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Randomized Control Trials

Postprandial endotoxemia may influence the development of type 2 diabetes mellitus: From the CORDIOPREV study

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SUMMARY

Background & aims: Insulin resistance (IR) and impaired beta-cell function are key determinants of type 2 diabetes mellitus (T2DM). Intestinal absorption of bacterial components activates the toll-like receptors inducing inflammation, and this in turn IR. We evaluated the role of endotoxemia in promoting inflammation-induced insulin resistance (IR) in the development of T2DM, and its usefulness as predictive biomarker.
Methods: We included in this study 462 patients from the CORDIOPREV study without T2DM at baseline. Of these, 107 patients developed T2DM according to the American Diabetes Association (ADA) diagnosis criteria after a median follow-up of 60 months (Incident-DIAB group), whereas 355 patients did not developed it during this period of time (Non-DIAB group).
Results: We observed a postprandial increase in lipopolysaccharides (LPS) levels in the Incident-DIAB at baseline ($P < 0.001$), whereas LPS levels were not modified in the Non-DIAB. Disease-free survival curves based on the LPS postprandial fold change improved T2DM Risk Assessment as compared with the previously described FINDRISC score (hazard ratio of 2.076, 95% CI 1.149–3.750 *vs.* 1.384, 95% CI 0.740–2.589). Moreover, disease-free survival curves combining the LPS postprandial fold change and FINDRISC score together showed a hazard ratio of 3.835 (95% CI 1.323–11.114), linked to high values of both parameters.
Conclusion: Our results suggest that a high postprandial endotoxemia precedes the development of T2DM. Our results also showed the potential use of LPS plasma levels as a biomarker predictor of T2DM development.
Clinical trials.gov.identifier: NCT00924937.

Abbreviations: IR, Insulin resistance; LPS, lipopolysaccharides; ADA, American Diabetes Association; LBP, LPS binding protein; CORDIOPREV, Coronary Diet Intervention with Olive Oil and Cardiovascular Prevention study; CHD, coronary heart disease; MED, Mediterranean diet; LF, low-fat diet; ISI, Insulin sensitivity index; IGI, insulinogenic index; Incident-DIAB, non-diabetic patients at baseline who developed T2DM after a median of 60 months of follow-up; Non-DIAB, non-diabetic patients at baseline who did not develop T2DM after the follow-up period; AUC, area under the curve.

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1. Introduction

Insulin resistance (IR) and impaired beta-cell function are key determinants of T2DM, although the underlying mechanisms and the precise temporal sequence vary extensively among populations [1]. In addition to the undisputed role that IR plays in the pathogenesis and prediction of the disease [2], it is also a therapeutic target once hyperglycemia is present [3]. Specifically, skeletal muscle IR is considered to be the initiating or primary defect that may be evident decades before b-cell failure and overt hyperglycemia develops [4].

Obesity is a major contributor to IR [5] and it has been associated to the chronic activation of inflammatory pathways causally linked to IR [6]. However, despite the association between obesity and T2DM, not all obese individuals develop T2DM, notwithstanding the fact that most patients with T2DM in Western countries are obese [7].

The molecular mechanisms responsible for activating inflammatory pathways in obesity are not well understood, but differences in phenotypic flexibility may condition the presence or absence of metabolic disorders such as IR and T2DM. Thus, it has been proposed that the capacity to timely respond and adapt to changing exposures may make the difference between remaining healthy or developing the disease [8,9].To evaluate this phenotypic flexibility, the body's homeostasis must be disrupted by tests such as a fat challenge or a glucose overload, and the responses measured using appropriate biomarkers [8,9].

In addition to the environmental changes, the organism is subjected to internal challenges such as those potentially posed by dysbiosis of the microbiota. Intestinal absorption of bacterial components such as the endotoxin lipopolysaccharides (LPS), bacterial DNA and flagellins, are known to activate the toll-like receptors, inducing inflammation, which may promotes IR [10]. However, this sequence of events is not well-established, and it is still unclear whether IR precedes the changes in the microbiota or vice-versa [11]. Moreover, several studies have shown that changes in the gut microbiota trigger the pathogenic mechanisms to promote the development of obesity, T2DM and metabolic syndrome [12].

A previous study has shown the association between endotoxemia and the risk of developing diabetes [13]. However, in this study, LPS was measured in a variable postprandial period (with a median time of 5 h an interquartile range of 3–7 h), after the ingestion of a non-standardized meal with dissimilar fat and carbohydrate contents (which has been reported to affect the postprandial LPS levels [14]). Furthermore, the fat content was not adjusted by the individual's body weight and T2DM diagnosis was not performed according to the American Diabetes Association (ADA) diagnosis criteria [15]. Therefore, the relationship observed between endotoxemia and T2DM incidence was subjected to several uncontrolled confounding factors. In order to shed light on the role of endotoxemia in promoting inflammation-induced IR leading to the development of T2DM, we investigated whether longitudinal fasting and postprandial measures of LPS and LPS binding protein (LBP) may improve the prediction of T2DM incidence, in the framework of the CORDIOPREV study. In addition, the inclusion in our experimental design of a postprandial fat challenge (mixed meal), which generates metabolic stress [16,17], may help classify individuals according to their phenotypic flexibility.

2. Material and methods

2.1. Study subjects

The Coronary Diet Intervention with Olive Oil and Cardiovascular Prevention study (CORDIOPREV; Clinical Trials.gov.Identifier: NCT00924937) is an ongoing prospective, randomized, open, controlled trial of 1002 patients receiving conventional treatment for coronary heart disease (CHD) who had their last coronary event over 6 months before enrollment in one of two different dietary models [a Mediterranean (MED) diet and a low-fat (LF) diet] over a period of 7 years. The patients were recruited between November 2009 and February 2012, mostly at the Reina Sofia University Hospital, Cordoba, Spain. The eligibility criteria, design and methods of the CORDIOPREV clinical trial have been reported elsewhere [18]. Briefly, patients were eligible if they were aged between 20 and 75 years, had established CHD without clinical events in the last 6 months, were thought to follow a long-term dietary intervention and did not have severe diseases or an estimated life expectancy of less than 5 years. All patients gave their written informed consent to participate in the study. The trial protocol and all amendments were approved by the local ethic committees, following the Helsinki declaration and good clinical practices. The experimental protocol conformed to international ethical standards.

We included in present work the 462 patients who had not been clinically diagnosed with T2DM at baseline in the CORDIOPREV-DIAB [19]. However, 79 were impair fasting glucose (IFG), 81 were impair glucose tolerance (IGT), 53 were both IFG and IGT, 180 had HbA1c in the range 5.7–6.4%, and 69 had no alteration in glucose metabolism at baseline. From a total of these 462 non T2DM patients at the beginning, 107 patients developed T2DM, according to the ADA diagnosis criteria [15], after a median of follow-up of 60 months. Baseline characteristics of the subjects in the study are shown in Supplemental Table 1.

2.2. Study design

The study design has been previously described [18]. Briefly, participants were randomized to receive two diets: a MED diet or a LF diet. The LF diet consisted of <30% total fat (<10% saturated fat, 12%–14% MUFA fat, and 6–8% PUFA fat), 15% protein, and a minimum of 55% carbohydrates. The MED diet comprised a minimum 35% of calories as fat (22% MUFA fat, 6% PUFA fat, and <10% saturated fat), 15% proteins, and a maximum of 50% carbohydrates. In both diets, the cholesterol content was adjusted to <300 mg/d.

2.3. Methodology of the two metabolic challenges

Two metabolic challenges, a fat overload and an oral glucose tolerance test, were performed on consecutive days at the beginning of the study and after 3 years of follow-up. Before starting the test, the patients had fasted (food/drugs) for 12 h and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test was given. Details are provided as Supplemental Materials and Methods.

2.4. Dietary assessment

At the beginning of the study and every year, each patient had a face-to-face interview with a nutritionist to fill in a 137-item semi-quantitative food frequency questionnaire, validated in Spain [20], and well as a validated 14-item questionnaire of adherence to the Mediterranean diet to produce a Mediterranean diet score [21]. MED and LF diets were designed to provide a wide variety of foods, including vegetables, fruit, cereals, potatoes, legumes, dairy products, meat and fish. Participants in both intervention groups received the same intensive dietary counseling. Nutritionists administered personalized individual interviews at inclusion and every 6 months, and quarterly group education sessions were held

with up to 20 participants per session and separate sessions for each group.

2.5. Clinical plasma parameters

Venous blood was collected in tubes containing EDTA at the times indicated above and used to analyze the participants' biochemical variables. Lipid variables, serum insulin and plasma glucose were determined as previously reported [19]. Insulin sensitivity index (ISI), HOMA-IR, insulinogenic index (IGI) and disposition indexes were calculated as previously described [19].

2.6. Measurement of plasma biomarkers

The endotoxin lipopolysaccharide (LPS) was measured in all the 462 patients at baseline and at 3 years of follow-up using the limulus amebocyte lysate test (QCL-1000 Chromogenic LAL (Lonzalberica S.A., Spain), as previously described [22]. LPS Binding Protein (LBP) levels were determined using a human LBP ELISA kit (HycultBiotech, Netherlands). Plasma concentrations of LBP, IL-6, MCP1 and TNF-a were measured in a subpopulation of 226 patients at baseline and at 3 years of follow-up (98 Incident-DIAB, 78 of which have already developed T2DM after 3 years of follow-up, and 128 Non-DIAB). Plasma levels of IL-6, MCP1 and TNF-a were determined using the Human IL-6 Quantikine HS ELISA Kit, Human CCL2/MCP-1 Quantikine ELISA Kit, and Human TNF-alpha/TNFSF1A Quantikine HS ELISA Kit (R&D Systems, Inc.).

2.7. Statistical analysis

All data presented are expressed as mean ± SEM. PASW statistical software, version 20.0 (IBM Inc., Chicago, IL, USA) was used for statistical analysis of the data. The normal distribution of variables was assessed using the Kolmogorov–Smirnov test. When values did not follow a normal distribution, they were $\log_{10}$ transformed. P values ≤ 0.05 were considered statistically significant. Details are provided as Supplemental Materials and Methods.

3. Results

3.1. Baseline characteristics of the participants

We observed that the values of BMI, weight, waist circumference, serum triacylglycerols (TAG), HbA1c, glucose, insulin and HOMA-IR were higher and the ISI, IGI and disposition index values were lower in the Incident-DIAB than in the Non-DIAB group (all, $P < 0.05$) (Table 1).

Table 1
Baseline characteristics of the population for type 2 diabetes mellitus incidence study. Means values ± S.E.M. Incident-DIAB: patients who developed T2DM but were non-diabetic at baseline. Non-DIAB: non-diabetic patients. BMI: body mass index. HbA1c: glycated hemoglobin A1c. ISI: insulin sensitivity index. IGI: insulinogenic index. One-way ANOVA *P*-values.

	Incident-DIAB	Non-DIAB	*P*-value
n	107	355	n/a
Men/women (n)	87/20	302/53	n/a
Age (years)	58.75 ± 0.87	57.33 ± 0.50	0.171
Weight (kg)	85.70 ± 1.47	82.49 ± 0.72	0.037
BMI (kg/m^2)	31.39 ± 0.47	29.88 ± 0.22	0.002
Waist circumference (cm)	105.28 ± 1.08	101.73 ± 0.57	0.003
Serum triacylglycerols (mg/dL)	132.60 ± 6.60	119.45 ± 3.24	0.059
Total cholesterol (mg/dL)	164.97 ± 3.41	160.65 ± 1.62	0.217
HDL-cholesterol (mg/dL)	43.52 ± 1.04	44.58 ± 0.53	0.355
LDL-cholesterol (mg/dL)	93.40 ± 2.66	91.10 ± 1.33	0.421
CRP (mg/L)	2.88 ± 0.29	2.51 ± 0.17	0.329
HbA1c (%)	6.03 ± 0.03	5.86 ± 0.02	<0.001
HbA1c (mmol/mol)	42.37 ± 0.36	40.51 ± 0.19	<0.001
Fasting glucose (mg/dL)	96.18 ± 1.04	92.59 ± 0.53	0.002
Fasting insulin (mU/L)	10.51 ± 0.66	8.34 ± 0.31	0.001
ISI	3.35 ± 0.20	4.32 ± 0.14	0.001
HOMA-IR	3.37 ± 0.30	2.58 ± 0.09	0.001
IGI	0.64 ± 0.30	1.08 ± 0.06	0.025
Disposition index	0.83 ± 0.05	1.03 ± 0.03	0.003

3.2. Influence of endotoxemia in T2DM development

To investigate the influence of endotoxemia in the development of T2DM, we measured the LPS and LBP plasma levels in the fasting state and 4 h after the intake of the mixed meal at the beginning of the study (Fig. 1). At baseline, no differences were found in fasting LPS plasma levels between groups; however, we did observe a postprandial increase in LPS plasma levels after the intake of the mixed meal in the Incident-DIAB group ($P < 0.001$), while no changes were observed in the Non-DIAB group (Fig. 2). When we analyzed the LBP plasma levels, we did not observe significant differences between the groups at baseline (Fig. 3).

Moreover, we also measured LPS and LBP plasma levels after the intake of a mixed meal administered at 3 years of follow-up between the 78 out of the 107 Incident-DIAB that had already developed T2DM at 3 years of follow-up and the Non-DIAB group. LBP plasma levels (fasting and postprandial measurements together) were higher in the Incident-DIAB group than in the Non-DIAB group ($P = 0.005$) (Fig. 3).

Finally, we investigated whether diet had an effect on the observed changes in endotoxemia in our population, as patients in each group were receiving a MED diet or a LF diet. No significant differences in LPS or LBP plasma levels were found after the consumption of LF or MED diets in the entire population or in the Incident-DIAB or Non-DIAB groups separately (data not shown).

3.3. Development of postprandial lipemia and T2DM

In order to assess whether differences in the postprandial TAG levels, as measured by the chylomicron formation rate, may determine the postprandial absorption of LPS, we evaluated TAG plasma levels after the intake of the mixed meal administered at baseline and at 3 years of follow-up. We observed higher postprandial TAG levels in the Incident-DIAB group than in the Non-DIAB group at baseline after 2, 3 and 4 h following the consumption of the mixed meal ($P = 0.045$, $P = 0.028$ and $P = 0.012$, respectively).

When we analyzed the postprandial TAG levels at 3 years of follow-up between the 78 out of the 107 Incident-DIAB that had already developed T2DM at 3 years of follow-up and the Non-DIAB group, we observed higher TAG plasma levels after 3 and 4 h of the intake of the mixed meal (statistical trend, $P = 0.092$ and $P = 0.052$, respectively). Moreover, we analyzed the AUC of TAG plasma levels and we observed higher levels in the 78 out of the 107 Incident-DIAB patients that had already developed T2DM at 3 years of follow-up than in the Non-DIAB group ($P = 0.028$) (Fig. 2).

3.4. Plasma levels of inflammatory cytokines in T2DM development

In order to assess the inflammatory status before and after T2DM development, we measured plasma levels of the inflammatory cytokines IL6, TNF-a, and MCP1. Whereas no differences between groups were observed at baseline, after 3 years of follow-up, we observed higher TNF-a levels in the 78 out of the 107 Incident-DIAB patients that had already developed T2DM at 3 years of follow-up than in the Non-DIAB group (fasting and postprandial values together) ($P = 0.032$) (Fig. 3).

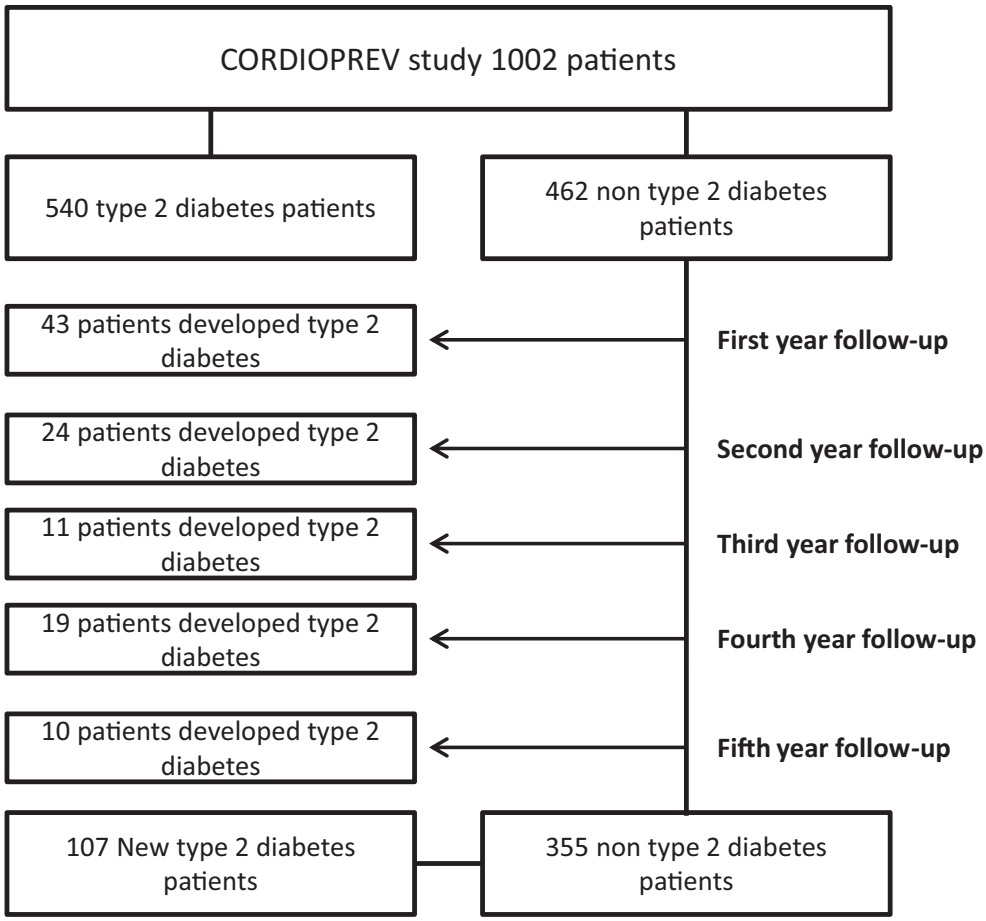


Fig. 1. Scheme of the study design.

We also analyzed the IL6 and MCP1 plasma levels, but no differences were found between groups and no effect was observed after the development of T2DM (Supplemental Fig. 1). In addition, when we analyzed the effects of diet on inflammatory cytokines, we observed no statistical differences between diets (data not shown).

3.5. Diabetes-free survival analysis

We performed a Cox proportional hazards regression analysis in order to determine the potential use of LPS postprandial levels as an independent predictor of T2DM development. To do that, the fold change between the postprandial and fasting LPS plasma levels was calculated and categorized by tertiles (ascending order). Once performed, the analysis was adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG plasma levels, HbA1c and ISI index. We observed a hazard ratio (HR) of 1.752 and 2.074 (95% CI 0.966–3.177 and 1.147–3.747, respectively) between the patients with lower LPS postprandial increase (tertile 1) and patients with intermediate (tertile2) and high (tertile 3) postprandial increase, respectively. In addition, we also categorized patients by HbA1c tertiles (ascending order), and we calculated the Cox proportional hazards regression analysis adjusted by age, gender, diet, BMI, HDL-c, and ISI index, obtaining an HR of 1.264 and 3.120 (95% CI 0.706–2.263 and 1.828–5.326, respectively) between the patients with lower HbA1c levels (tertile 1) and patients with intermediate (tertile 2) and high (tertile 3) HbA1c levels, respectively. Moreover, we also categorized the AUC of TAG by tertiles (ascending order), and we calculated the Cox proportional hazards regression analysis adjusted by age, gender, diet, BMI, HDL-c, HbA1c and ISI index, obtaining an HR of 1.452 and 1.556 (95% CI 0.802–2.628 and 0.853–2.840, respectively) between the patients with lower AUC of TAG (tertile 1) and patients with intermediate (tertile 2) and high (tertile 3) AUC of TAG, respectively. When we performed the Cox proportional hazards regression with the FINDRISC Score [23], an index that identifies individuals at high risk for T2DM, adjusted by diet, HDL-c and AUC of TAG, HbA1c and ISI index, we observed an HR of 1.519 and 1.673 (95% CI 0.860–2.683 and 0.934–2.998, respectively) between the patients with lower FINDRISC Score (tertile 1) and patients with intermediate (tertile 2) and high (tertile 3) FINDRISC Score, respectively (Fig. 4).

In addition, we combined the LPS postprandial fold change, HbA1c levels and the AUC of TAG with the FINDRISC score, dividing the latter by the median, which produced the following groups: group 1, with low FINDRISC (lower than median) and low LPS (tertile 1); group 2, with low FINDRISC (lower than median) and intermediate-high LPS (tertiles 2 and 3); group 3, with high FINDRISC (higher than median) and low LPS (tertile 1); and group 4, with high FINDRISC (higher than median) and intermediate-high LPS (tertiles 2 and 3). Cox proportional hazards regression were calculated for the LPS postprandial fold change combined with the FINDRISC, and adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG plasma levels, HbA1c and ISI index. The addition of the LPS postprandial fold change to the FINDRISC score to assess the risk of T2DM development increased to an HR of 3.977 (95% CI 1.372–11.527) between the patients with lower FINDRISC score and low LPS postprandial fold change, and patients with a high FINDRISC score and intermediate-high LPS postprandial fold change. We followed the same strategy, combining the HbA1c levels with

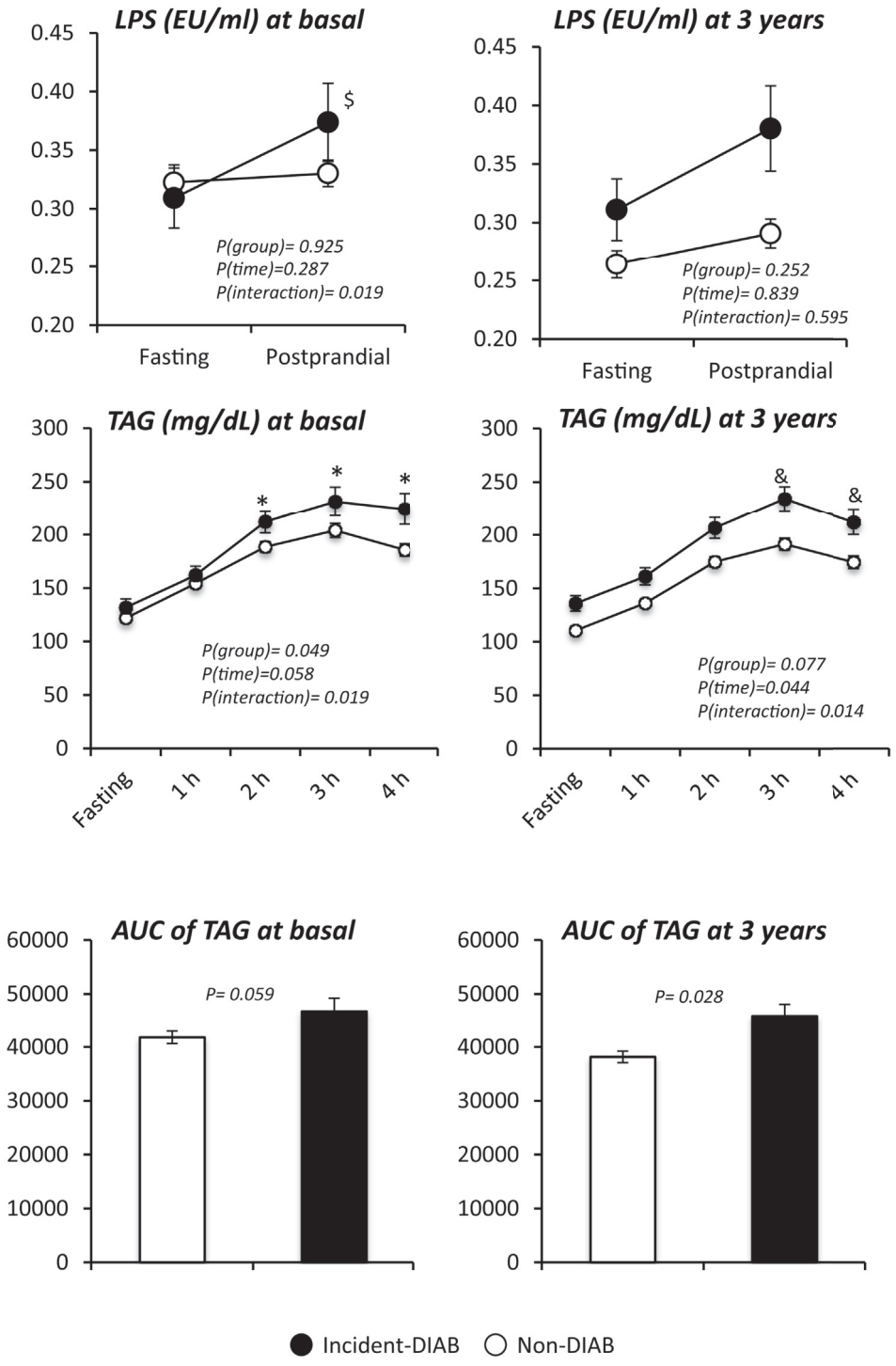


Fig. 2. Fasting and postprandial LPS and TAG circulating levels. Mean (±S.E.M.) of LPS (EU/mL) and TAG (mg/dL) plasma levels at 12-h fasting and after the administration of the mixed meal. Left panel, baseline: Incident-DIAB, patients who developed T2DM after a median of follow-up of 60 months as compared with Non-DIAB group, patients who did not develop T2DM after the follow-up period. Right panel, 3 years of follow-up: comparison between the Incident-DIAB patients who had already developed T2DM at this time (78 out of 107 patients) and the Non-DIAB group. AUC: area under the curve calculated by the trapezoidal method of TAG levels after the administration of the mixed meal at baseline and at 3 years; univariate analysis of AUC (area under curve) with age, gender and BMI as co-variables was used to determine the statistical differences between groups. LPS and TAG levels were analyzed by ANOVA for repeated measures *P*-values adjusted by age, gender and BMI. $^{\$}P < 0.05$ between postprandial and fasting in the Post-Hoc Bonferroni's multiple comparison tests. $^{*}P < 0.05$ and $^{\&}P < 0.1$ between groups in the Post-Hoc Bonferroni's multiple comparison tests. LPS, TAG, and AUC values were log transformed before statistical analysis.

the FINDRISC score, and we observed an HR of 2.890 (95% CI 1.456–5.737) between the patients with lower FINDRISC score and low HbA1c levels and patients with high FINDRISC and intermediate-high HbA1c levels. Moreover, combining the AUC of TAG with the FINDRISC score, and we observed an HR of 2.102 (95% CI 0.886–4.984) between the patients with lower FINDRISC score and low AUC of TAG and patients with high FINDRISC and intermediate-high AUC of TAG (Fig. 5).

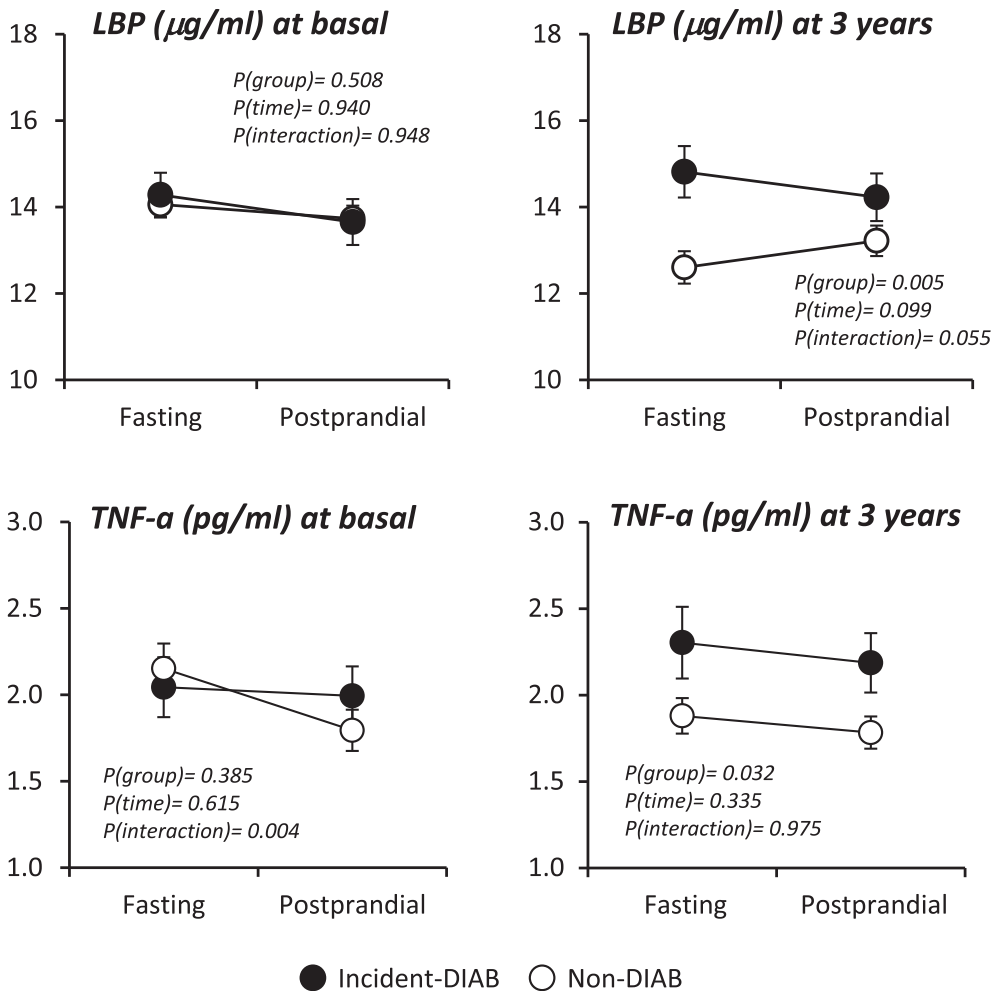


Fig. 3. Fasting and postprandial levels of LBP and TNF-a. Mean (±S.E.M.) of LBP (μg/mL) and TNF-a (pg/mL) plasma levels at 12-h fasting and after the administration of the mixed meal. Left panel, baseline: Incident-DIAB, patients who developed T2DM after a median of follow-up of 60 months as compared with Non-DIAB group, patients who did not develop T2DM after the follow-up period. Right panel, 3 years of follow-up: comparison between the Incident-DIAB patients who had already developed T2DM at this time (78 out of 107 patients) and the Non-DIAB group. ANOVA for repeated measures *P*-values adjusted by age, gender and BMI. TNF-a values were log transformed before statistical analysis.

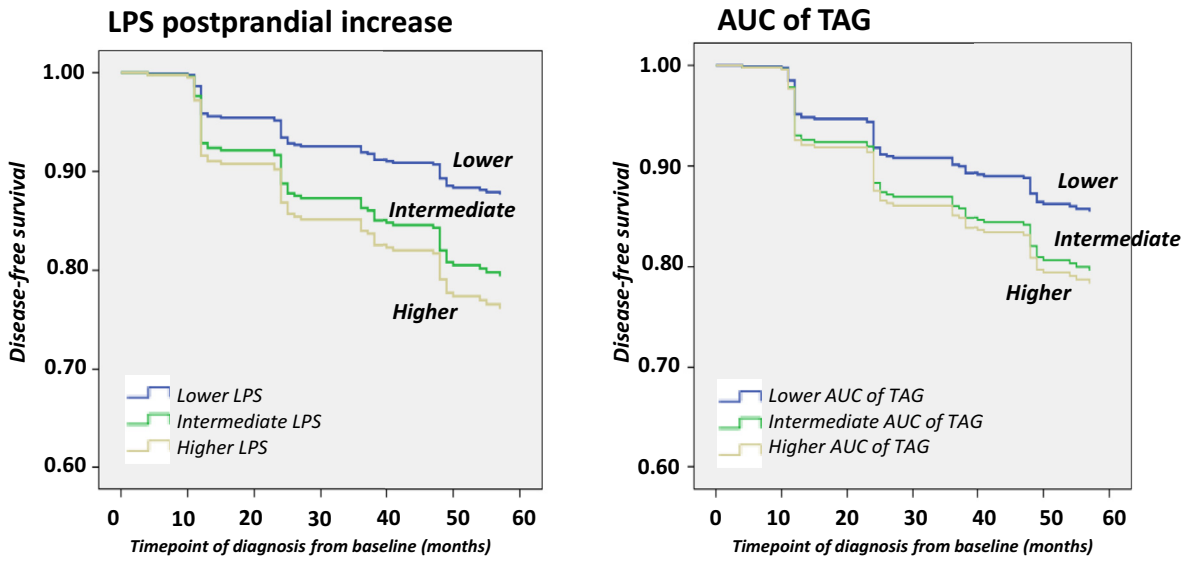
4. Discussion

Our data show a significant increase in baseline postprandial plasma levels of LPS and TAG following a mixed meal, among subjects with incident T2DM, as compared to those who remained T2DM-free after a median of follow-up of 60 months. These differences in LPS and TAG were not observed in the fasting state, which supports the usefulness of a metabolic challenge to discriminate among individuals with different phenotypic flexibility, which in turn may predict the presence or absence of metabolic disorders such as IR and T2DM [8,9].

Our intestines harbor a vast microbial community [24], which acts collectively as an organ that is fully integrated in host metabolism and is involved in energy extraction from nutrients, regulates innate and adaptive immunity and is involved in energy balance [25]. It has been depicted that the gut lumen contains more than 1 g of LPS, a component of the Gram (-) bacterial cell wall) [26]. LPS can enter systemic circulation via two major routes: the paracellular route, through tight junctions between intestinal epithelial cells (IEC) [27]; and the transcellular route, through epithelial cells [28] as a result of raft recruitment of LPS-related signaling proteins leading to signaling and endocytosis [29]. In addition, a transcellular mechanism involving LPS internalization by IEC through the apical surface and LPS transport to the Golgi, to be further incorporated into chylomicrons, has been proposed [30]. The latter would help explain the postprandial inflammatory response observed after food intake, which is closely associated with the postprandial increase of TAG in plasma [17].

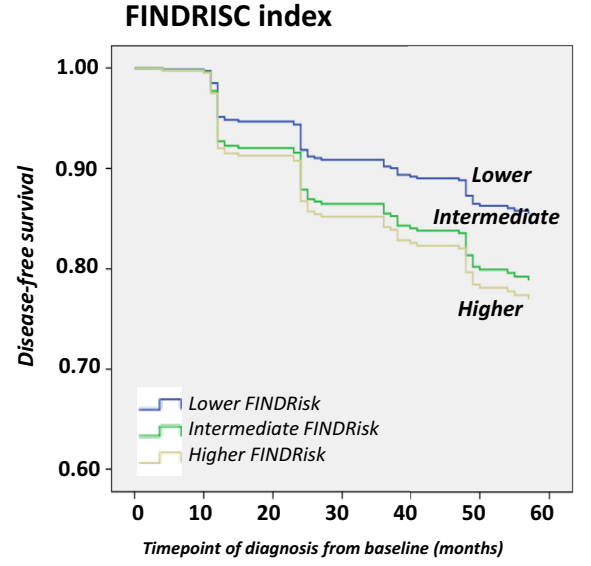
Our results suggest that a higher postprandrial TAG increase in patients who developed T2DM may facilitate higher LPS absorption through the transcellular mechanism, as chylomicron formation promotes intestinal absorption of lipopolysaccharides [30]. The fact that these differences were observed at baseline suggests that higher postprandial endotoxemia precedes the classical diagnosis of T2DM by years. Moreover, the higher LPS levels observed in the study performed at 3 years of follow-up (when 78 out of the 107 Incident-DIAB had already developed T2DM), supports the notion that once diabetes is established, there is additional impairment of the integrity of the intestinal barrier. Thus, it is likely that, at this time point, LPS is absorbed by the paracellular route through tight junctions in addition to the fat-induced LPS absorption involving chylomicron formation.

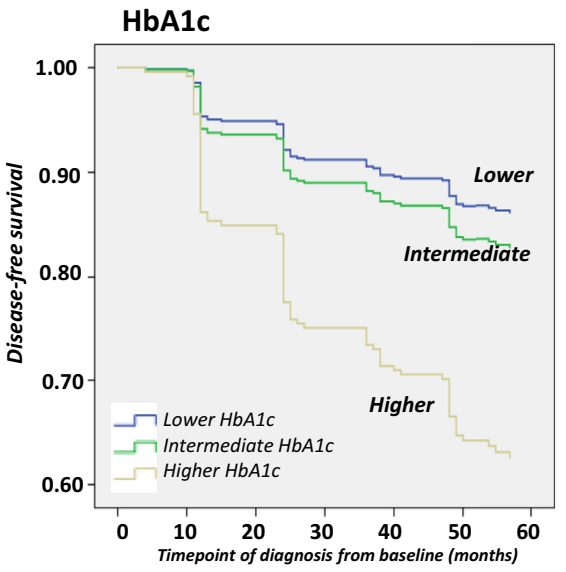
Furthermore, these changes in the intestinal barrier integrity may be associated with changes in the gut microbiota, which appears to be linked with pathogenic mechanisms that promote the development of obesity and T2DM [12,31], inasmuch as changes in



	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
FC_LPS_basal T1-T2	1.752	0,966	3.177
FC_LPS_basal T1-T3	2.074	1.147	3.747

	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
AUC_TAG_basal T1-T2	1.452	0.802	2.628
AUC_TAG_basal T1-T3	1.556	0.853	2.840





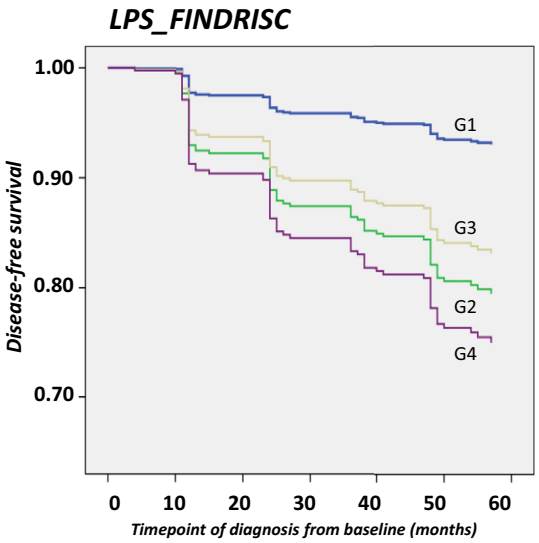
	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
FINDRISC_basal T1-T2	1.519	0.860	2.683
FINDRISC_basal T1-T3	1.673	0.934	2.998

	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
HbA1c_basal T1-T2	1.264	0.706	2.263
HbA1c_basal T1-T3	3.120	1.828	5.326

Fig. 4. Disease-free survival by COX proportional hazards regression analysis according to the postprandial increase in LPS after the fat challenge. The fold change between postprandial and fasting LPS plasma levels at baseline was calculated and patients were categorized by tertiles (ascending order). The LPS model was adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG and HbA1c and ISI index, the AUC TAG model was adjusted by age, gender, diet, BMI, HDL-c and HbA1c and ISI index, and the HbA1c model was adjusted by age, gender, diet, BMI, HDL-c and ISI index. FINDRISC model was not adjusted to avoid over-fitting. The hazard ratio (HR) between tertile 1 and tertile 2; and between expression tertile 1 and tertile 3 was calculated. T1: tertile 1; T2: tertile 2; T3: tertile 3.

gut microbiota composition have been shown to affect intestinal permeability [32,33]. This idea is supported by the fact that plasma levels of LBP, an acute phase protein that responds to invasive bacterial infection [34], were higher among those patients who had developed T2DM, possibly as consequence of changes in the gut microbiota composition.

Initially, low levels of LBP were thought to potentiate cell responses to LPS whereas high LBP concentrations were inhibitory [35]. Further studies have shown that high LBP levels appear to be associated with an increased risk of bacterial infection and immune and hemodynamic derangement, rather than suppression of these responses [36]. The latter is also supported by our study, which



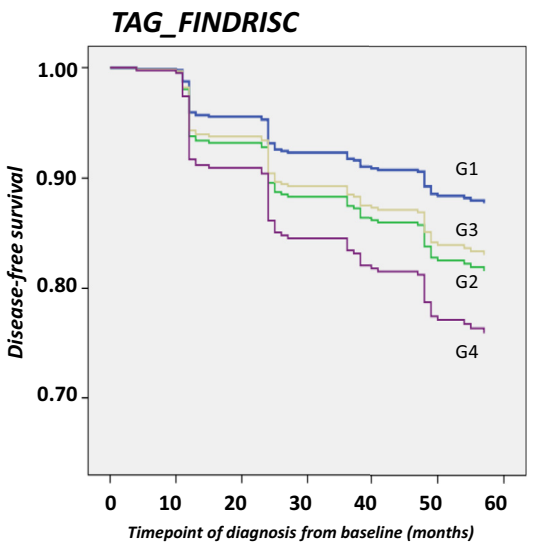
	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
LPS_FINDRISC G1-G2	3.181	1.097	9.222
LPS_FINDRISC G1-G3	2.558	0.811	8.066
LPS_FINDRISC G1-G4	3.977	1.372	11.527

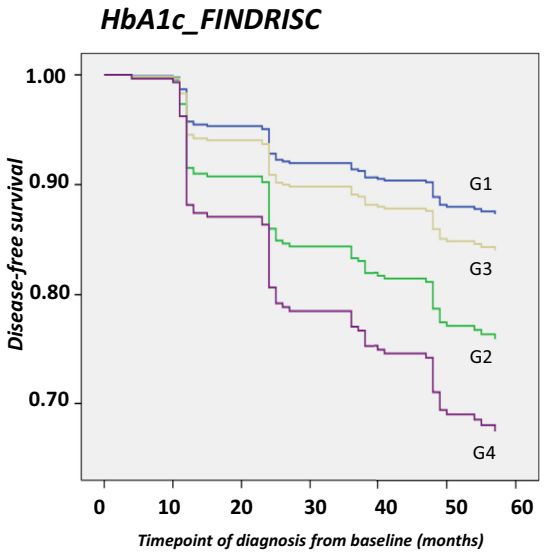
Group 1: Low FINDRISC-Low LPS

Group 2: Low FINDRISC-Intermediate/High LPS

Group 3: High FINDRISC-Low LPS

Group 4: High FINDRISC-Intermediate/High LPS





	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
TAG_FINDRISC G1-G2	1.555	0.654	3.701
TAG_FINDRISC G1-G3	1.419	0.529	3.811
TAG_FINDRISC G1-G4	2.102	0.886	4.984

Group 1: Low FINDRISC-Low TAG

Group 2: Low FINDRISC-Intermediate/High TAG

Group 3: High FINDRISC-Low TAG

Group 4: High FINDRISC-Intermediate/High TAG

	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
HbA1c_FINDRISC G1-G2	2.028	0.984	4.177
HbA1c_FINDRISC G1-G3	1.283	0.604	2.728
HbA1c_FINDRISC G1-G4	2.891	1.456	5.737

Group 1: Low FINDRISC-Low HbA1c

Group 2: Low FINDRISC-Intermediate/High HbA1c

Group 3: High FINDRISC-Low HbA1c

Group 4: High FINDRISC-Intermediate/High HbA1c

Fig. 5. Disease-free survival by COX proportional hazards regression analysis according to the postprandial increase in LPS and TAG after the fat challenge in combination with FINDRISC. Patients were categorized by the fold change in LPS plasma levels between the postprandial and fasting periods at baseline into tertiles (ascending order) and by the FINDRISC score divided by the median. Patients were categorized in the following groups: group 1, with low FINDRISC (lower than median) and low LPS (tertile 1); group 2, with low FINDRISC (lower than median) and intermediate-high LPS (tertile 2 and 3); group 3, with high FINDRISC (higher than median) and low LPS (tertile 1); and group 4, with high FINDRISC (higher than median) and intermediate-high LPS (tertile 2 and 3). LPS postprandial fold change combined with the FINDRISC model was adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG plasma levels, HbA1c and ISI index. The same was performed combining the AUC of TAG and HbA1c with FINDRISC score, and adjusted by age, gender, diet, BMI, HDL-c, HbA1c (only in AUC of TAG model), and ISI index.

showed an increase in TNF-a plasma levels in parallel with the LBP increase. Based on this, LBP plasma levels may serve as a dysbiosis marker during the development of T2DM.

In summary, our results show that high postprandial triglyceridemia-associated endotoxemia precedes the diagnosis of diabetes by years. This idea is also supported by our disease-free survival analysis, which shows that patients with a low LPS postprandial increase are at a low risk of disease as compared with those categorized as having an intermediate or high LPS postprandial increase. Moreover, compared with a previous study in the

FINDRISC97 cohort, which associated endotoxemia and T2DM development, our study showed a higher predictive value, even taking into account that our study population was smaller (462 in CORDIOPREV, hazard ratio of 2.074, 95% CI 1.156–3.619 *vs*. 7169 subjects in FINDRISC97, 1.596, 95% CI 0.870–2.927 [13]). This difference in the prediction may lie in the fact that we administered a standard mixed meal, measured LPS before and 4 h after the meal, and used the LPS fold change (defined as the postprandial/fasting ratio) as inputs for T2DM prediction. By contrast, in the study performed with the FINDRISC97 cohort, LPS was measured within a variable time range after the ingestion of a meal. In fact, participants in the FINDRISC97 study were asked to fast 4 h before blood extraction (5 h as a median time of blood sampling after meal ingestion, with an interquartile range of 3–7 h), which may have resulted in highly variable LPS levels [13]. In contrast to the CORDIOPREV study, in which the meals were the same for all participants and the fat content was adjusted by body weight [18], the participants of FINDRISC97 were only asked to avoid heavy meals. Thus, the meal was not standardized between participants in terms of fat or carbohydrate content, yet these parameters have been described to affect postprandial LPS levels [14].

In addition, in the CORDIOPREV study, the diagnosis of T2DM was performed according to the ADA diagnosis criteria [15], and assessed by clinicians at inclusion and every year, whereas the diagnosis in the FINDRISC97 cohort study was assessed by a self-administered questionnaire sent to the participants by mail, and physical measurements and blood sampling were carried out in primary care centers [37]. A more accurate T2DM diagnosis in the CORDIOPREV study, mainly in the diagnosis of the new cases of T2DM, may also be proven to increase the effectiveness of our dynamic test in the prediction of T2DM development.

Our results indicate that LPS postprandial changes improve the prediction of the risk of developing T2DM as compared with FINDRISC, a diabetes risk score based on anthropometric measures, blood pressure, physical activity and dietary habits. This should prove very useful to identify individuals at high risk for T2DM [23], and suggests that dynamic tests, such as a fat challenge, are more efficient than steady state-based measurements in assessing the phenotypic flexibility of an individual and the subsequent risk of developing metabolic disease. Notably, although individually HbA1c level was more efficient in T2DM risk prediction than the LPS postprandial increase, the LPS-FINDRISC combination was more efficient in T2DM risk prediction than the HbA1c-FINDRISC, and a high LPS postprandial fold change predictsT2DM development in patients with both high and low FINDRISC score. Moreover, our results are consistent with those obtained in animal models showing that subcutaneous infusion of LPS or high fat diet-induced endotoxemia triggers IR [38,39], and it also agrees with human studies pointing to IR as the initiating or primary defect in T2DM [4]. Our study also showed that the patients who developed T2DM after a median of follow-up of 60 months had higher postprandial endotoxemia together with lower insulin sensitivity at baseline, as shown by the insulin sensitivity indexes investigated. Taken together, our results suggest that an alteration in the intestinal barrier takes place before T2DM development, presumably by changes in gut microbiota, as evidenced by LBP plasma levels, which increased the LPS intestinal absorption, with the subsequent increases in TNF-a plasma levels. This is especially important, as TNF-a has been shown to cause IR by increasing serine phosphorylation on insulin receptor substrate-1 leading to its inactivation [40]. Thus, our results support the role of the intestine in the development of T2DM, as increased intestinal permeability may allow the absorption of pro-inflammatory bacterial components which induce inflammation and subsequently IR [10].

Our study has limitations. One limitation lies in the fact that the prevention of T2DM was not the primary endpoint of the CORDIOPREV trial, but was rather a secondary analysis conducted in the subgroup of cardiovascular patients without T2DM at baseline. In fact, the study included a large number of patients with acute myocardial infarction (AMI), which limits our findings to people with these characteristics and precludes its generalization to healthy individuals. Although diabetes prediction is very important, since patients with AMI and T2DM have a considerably higher risk of developing a new cardiovascular event than those without T2DM [41], validation in a cohort without cardiovascular disease and closer to general population would allow us to apply these methods to the general population.

Our results show that an elevated postprandial endotoxemia precedes the development of T2DM in patients with acute myocardial infarction and probably plays a role in promoting inflammation-induced insulin resistance and/or beta-cell dysfunction. In addition, our results also support the role of LPS plasma levels as a predictor biomarker and as a pathogenic factor in T2DM development. Further studies are needed to assess the potential changes in the integrity of the intestinal barrier that may precede T2DM development and the role of gut microbiota in this process.

Statement of authorship

A.C., R. J-L., J.F. A.-D., O.A. R.-Z., S. G.-C., J. L.-M. performed the experiments. A.C., R. J-L., J D-L, P.P-.M. drafted the manuscript. R B-R, J D-L, P.P-.M. performed the data analysis and results interpretation. J D-L, P.P-.M., B.v.O., J.M.O., M. M.-M., F.P.-J. and J.L.-M. contributed to the writing of the manuscript and revised it critically for important intellectual content. A.C., R. J-L., F.P.-J. and J.L.-M. conceived and designed the experiments. F.P.-J. and J.L.-M. have the responsibility for the contents of the article.

Conflict of interest

The author reports no conflicts of interest in this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.03.016.

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