <6 <7 <9 <11 ≥12
Fasing duration (hours)

FIGURE 2

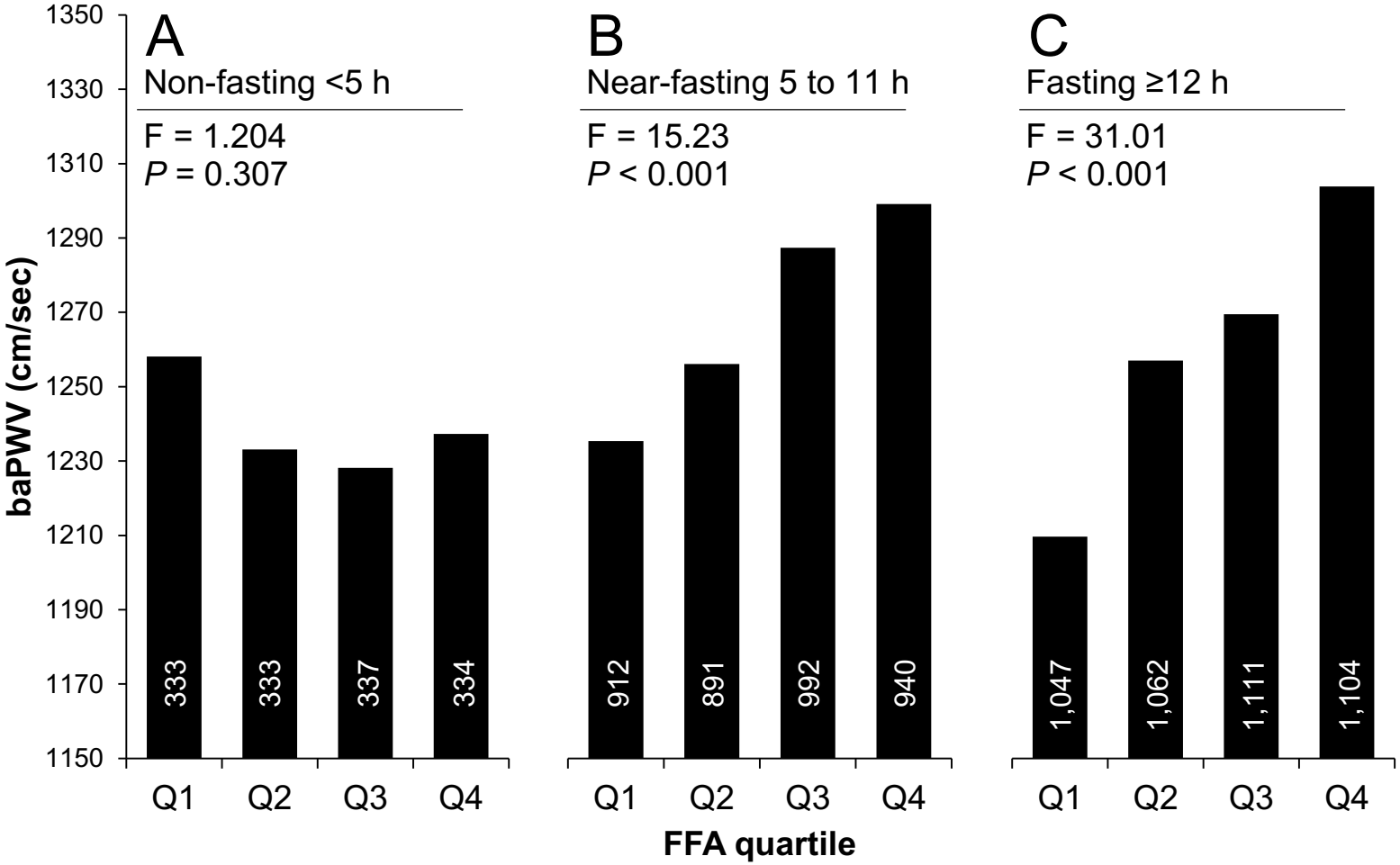
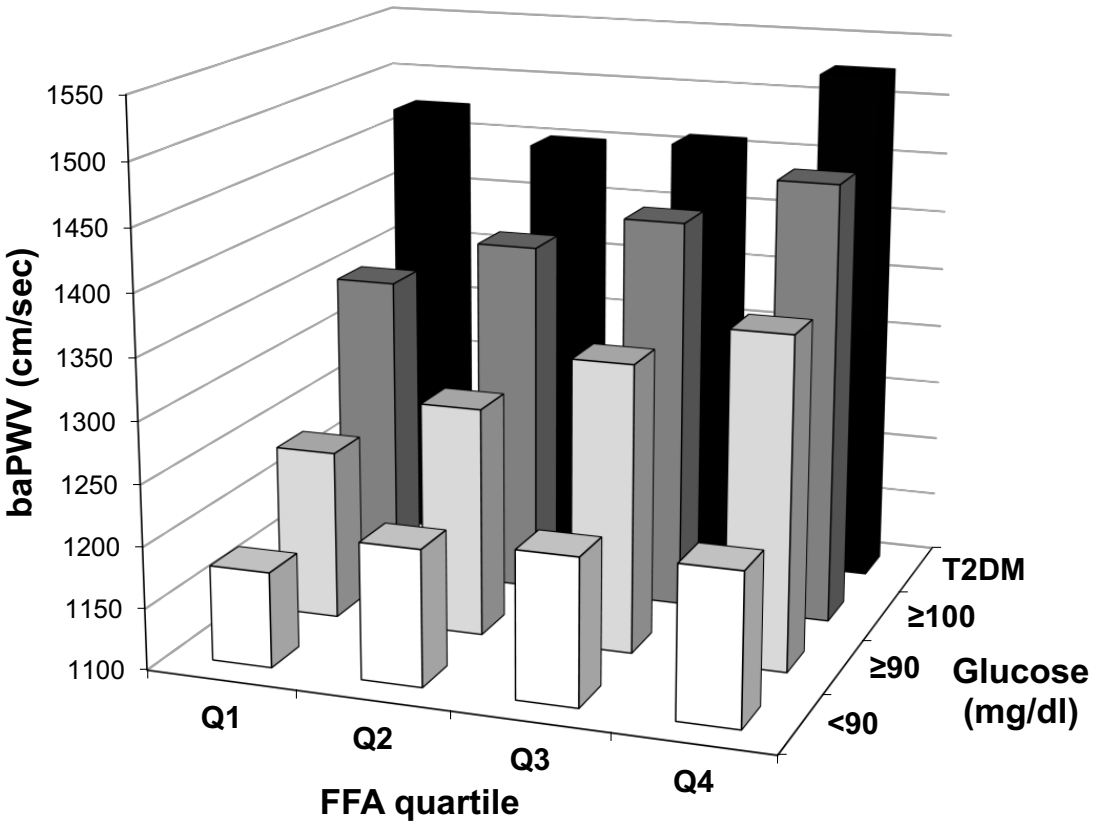

A
Non-fasting <5 h
F = 1.204
P = 0.307
B
Near-fasting 5 to 11 h
F = 15.23
P < 0.001
C
Fasting ≥12 h
F = 31.01
P < 0.001
baPWV (cm/sec)
1350
1330
1310
1290
1270
1250
1230
1210
1190
1170
1150
333
333
337
334
912
891
992
940
1,047
1,062
1,111
1,104
Q1
Q2
Q3
Q4
FFA quartile

FIGURE 3


1550
1500
1450
1400
1350
1300
1250
1200
1150
1100
baPWV (cm/sec)
Q1
Q2
Q3
Q4
FFA quartile
T2DM
≥100
≥90
<90
Glucose
(mg/dl)

Supplementary Table 1. Clinical characteristics of study subjects by fasting condition

	Non-fasting (n=1,337)	Near-fasting (n=3,735)	Fasting (n=4,324)	*P*
Age (years old)	52±14	54±13	53±13	<0.001
Sex (male %)	32.8	32.9	31.6	0.419
BMI (kg/m^2)	22.3±3.3	22.4±3.3	22.1±3.2	0.005
Current smoking (%)	20.2	13	14.1	<0.001
Blood pressure				
SBP (mmHg)	122±18	124±18	123±18	<0.001
DBP (mmHg)	75±11	76±11	76±11	<0.001
Antihypertensive treatment (%)	14.5	16.7	15.6	0.169
Hypertension (%)	26.8	31.1	29.5	0.011
Metabolic parameters				
Glucose (mg/dl)	92±20	89±11	90±14	<0.001
Insulin (μU/ml)	8.51±9.63	4.63±3.63	5.03±3.08	<0.001
HbA1c (%)	5.4±0.5	5.5±0.5	5.5±0.5	0.029
Antihyperglycemic treatment (%)	2.2	2.5	2.4	0.803
Type 2 diabetes (%)	3	3.7	4	0.251
Total cholesterol (mg/dl)	203±34	208±35	207±34	<0.001
HDL cholesterol (mg/dl)	64±17	66±17	66±17	0.001
LDL cholesterol (mg/dl)	120±30	124±32	123±31	<0.001
Triglyceride (mg/dl)	111±72	100±69	91±62	<0.001
Albumin (g/dl)	4.46±0.24	4.49±0.22	4.47±0.22	<0.001
FFA (mEq/l)	0.61±0.32	0.84±0.29	0.69±0.24	<0.001
hsCRP (μg/ml)	0.88±4.01	0.90±3.32	0.86±3.26	0.832
baPWV (cm/sec)	1,239±220	1,270±229	1,261±231	<0.001

BMI, body mass index; HbA1c, glycosylated hemoglobin A1c; FFA, free fatty acid; hsCRP, high sensitive C-reactive protein; baPWV, brachial-to-ankle pulse wave velocity.
Fasting condition at blood sampling was defined as follows; fasting, ≥12 h; near-fasting, 5 to 11 h; non-fasting, <5 h. Hypertension was defined as any or all of brachial systolic blood pressure (SBP) ≥140 mmHg, diastolic BP (DBP) ≥90 mmHg, or taking antihypertensive medication. Type 2 diabetes was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, HbA1c ≥6.5 %, and use of hypoglycemic treatment.

Supplementary Table 2. Insulin levels classified by fasting duration and FFA quartile

			Fasting duration (hours)									
			<1	<2	<3	<4	<5	<6	<7	<9	<11	≥12
Total		n	122	220	427	568	865	1245	987	450	188	4,324
		mean	15.44	13.37	7.30	6.04	4.86	4.64	4.38	4.38	5.48	5.03
		(SD)	(13.91)	(12.95)	(7.58)	(6.54)	(3.95)	(3.90)	(3.07)	(2.85)	(4.42)	(3.08)
FFA quartile	Q1	n	29	49	103	132	211	302	234	111	46	1,047
		mean	22.44	19.59	13.55	11.07	6.23	5.61	4.67	4.83	4.83	5.19
		(SD)	(13.23)	(15.30)	(10.93)	(10.58)	(6.15)	(6.32)	(4.19)	(3.44)	(3.69)	(3.21)
	Q2	n	31	61	108	152	212	313	249	114	44	1,062
		mean	23.87	17.48	6.52	5.22	4.70	4.21	4.32	3.76	5.53	5.05
		(SD)	(17.42)	(12.91)	(6.61)	(4.68)	(3.11)	(2.51)	(2.74)	(2.20)	(4.56)	(3.12)
	Q3	n	32	55	109	136	218	307	252	111	46	1,111
		mean	9.46	11.66	4.69	4.10	4.33	4.18	4.25	4.58	6.33	4.97
		(SD)	(6.64)	(11.90)	(3.35)	(2.64)	(2.90)	(2.81)	(2.49)	(3.03)	(5.60)	(2.91)
	Q4	n	30	55	107	148	224	323	252	114	52	1,104
		mean	6.33	4.97	4.72	4.17	4.22	4.57	4.32	4.37	5.27	4.92
		(SD)	(5.16)	(4.08)	(2.85)	(2.41)	(2.24)	(2.55)	(2.61)	(2.49)	(3.66)	(3.07)

FFA, free fatty acids.

Unit of insulin is μU/ml. Quartile of FFA was calculated within each timeslot.

Supplementary Table 3. Association of FFA with glycemic parameters

		FFA (mEq/l)								
		Non-fasting (<5 h)			Near-fasting (5 to 11 h)			Fasting (≥12 h)		
		N	Mean±SD	*P*	N	Mean±SD	*P*	N	Mean±SD	*P*
Insulin quartile	Q1	331	0.72±0.25	<0.001	927	0.83±0.26	<0.001	1081	0.69±0.25	0.215
	Q2	336	0.73±0.31		935	0.86±0.29		1081	0.69±0.24	
	Q3	336	0.63±0.32		939	0.87±0.28		1081	0.70±0.24	
	Q4	334	0.36±0.22		934	0.81±0.31		1081	0.68±0.24	
Type 2 diabetes	T2DM	40	0.54±0.33	0.156	139	0.78±0.29	0.008	172	0.71±0.22	0.385
	NGT	1297	0.61±0.32		3596	0.84±0.28		4152	0.69±0.24	
Antihyperglycemic treatment	Yes	29	0.50±0.33	0.063	93	0.75±0.28	0.001	103	0.68±0.21	0.597
	No	1,308	0.61±0.32		3,642	0.84±0.28		4,221	0.69±0.24	
HbA1c quartile	Q1	404	0.63±0.32	0.164	987	0.84±0.31	0.817	1145	0.69±0.26	0.244
	Q2	351	0.60±0.31		950	0.84±0.28		1120	0.68±0.24	
	Q3	267	0.61±0.33		1094	0.84±0.28		1295	0.69±0.23	
	Q4	315	0.58±0.31		704	0.83±0.27		764	0.71±0.22	

FFA, free fatty acids; T2DM, type 2 diabetes; NGT, normal glycemic tolerance; HbA1c, glycosylated hemoglobin A1c

Values are mean ± standard deviation. Type 2 diabetes was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, HbA1c ≥6.5%, and use of hypoglycemic treatment. Quartiles of insulin and HbA1c were calculated within each subgroup. Statistical significance was assessed by analysis of variance.

Supplementary Table 4. Multiple linear regression analysis for serum FFA level (n=9.396)

		FFA (mEq/l)	
		β	*P*
Age (years old)		0.100	<0.001
Sex (male)		-0.232	<0.001
BMI (kg/m^2)		0.025	0.034
Current smoking		-0.036	<0.001
SBP (mmHg)		0.062	<0.001
Antihypertensive treatment		0.013	0.212
Albumin (g/dl)		0.226	<0.001
HDL cholesterol (mg/dl)		0.112	<0.001
LDL cholesterol (mg/dl)		-0.001	0.952
Triglyceride (mg/dl)		0.016	0.133
Glucose (mg/dl)		-0.012	0.246
Antihyperglycemic treatment		-0.010	0.301
Insulin (log-normalized)		-0.142	<0.001
hsCRP (log-normalized)		0.072	<0.001
Fasting condition	Fasting (≥12 h)	reference	
	Near-fasting (5 to 11 h)	0.340	<0.001
	Non-fasting (<5 h)	-0.200	<0.001

FFA, free fatty acid; BMI, body mass index; SBP, systolic blood pressure; hsCRP, high sensitive C-reactive protein; baPWV, brachial-to-ankle pulse wave velocity.
Fasting condition at blood sampling was defined as follows; fasting, ≥12 h; near-fasting, 5 to 11 h; non-fasting, <5 h.

Supplementary Table 5. Multiple linear regression analysis for serum FFA level by fasting status

	FFA (mEq/l)					
	Non-fasting (<5 h)		Near-fasting (5 to 11 h)		Fasting (≥12 h)	
	β	*P*	*β*	*P*	*β*	*P*
Age (years old)	-0.004	0.882	0.160	<0.001	0.115	<0.001
Sex (male)	-0.221	<0.001	-0.227	<0.001	-0.238	<0.001
BMI (kg/m^2)	0.134	<0.001	-0.024	0.236	-0.040	0.033
Current smoking	-0.014	0.577	-0.040	0.014	-0.053	0.001
SBP (mmHg)	0.032	0.236	0.072	<0.001	0.055	0.002
Antihypertensive treatment	0.049	0.050	-0.009	0.602	0.021	0.193
Albumin (g/dl)	0.227	<0.001	0.218	<0.001	0.209	<0.001
HDL cholesterol (mg/dl)	0.077	0.005	0.135	<0.001	0.134	<0.001
LDL cholesterol (mg/dl)	0.082	0.001	-0.006	0.697	-0.036	0.020
Triglyceride (mg/dl)	0.007	0.812	-0.006	0.732	0.048	0.005
Glucose (mg/dl)	0.017	0.571	-0.031	0.068	0.024	0.158
Antihyperglycemic treatment	0.005	0.852	-0.030	0.061	-0.007	0.639
Insulin (log-normalized)	-0.481	<0.001	-0.006	0.749	-0.036	0.046
hsCRP (log-normalized)	0.058	0.020	0.049	0.005	0.096	<0.001

baPWV, brachial-to-ankle pulse wave velocity; BMI, body mass index; SBP, systolic blood pressure; hsCRP, high-sensitive C-reactive protein; FFA, free fatty acid.

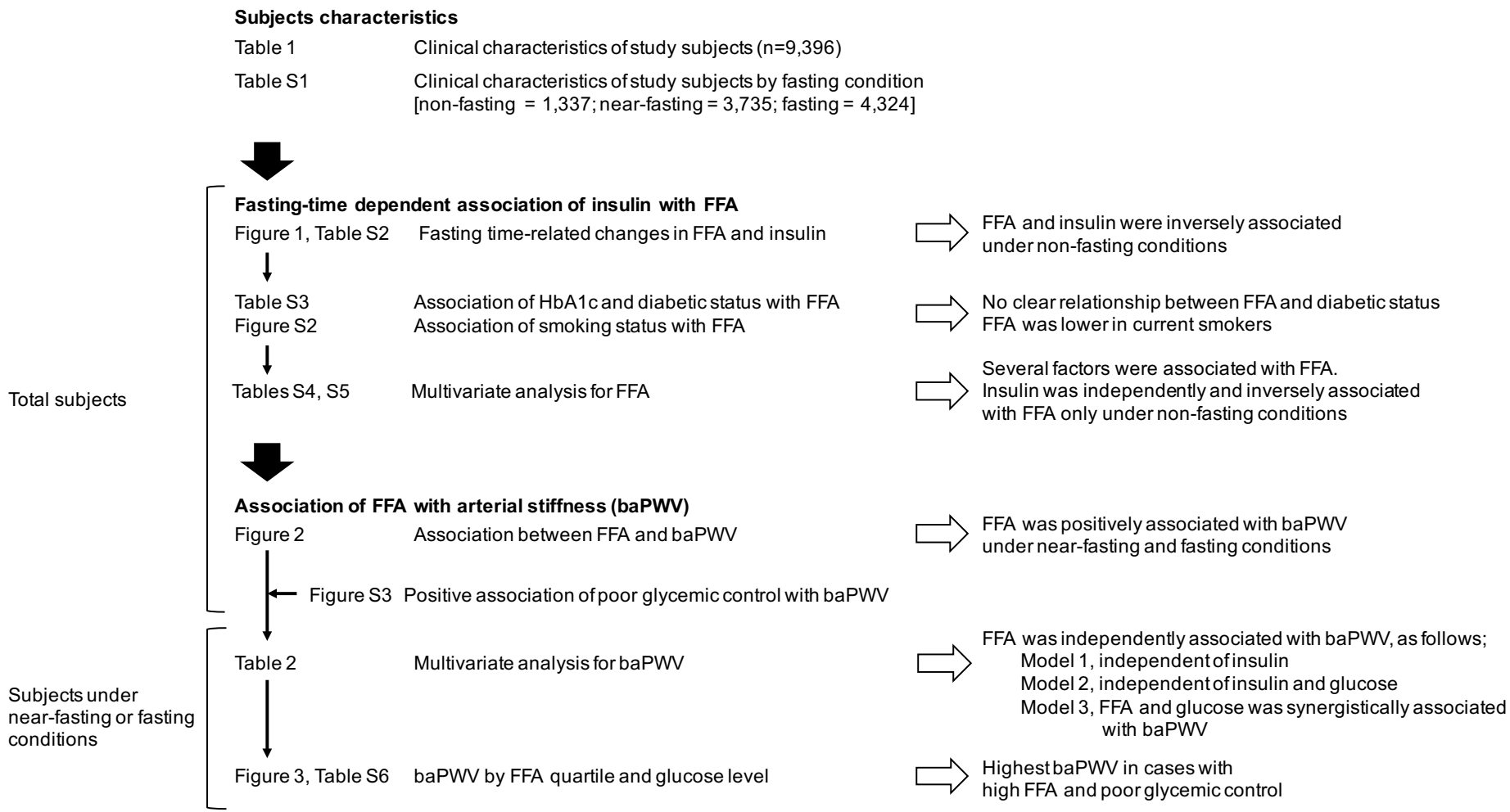
Fasting condition at blood sampling was defined as follows; fasting, ≥12 h; near-fasting, 5 to 11 h; non-fasting, <5 h.

Supplementary Table 6. Mean baPWV by FFA quartile and glycemic control level in subjects under fasting or near-fasting condition (n=8,059)

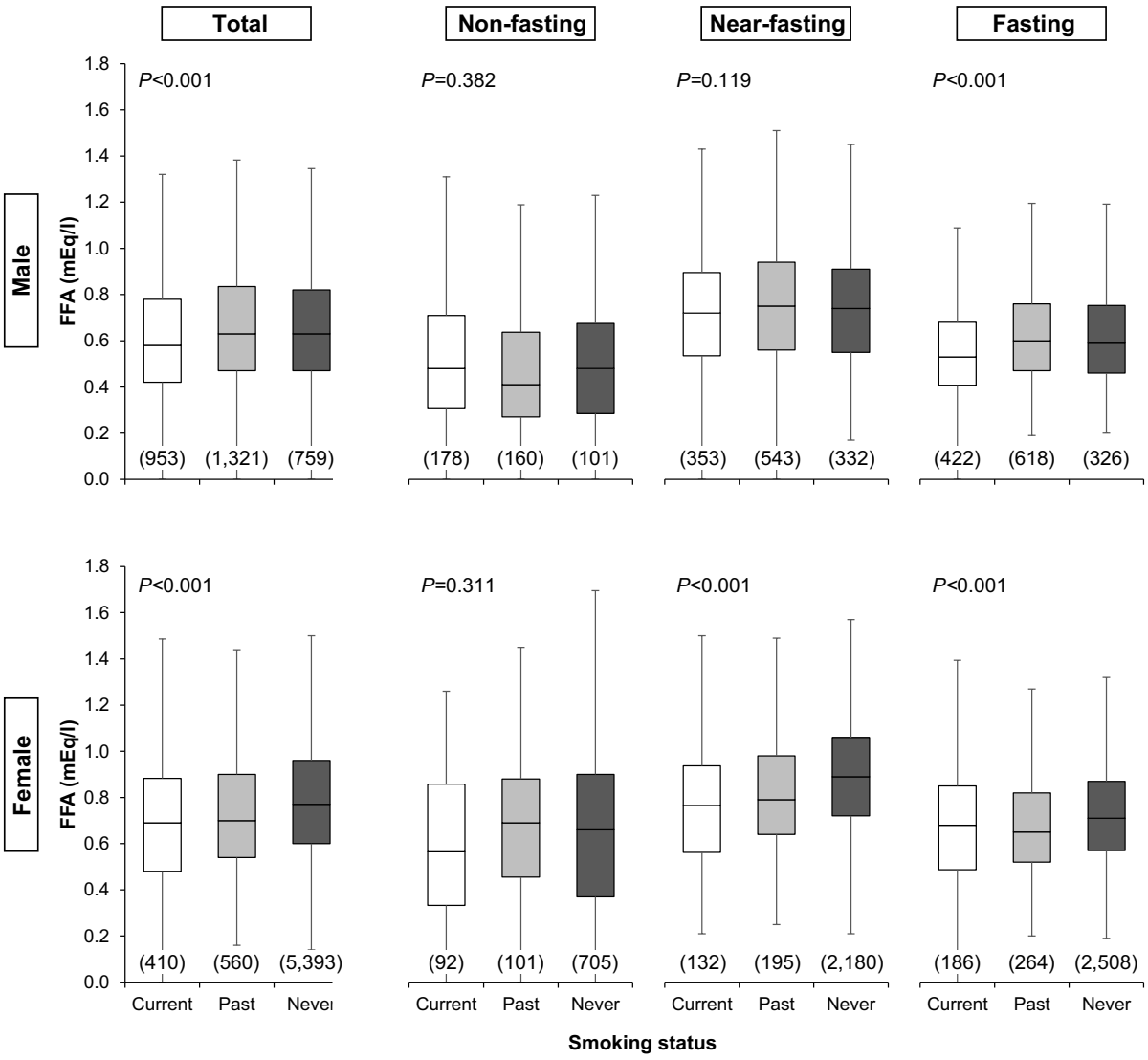
		FFA quartile							
		Q1		Q2		Q3		Q4	
		n	Mean±SD	n	Mean±SD	n	Mean±SD	n	Mean±SD
Glucose (mg/dl)	<90	1167	1178±183	1147	1211±227	1236	1219±206	1145	1223±211
	≥90	550	1238±197	577	1287±200	629	1335±227	629	1369±263
	≥100	153	1351±212	154	1391±239	163	1420±195	198	1460±249
T2DM		89	1474±237	75	1450±203	75	1460±191	72	1525±233

FFA, free fatty acid; T2DM, type 2 diabetes.

Values are mean ± standard deviation. FFA quartile was calculated within subgroups stratified by fasting condition (fasting and near-fasting) and then combined. T2DM was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, glycosylated hemoglobin A1c ≥6.5 %, and use of hypoglycemic treatment. Remaining subjects who did not receive hypoglycemic medication were subdivided by plasma glucose level.



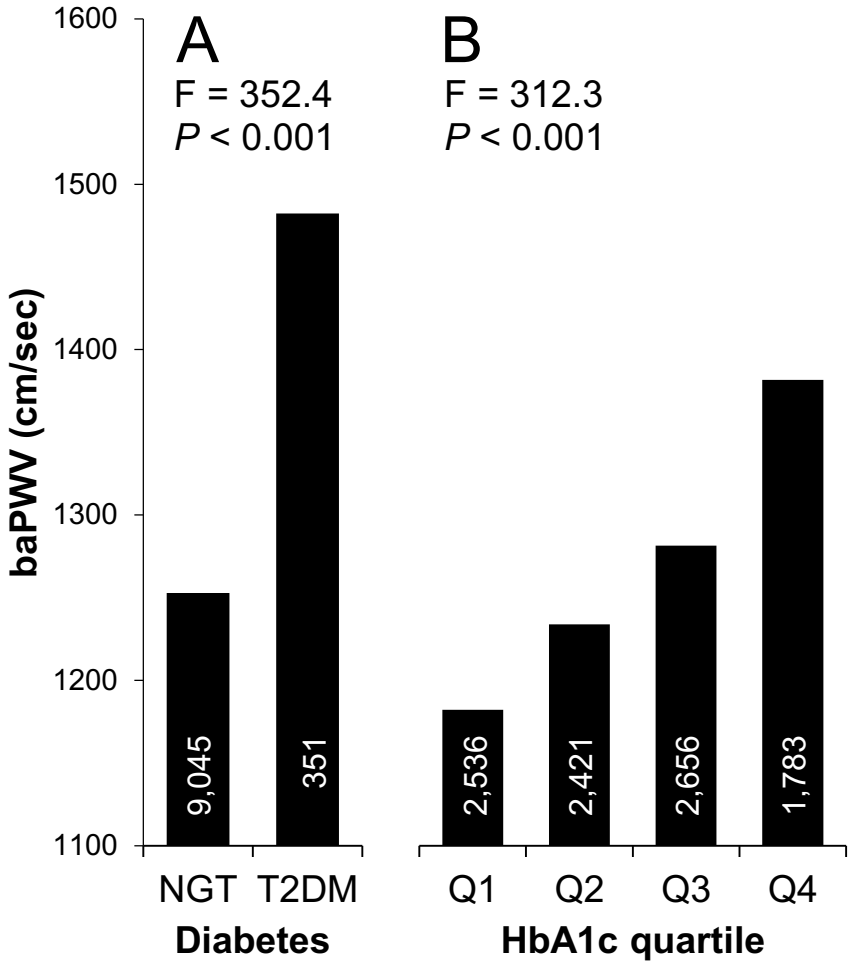
Supplementary Figure 1. Summary of present findings



Supplementary Figure 2. Smoking status and serum FFA levels.

Number of subjects in each sub-group are shown in parentheses. Statistical significance was assessed by analysis of variance.

Fasting condition at blood sampling was defined as follows; fasting, ≥12 h; near-fasting, 5 to 11 h; non-fasting, <5 h.



Supplementary Figure 3. Effect of glycemic control on arterial stiffness.

baPWV, brachial-to-ankle pulse wave velocity; T2DM, type 2 diabetes; HbA1c, glycosylated hemoglobin A1c.

Type 2 diabetes was defined as any or all of fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, HbA1c ≥6.5 %, and use of hypoglycemic treatment. Quartile of HbA1c was calculated within subgroups stratified by fasting condition (non-fasting, near-fasting, fasting) and then combined. Statistical significance was assessed by analysis of variance. Number of subjects in each subgroup are shown in column.