Incretin treatment and atherosclerotic plaque stability: Role of adiponectin/APPL1 signaling pathway

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ABSTRACT

Aims: Glucagon like peptide 1 (GLP-1) analogues and dipeptidyl peptidase IV (DPP-4) inhibitors reduce atherosclerosis progression in type 2 diabetes mellitus (T2DM) patients and are associated with morphological and compositional characteristics of stable plaque phenotype. GLP-1 promotes the secretion of adiponectin which exerts anti-inflammatory effects through the adaptor protein PH domain and leucine zipper containing 1 (APPL1). The potential role of APPL1 expression in the evolution of atherosclerotic plaque in TDM2 patients has not previously evaluated.
Methods: The effect of incretin therapy in the regulation of adiponectin/APPL1 signaling was evaluated both on carotid plaques of asymptomatic diabetic (n = 71) and non-diabetic patients (n = 52), and through in vitro experiments on endothelial cell (EC).
Results: Atherosclerotic plaques of T2DM patients showed lower adiponectin and APPL1 levels compared with non-diabetic patients, along with higher oxidative stress, tumor necrosis factor-α (TNF-α), vimentin, and matrix metalloproteinase-9 (MMP-9) levels. Among T2DM subjects, current incretin-users presented higher APPL1 and adiponectin content compared with never incretin-users. Similarly, in vitro observations on endothelial cells co-treated with high-glucose (25 mM) and GLP-1 (100 nM) showed a greater APPL1 protein expression compared with high-glucose treatment alone.
Conclusions: Our findings suggest a potential role of adiponectin/APPL1 signaling in mediating the effect of incretin in the prevention of atherosclerosis progression or plaque vulnerability in T2DM.

1. Introduction

Cardiovascular disease (CVD) based on atherosclerosis is the major cause of mortality in subjects with type 2 diabetes mellitus (T2DM) (Grundy et al., 1999). T2DM is well known to accelerate the clinical course of atherosclerosis, an inflammatory disease of the arterial wall, in which leukocytes and oxidized lipoproteins accumulate, leading to the formation of atherosclerotic plaques (Creager, Luscher, Cosentino, & Beckman, 2003). The conversion of a stable plaque to a vulnerable plaque involves many processes, including inflammation, cellular breakdown, expansion of the cellular component, thinning of the fibrous cap, formation of a large lipid core, and intraplaque hemorrhage. Inflammatory events secondary to diabetes play a key role in the plaque erosion and fissuring (Menegazzo et al., 2015).

Several new pharmacological compounds have been developed to treat T2DM patients including glucagon like peptide 1 (GLP-1) analogues and dipeptidyl peptidase IV (DPP-4) inhibitors (Kim & Egan, 2008). Recent studies suggest that treatment with DPP-4 inhibitors and GLP-1 analogues has a protective effect on CVD (Ussher & Drucker, 2012). In particular, DPP-4 inhibitors have been recently shown to reduce atherosclerosis progression in T2DM patient probably through the reduction of over daily inflammation and oxidative stress (Barbieri et al., 2013). Furthermore, more recent studies demonstrated the incretin treatment to be associated with morphological and compositional characteristics of a potential stable plaque phenotype (Balestrieri et al., 2015; Burgmaier et al., 2013). In particular, it has been demonstrated that GLP-1 reduces plaque inflammation and increase phenotypic characteristics of plaque

stability in a murine model of atherosclerosis (Balestrieri et al., 2015; Burgmaier et al., 2013; Gaspari, Welungoda, Widdop, Simpson, & Dear, 2013). The mechanisms underlying the decreased risk of developing cardiovascular disease are still poorly understood. Interestingly, treatment with exendin-4, an agonist of the GLP-1 receptor, promotes increases of adiponectin expression and plasma levels in high fat-fed rats (Li et al., 2008). Furthermore, administration of liraglutide prevented hypoadiponectinemia-induced increases in plasma insulin, free fatty acids, triglycerides and total cholesterol in high fat diet APOE−/− mice (Li et al., 2011). Liraglutide also attenuated hypoadiponectinemia-induced deterioration in peripheral and hepatic insulin sensitivity and alterations in key regulatory factors implicated in glucose and lipid metabolism (Li et al., 2011). In Japanese T2DM patients, serum adiponectin levels increased after three-month treatment with sitagliptin without change of body weight (Nonaka et al., 2008). Furthermore, treatment of T2DM patients with sitagliptin reversed vascular endothelial dysfunction, as evidenced by the increase in the flow mediated dilation (FMD) (Kubota et al., 2012). The changes in the FMD significantly correlated with those of the plasma adiponectin.

Numerous studies have shown that low plasma adiponectin concentrations are associated with increased plaque vulnerability in patients with coronary artery disease (Sawada et al., 2010). The anti-atherosclerotic effects of adiponectin have been demonstrated at several stages of plaque development, ranging from endothelial dysfunction and plaque initiation to plaque progression and rupture (Sawada et al., 2010).

Adiponectin exerts its effects through two membrane receptors, ADIPOR1 and ADIPOR2 (Nonaka et al., 2008). Adaptor protein PH domain and leucine zipper containing 1 (APPL1) and adaptor protein PH domain and leucine zipper containing 2 (APPL2) are the first identified adaptor proteins that interact directly with adiponectin receptors (Deepa & Dong, 2009). They are highly expressed in insulin target tissues, including skeletal muscle, liver and adipose tissue and mediate adiponectin effects on metabolism via binding to N terminus of adiponectin receptors (Deepa & Dong, 2009). Adiponectin signaling through APPL1 is necessary to exert its anti-inflammatory and cytoprotective effects on EC (Mao et al., 2006; Wang et al., 2011).

Furthermore, APPL1 also acts as a mediator of other pathways by interacting directly with membrane receptors or signaling proteins, playing critical roles in cell proliferation, apoptosis, cell survival, endosomal trafficking, and chromatin remodeling (Han, 2012) and represents an important key regulator of insulin signaling and secretion (Ryu et al., 2014). To this regard, it has been shown that knockout (KO) of APPL1 in mice reduced insulin and adiponectin signaling and led to systemic insulin resistance. Indeed, both insulin resistance (IR) and hyperglycemia play important roles in atherogenesis promoting, both early stages of lesion formation and clinically relevant advanced plaque progression and contributing to atheroma plaque vulnerability which is partly due to augmented inflammatory pathway expression within the plaque (Martínez-Hervás et al., 2014).

No evidence exist about a potential role of APPL1 expression in the evolution of atherosclerotic plaque in T2DM patients. We hypothesized that diabetes may affect the anti-inflammatory and cytoprotective effects of APPL1 on EC favoring the instability of atherosclerotic plaques phenotype and that an incretin-induced increase in adiponectin/APPL1 signaling may contribute to the stabilization of plaque, thus, preventing atherosclerosis progression in T2DM patients. Thus, this study was designed to identify differences in adiponectin, APPL1 expression, as well as in inflammatory infiltration, in carotid plaques of asymptomatic diabetic and non-diabetic patients. Indeed, the effects of incretin therapy on APPL-1 expression in carotid plaques of diabetic patients, were also investigated. Furthermore, the incretin effect on APPL-1 expression was also evaluated by a set of in-vitro experiments on endothelial cell (EC).

2. Research design and methods

Patients were recruited from the outpatient Department of Cardiology and Cardiovascular Surgery of the Cardarelli Hospital, Naples, Italy, from January 2009 to December 2014. Among them 71 type 2 diabetic and 52 non-diabetic patients (non-diabetic group) with asymptomatic carotid stenosis (according to North American Symptomatic Carotid Endarterectomy Trial classification), enlisted to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis (Young et al., 1996) were selected. Asymptomatic patients underwent a baseline clinical examination, medical history, and computed tomography or MRI. Carotid sonography was performed on a single ultrasound machine (Aloka 5500). Diabetes was diagnosed according to American Diabetes Association criteria (Moghissi et al., 2009). T2DM patients answered a specific questionnaire about medicines used for diabetes treatment, the date of the beginning and end of treatment, route of administration, and duration of use. Questionnaire was developed for producing data about patients medication use for treating diabetes. Structured closed-ended questionnaire was prepared in Italian language and was administered by trained experienced clinicians. The collected data were reviewed and checked for completeness and consistency by diabetologist. Questions were simple, clear and easy to understand. Questionnaire was completed by all the patients enrolled and the collected data were compared with information from clinical chart or general practice doctor's prescription. T2DM patients who never used incretin, such as GLP-1 agonists and DPP-4 inhibitors, were classified as "never incretin-users." T2DM patients who had already used GLP-1 agonists or DPP-4 inhibitors were classified as "current incretin-users." Among the 71 T2DM patients enrolled, 38 were current incretin-users, and 33 were never incretin-users. Information on duration of treatment was available for all current incretin-users. A duration of incretin treatment was 38 ± 6 months. All patients had no clinical or laboratory evidence of heart failure, previous stroke, valvular defects, malignant neoplasms, or secondary causes of hypertension nor neurologic symptoms or cerebral lesions as assessed by computed tomography. During the observational period, dietary counseling was added to the previous therapy. The study was approved by the ethics committee of Cardarelli Hospital, and informed written consent was obtained for each patient. The study has been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008.

2.1. Laboratory analysis

After an overnight fast, plasma glucose, HbA1c, serum lipids were measured by enzymatic assays. Adiponectin plasma levels were measured with Orgenium Laboratories' human ELISA test. For estimation of circulating intact GLP-1, plasma immunologic active form of GLP-1 (7-36) was determined using a specific ELISA kit (Active GLP-1 7-36, Epitope). Blood samples were collected in ice-cooled blood collection system for plasma GLP-1 preservation tubes (BD P700) and immediately, were centrifuged at 2500 rpm for 10 minutes in refrigerated centrifuge. Samples were stored at −80 °C. In particular GLP-1 levels (Active GLP-1 7-36, Epitope) were measured after an overnight fast and after breakfast. Standardized breakfast contained 419 kcal (57% carbohydrate, 17% protein, and 26% fat). After breakfast, blood samples for measurement of GLP-1 were obtained every 30 min along 2 h. The mean of the four GLP-1 evaluations was defined as post-prandial GLP-1 value.

2.2. Plaque phenotype

In order to characterize plaque phenotype, inflammatory infiltration, matrix metalloproteinases (MMPs), TNF-α, nitrotyrosine, and vimentin content have been evaluated.

Stable plaques are rich in VSMC and collagen with few inflammatory cells whereas unstable high risk plaques that are prone to rupture contain few VSMCs, more macrophages and little collagen (Adiguzel, Ahmad, Franco, & Bendeck, 2009; Dhume & Agrawal, 2003; Spagnoli, Bonanno, Sangiorgi, & Mauriello, 2007). The stability of atherosclerotic plaques depends on the balance between the matrix formation and degradation of extracellular matrix (ECM) (Molloy et al., 2004; Rao, Kansal, Stoupa, & Agrawal, 2014). The activated macrophages and T-lymphocytes present in atherosclerotic plaques secrete matrix metalloproteinases (MMPs) that degrade extracellular matrix proteins, and weaken the fibrous cap, leading to myocardial infarction and stroke. Indeed, VSMCs may exhibit a differential protein content as a result of atherosclerosis development. Vimentin is a structural component of microtubules and intermediate filaments. Strong vimentin staining has been detected in the active areas of inflammation at the edge of necrotic and inflammatory areas and at the plaque shoulder. The non-lesion side of the artery showed minimal immunoreactivity. The cells involved in the atherosclerotic plaques secrete inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-12 (IL-12) (Tedgui & Mallat, 2006). Among these, TNF-α, a powerful pleiotropic cytokine with multiple cellular functions, plays a role in the inflammation, initiation, development, susceptibility, severity, and response to treatment, etc. Interestingly the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) can trigger secretion of vimentin (Rao, Rai, Stoupa, Subramanian, & Agrawal, 2016). Activation of inflammatory cells is also associated with an increase in oxidative stress potentially promoting plaque rupture. Beckman et al. first detected protein nitrotyrosine formation in early subintimal fatty streaks and in foamy macrophages within lesions of human coronary arteries in 1994 (Beckmann et al., 1994). In vitro studies demonstrated that nitrotyrosine is a highly specific marker of LDL oxidized. Nitration of LDL is thought to interfere with cholesterol transport and trigger the release of TNF-α, which can amplify the inflammatory response (Smythe, Skinner, Bruckdorfer, Haskard, & Landis, 2003).

2.3. Atherectomy specimens

Specimens taken during the carotid endoarterectomy were cut perpendicular to the long axis into two halves. The first half was frozen in liquid nitrogen for the following ELISA analysis. A portion of the other half specimen was immediately immersion-fixed in 10% buffered formalin. Sections were serially cut at 5 μm, mounted on lysine-coated slides, and stained with hematoxylin and eosin and with the trichrome method. Carotid artery specimens were analyzed by light microscopy.

2.4. Immunohistochemistry

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Similar regions of the plaque were analyzed (Fig. A.1). Serial sections were incubated with specific antibodies against adiponectin (1:50, Santa Cruz), APPL1 (1:500, Santa Cruz), nitrotyrosine (1:50 Santa Cruz), CD3 (1:25, Dako), matrix metalloproteinase-9 (MMP-9) (1:50, Santa Cruz), tumor necrosis factor-α (TNF-α) (1:20 R&D), CD68 (1:50, Santa Cruz), and vimentin (1:400, Dako). The sections were processed and colored using the automatic immunostainer "Bench-Mark 7600"; the positivity of sections is given by the binding of a chromogen, diaminobenzidine, to the site of the antigen–antibody reaction which develops a brown coloration. Quantitative immunohistochemistry analysis was performed by quantitative 24-bit color image analysis system (IM500; Leica Microsystem AG) and ImageJ software. Results are expressed as percentage of positivity.

2.5. Biochemical assays

Plaque content of MMP9, TNF-α, and nitrotyrosine was evaluated by ELISA assays. Specific kits were used to quantify MMP-9 (Santa Cruz), TNF-α (R&D Systems), and nitrotyrosine (Imgenex) levels according to the calibration curves provided by manufacture's protocol. Total protein were determined by Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951). Each determination was repeated at least three times.

2.6. Endothelial cell culture and treatments

Human aortic ECs (HAECs) (Lonza Cologne, Walkersville, MD, USA) were cultured with EGM-2MV BulletKit (Lonza) supplemented with 5% FBS, 0.6% HEPES, at 37 °C in a 5% Co_2 humidified atmosphere, and used between passage 4 and 7. Short-term exposure to high-glucose (25 mM) was carried out in the presence or absence of GLP-1 receptor agonist (Exenatide, Byetta) (100 nM) in complete culture media for 3 days. To this end, ECs were cultured in six-well plates (Costar, Corning, NY, USA) at 70–80% confluence. GLP-1 receptor agonist was added 30 min before starting the high-glucose treatment and left in the culture media throughout the high-glucose treatment. Control cells were cultured for 3 days under basal conditions.

2.7. Confocal laser-scanning microscopy

Immunofluorescence detection by confocal laser-scanning microscope analysis (Zeiss LSM 700) was performed on deparaffinized atherosclerotic plaque sections from patients enrolled and on in vitro-cultured EC, as previously described (Balestrieri et al., 2015). Samples were incubated overnight at 37 °C with antibodies against APPL1 (1:500) (Abcam, Cambridge, UK), von Willebrand factor (1:500) (Abcam, Cambridge, UK), and vimentin (1:1000) (Sigma, St. Louis, MO, USA). Secondary antibodies were Alexafluor 633 (1:1000) or Alexafluor 488 (1:1000) (Invitrogen, Life Technologies Italia, Monza, Italy). The fluorescence intensity was quantified with ImageJ software and expressed as arbitrary fluorescence units (AFU).

2.8. Western blot analysis

Protein extracts of plaque tissues and EC were prepared as previously described (Balestrieri et al., 2015). Briefly, tissues (400 mg) were washed twice in PBS and cut into small pieces. Protein extraction was performed by adding 800 μl of 2D lysis buffer (ratio weight/volume 1:2) (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl, pH 8.8). Samples were homogenized using a tissue homogenizer (Precellys 24, Bertin Technologies, Bertin Pharma, Montigny-le-Bretonneux, France). Tubes containing homogenized tissue were placed on a tube shaker for 30 min and then centrifuged at 800 $\times g$ for 10 min at 4 °C to collect the supernatant. An aliquot of the supernatant was used for total protein determination by the Lowry method (Lowry et al., 1951). Protein extracts (15 μg) were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by Trans-Blot Turbo Transfer System (BioRad). Membranes were incubated overnight at 4 °C with antibodies against APPL1 (1:3000) (Abcam, Cambridge, UK cod 59592) followed by incubation with secondary antibody. Membranes were normalized with a polyclonal antibody against γ-tubulin protein (1:1000) (GTU-88) (Sigma, St. Louis, MO, USA). Band detection was performed by the enhanced chemiluminescence kit (ECL, Amersham, Aylesbury, UK) and semiquantitative densitometry by Scan LKB (Amersham Pharmacia).

2.9. Statistical analysis

All variables are presented as means ± standard deviation (SD). The in vitro data shown were mean values of at least four independent experiments and expressed as mean ± SD. In order to investigate differences between study groups, sample size was estimated by GPOWER software. The resulting sample size, estimated according to a global effect size of 0.30 with type I error of 0.05 and a power of 80% was 111 patients.

Analysis of variance (ANOVA) with Scheffe's test was used for analyze differences among different groups. Statistical significance was set at a level of $P < 0.05$. Pearson product-moment correlations were calculated to test associations among variables. Statistical analyses were performed using the SPSS statistical package.

3. Results

Clinical characteristics of the study population are presented in Table 1. Percentage of carotid diameter reduction, risk factors, and concomitant non-hypoglycemic therapy did not differ among the groups. In T2DM patients, mean plasma glucose, BMI and HbA1c levels did not differ between never and current incretin-users. However,

fasting plasma adiponectin, basal and post-prandial GLP-1 levels were higher in current compared to never incretin-users ($P < 0.01$). Both basal and post prandial plasma GLP-1 correlated positively with plasma adiponectin levels (respectively $r = 0.52$; $p < 0.01$ and $r = 0.65$; $p < 0.01$) and negatively with plaque TNF-α (respectively $r = -0.60$; $p < 0.01$ and $r = -0.78$; $p < 0.01$), MMP9 (respectively $r = -0.57$; $p < 0.01$ and $r = -0.82$; $p < 0.01$) and nitrotyrosine (respectively $r = -0.57$; $p < 0.01$ and $r = -0.78$; $p < 0.01$) content.

3.1. Plaque composition

Compared with non-diabetic patients, both diabetic incretin-users (n = 38) and never incretin-users (n = 33) had a significantly greater portion of plaque area occupied by macrophages and T-cells (Table 1 and Fig. 1). Among diabetic patients, current incretin-users group had a significant smaller portion of plaque area occupied by macrophages ($P < 0.01$) and T-cells ($P < 0.01$) compared with the never incretin-user group (Table 1 and Fig. 1). Both immunohistochemistry and ELISA revealed markedly higher staining and levels of TNF-α in both current ($p < 0.05$) and never incretin-users ($p < 0.05$) compared with non-diabetic lesions. Among diabetic patients, staining and levels of TNF-α were significantly more abundant in

Table 1
Clinical characteristic of study population (n = 123).

Patients characteristics	Non diabetic (N = 52)	Never incretin-users (N = 33)	Current incretin-users (N = 38)
Age, years	73.7 ± 4.5	71.3 ± 5.8	72.2 ± 4.5
Sex (female/male)	28/24	19/14	24/14
BMI (kg/m2)	28.5 ± 1.7	30.6 ± 1.9	29.5 ± 2.4
Systolic blood pressure (mm Hg)	129 ± 12.8	129 ± 21.3	128 ± 13.3
Diastolic blood pressure (mm Hg)	77 ± 5.7	77.3 ± 6.3	79.4 ± 6.6
Total cholesterol (mmol/l)	5.53 ± 1.04	5.71 ± 1.17	5.68 ± 0.95
HDL cholesterol (mmol/l)	1.39 ± 0.42	1.26 ± 0.71	1.27 ± 0.24
Triglycerides (mmol/l)	1.85 ± 0.23	2 ± 0.27	1.86 ± 0.30
HbA1C (%)	5.2 ± 0.5*†	8.3 ± 0.6	7.9 ± 0.8
Blood glucose (mmol/l)	4.9 ± 0.32*†	8.9 ± 1.14	8.8 ± 1.16
Insulin (μU/ml)	11.22 ± 2.24*†	14.03 ± 1.04	13.31 ± 1.67
Basal GLP-1 (pmol/l)	10.3 ± 1.26*†	7.9 ± 1.77	9 ± 0.82*
Postprandial GLP-1 (pmol/l)	27.1 ± 3.05*†	16.4 ± 2.66	25 ± 3.09*
Fasting adiponectin (μg/ml)	9.65 ± 1.5*†	5.68 ± 1.26	8.78 ± 0.73*
CRP (mg/dl)	1.66 ± 0.17*†	2.06 ± 0.30	2.04 ± 0.11
Coronary artery disease	26 (50)	19 (58)	23 (62)
Hypertension	22 (42)	16 (48)	19 (50)
Hypercholesterolemia	18 (38)	14 (43)	17 (44)
Cigarette smoking	17 (32)	10 (30)	10 (26)
Drug use (%)			
Aspirin	42 (80)	29 (90)	36 (94)
Warfarin	4 (8)	2 (6)	2 (6)
β-Blocker	16 (30)	8 (25)	11 (28)
Calcium-channel blocker	6 (12)	5 (16)	6 (16)
Statin	35 (68)	25 (77)	29 (76)
ACE inhibitor	25 (48)	20 (62)	24 (64)
Diuretic agent	17 (32)	9 (28)	10 (26)
AT-2 antagonist	21 (40)	12 (36)	13 (34)
Insulin	–	5 (16)	4 (12)
Sulfonylureas	–	5 (16)	5 (14)
Metformin	–	24 (73)	23 (62)
PPAR-γ agonists	–	–	–
Acarbose	–	5 (15)	7 (18)
GLP-1agonists	–	–	5 (14)
DPP-4 inhibitors	–	–	33 (88)
Plaque characteristics			
Macrophage-rich areas, %	6.1 ± 1.4*†	24.8 ± 2.6	17.1 ± 1.9*
T-cells per mm2 section area	16.4 ± 6.4*†	72.5 ± 14.9	29.1 ± 7.3*
Vimentin, %	8.9 ± 2.3*†	28.3 ± 2.03	19.2 ± 1.9*
MMP-9, μg/mg	4.2 ± 1.5*†	17.9 ± 1.2	8.9 ± 1.1*
Nitrotyrosine, nmol/pg	1.8 ± 0.6*†	6.5 ± 0.8	3.4 ± 0.7*
TNF-α, pg/mg	24.4 ± 4.18*†	94.4 ± 6.02	61.4 ± 6.6*

Data are presented as mean ± SD, or number (%). CRP = C-reactive protein; IHD = ischemic heart disease; BMI = body mass index; ACE = angiotensin-converting enzyme; HDL = high-density lipoprotein; peroxisome proliferator–activated receptor = PPAR-γ; GLP-1 = glucagon-like peptide-1; DPP-4 = dipeptidyl-peptidase-4.
  * $P < 0.05$ compared with never current user group.
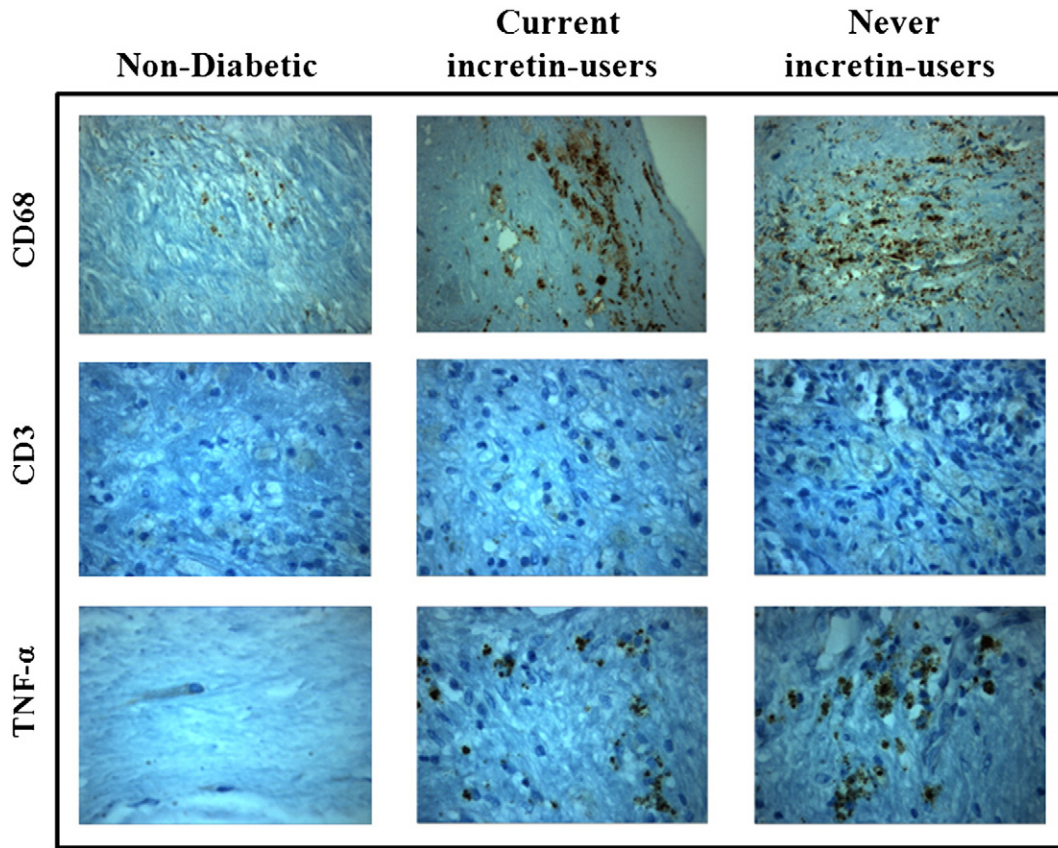 † $P < 0.05$ compared with current user group.

Fig. 1. Inflammation in atherosclerotic plaques. Representative images of immunochemical analysis of macrophages (CD68) (X400), lymphocytes (CD3) (X400) and TNF-α (X400) in control, current incretin-user, and never incretin-user asymptomatic plaques. Similar regions of plaque are shown. These results are typical of control, current incretin-user, and never incretin-user asymptomatic plaques.

lesions from never incretin-users than lesions from the current incretin-users group ($P < 0.001$) (Table 1 and Fig. 1). MMP-9 levels were more abundant in diabetics current ($P < 0.01$) and never incretin ($P < 0.01$) than in non-diabetic lesions. In particular, MMP-9 levels were more abundant in lesions from diabetic never incretin-users than lesions from the current incretin-user group. As far as vimentin, higher content was found in plaques of both current ($P < 0.01$) and never incretin-users ($P < 0.01$) compared with non-diabetic patients (Table 1 and Fig. 2). Content of vimentin of plaques from never incretin-users was higher than lesions from the current incretin-user group ($P < 0.001$) (Fig. 2). Higher nitrotyrosine levels were found in diabetic than in non-diabetic plaques ($P < 0.001$vs. both current and never incretin-users). Among diabetic plaques, nitrotyrosine levels were significantly higher in never incretin-users than in current incretin-users ($P < 0.01$) (Fig. 2).

3.2. APPL1 protein levels in atherosclerotic plaques from diabetic and non-diabetic patients

Both immunohistochemistry (Fig. 2) and confocal laser-scanning microscopy analyses (Fig. 3, panel A and B) revealed that levels of APPL1 were consistently lower in plaques from diabetic patients compared to plaques from non-diabetic patients and, specifically, in plaques from diabetic never incretin-users ($P < 0.01$). APPL1 immunofluorescence expression levels were found consistently lower in plaques from diabetic never incretin-users compared to non-diabetic patients (20.05 ± 1.8 vs. 31.05 ± 2.01 arbitrary fluorescence units (AFU)) ($P < 0.05$). Levels of APPL1 in plaques from current incretin-users were significantly higher than those observed in never incretin-users (26.58 ± 1.5 vs. 20.05 ± 1.8 AFU) ($P < 0.05$)

(Fig. 3 panel A). Likewise, Western blot analysis of APPL1 protein levels in atherosclerotic plaques from non-diabetic and diabetic patients (current and never incretin-users) showed a similar trend (Fig. 3 panel B). Indeed, never incretin-users APPL1 arbitrary units (AU) were 48.2% lower than non-diabetic patients ($P < 0.01$) whereas APPL1 levels of current incretin-users were 21% lower than non-diabetic patients ($P < 0.05$). Moreover, immunofluorescence co-expression of APPL1 and von Willebrand factor suggested that APPL1 is expressed at endothelial levels and confirmed the lower APPL1 expression level in plaques from diabetic never incretin-users compared to current incretin-users and non-diabetic patients (Fig. A.2). Finally, adiponectin levels have been found to be lower in plaques from diabetic patients compared to plaques from non-diabetic patients and, specifically, in plaques from diabetic never incretin-users ($P < 0.01$) (Fig. 2).

3.3. High-glucose (hGluc) and GLP-1 receptor agonist effect on APPL1 expression in EC

The effect of high-glucose (hGluc) concentration and GLP-1 receptor agonist was evaluated in vitro on cultured EC. Confocal laser scanning microscopy revealed that short-term exposure to hGluc down-regulates APPL1 protein after 18 h ($P < 0.05$) and 24 h ($P < 0.01$) of exposure (Fig. 4). Interestingly, in EC treated hGluc + GLP-1, the APPL1 protein levels were near to control values. Similar results were obtained when the expression levels of APPL1 were evaluated by Western blot analysis (Fig. A.3). Indeed, short-term exposure to hGluc down-regulates APPL1 protein after 18 h (0.86 ± 0.03 arbitrary units vs. 1.067 ± 0.014 in control cells) ($P < 0.05$) and 24 h (0.75 ± 0.02 arbitrary vs. 0.51 ± 0.01 in control cells) ($P < 0.01$)
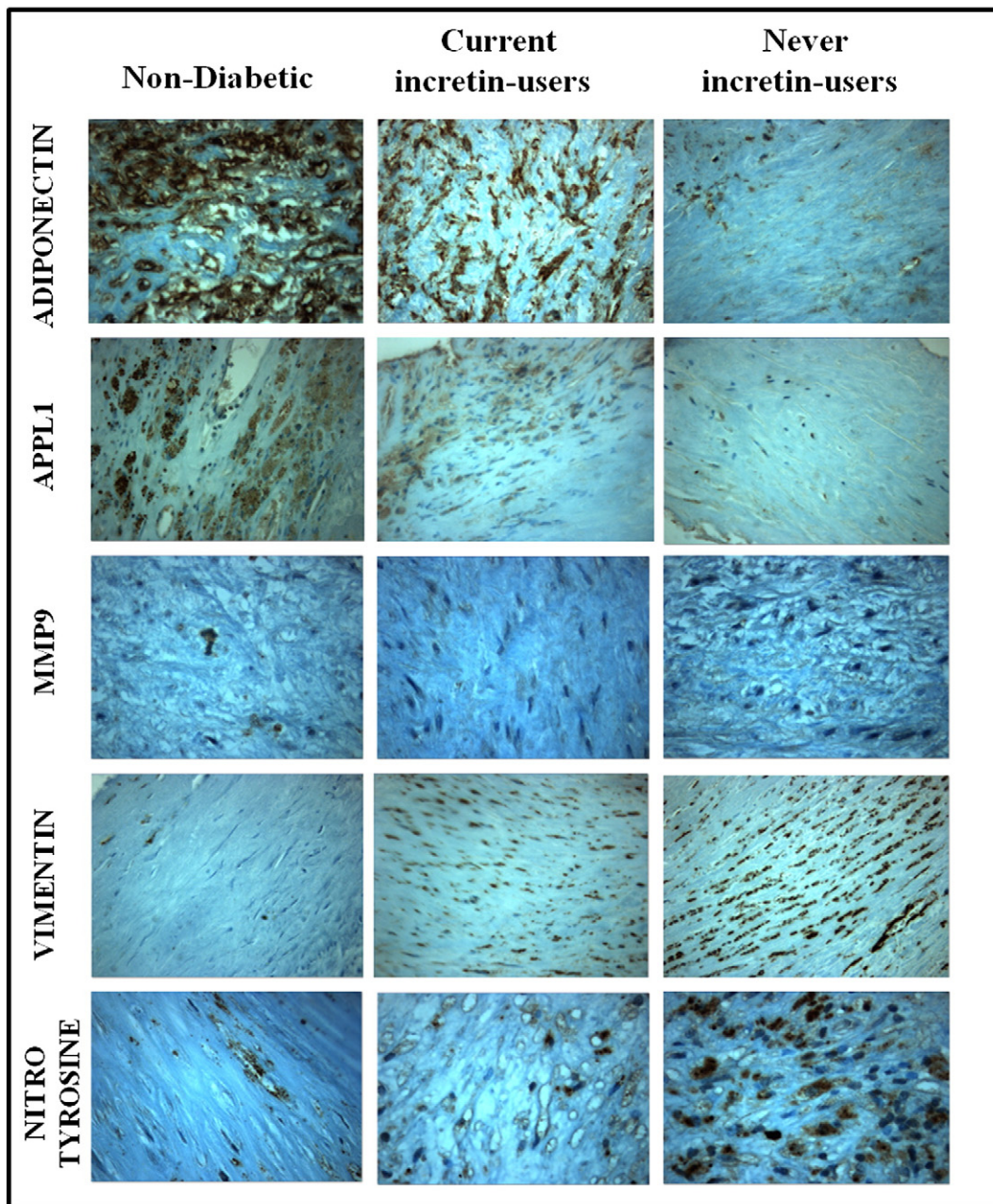
Fig. 2. Atherosclerotic plaque phenotypes. Representative images of immunochemical analysis of APPL1 (X400), adiponectin (X400), MMP-9 (X400), vimentin (X400), and nitrotyrosine (X400) in control, current incretin-user, and never incretin-user asymptomatic plaques. Similar regions of plaque are shown. These results are typical of control, current incretin-user, and never-incretin user asymptomatic plaques.

of exposure (Fig. A.3) whereas in hGluc + GLP-1-treated EC the APPL1 protein levels were significantly higher that observed in cells treated with hGluc alone ($P < 0.05$ vs. hGluc).

4. Discussion

This study provides the first evidence of the relationship between the APPL1 signaling pathway and the inflammatory process at atherosclerotic plaque levels in T2DM patients. Indeed, results demonstrated that atherosclerotic plaques of diabetic patients have lower adiponectin and APPL1 levels, compared with atherosclerotic plaques of non-diabetics. Moreover, levels of adiponectin and APPL1 in the atherosclerotic plaques of diabetic patients were associated with higher oxidative stress, pro-inflammatory cytokine TNF-α, and MMP-9 levels, as well as higher interstitial vimentin content.

Interestingly, among diabetic subjects, those current incretin-users showed higher APPL1 and adiponectin content compared with never incretin-users.

APPL1 and APPL2 are the first identified adaptor proteins that directly interact with adiponectin receptors (Deepa & Dong, 2009). They are both highly expressed in insulin target tissues, including skeletal muscle, liver and adipose tissue and mediate adiponectin signaling and its effects on metabolism (Deepa & Dong, 2009). APPL1 positively mediates adiponectin signaling in cells and its interaction with ADIPOR1 is inhibited by overexpression of APPL2 (Wang et al., 2009).

Low plasma adiponectin concentration has been found associated with increased plaque vulnerability in patients with coronary artery disease (Sawada et al., 2010). The anti-atherosclerotic effects of adiponectin have been demonstrated at several stages in plaque
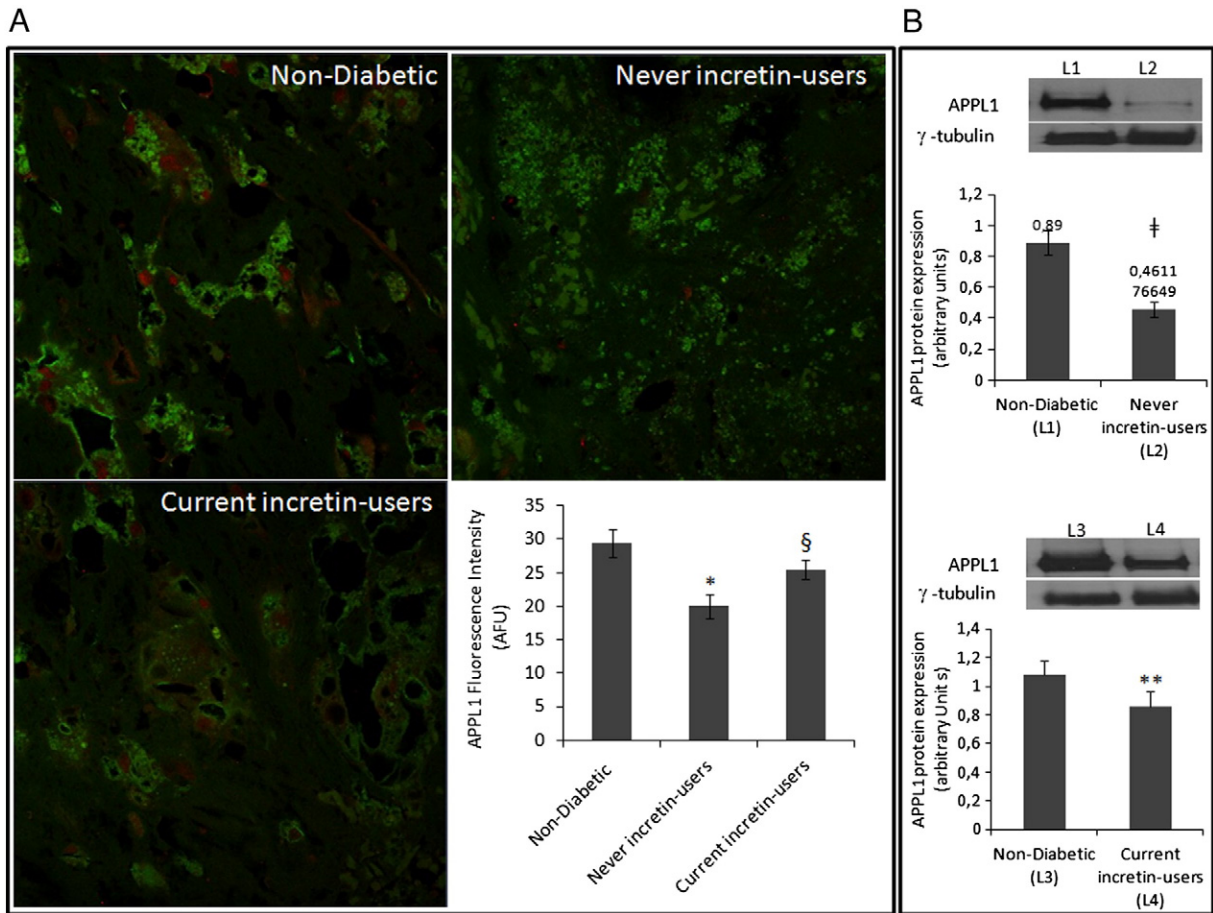
Fig. 3. APPL1 expression in atherosclerotic plaques of diabetic and non-diabetic patients. (A) Representative confocal images of APPL1 (red) and vimentin (green). Bar graph of APPL1 arbitrary fluorescence units (AFU). (B) Western blot analysis of APPL1 protein levels in atherosclerotic plaque homogenates from non-diabetic patients (L1, L3), diabetic never-incretin-users (L2), and diabetic current incretin-users (L4). Insets, representative images of Western blot analysis. Data are mean ± SD with (A) [**]$P < 0.01$ vs. non-diabetic, [*]$P < 0.05$ vs. non-diabetic, [§]$P < 0.05$ vs. never incretin-users, (B) [‡]$P < 0.01$ vs. non-diabetic, [**]$P < 0.05$ vs. non-diabetic.

development, ranging from endothelial dysfunction and plaque initiation to plaque progression and rupture (Sawada et al., 2010; Zhu, Cheng, Vanhoutte, Lam, & Xu, 2008). Adiponectin exerts its vasculoprotective effects through its direct actions in the vascular system, such as increasing endothelial nitric oxide (NO) production, inhibiting EC activation and endothelium–leucocyte interaction, enhancing phagocytosis, and suppressing macrophage activation, macrophage-to-foam cell transformation and platelet aggregation (Zhu et al., 2008). In addition, adiponectin reduces neointima formation through an oligomerization-dependent inhibition of smooth muscle proliferation. Adiponectin signaling through APPL1 exerts anti-inflammatory and cytoprotective effects on EC (Cheng et al., 2007), linking adiponectin receptors and the downstream signaling events leading to increased NO production (Cheng et al., 2007; Zhu et al., 2008). T2DM is well known to lead to increased vulnerability for plaque disruption (Creager et al., 2003) and to be associated with reduced adiponectin plasma levels (Kadowaki et al., 2006). To date, no studies have established the direct involvement of APPL1 in the atherogenic process and in evolution versus instability of atherosclerotic plaque, in T2DM patients. In this study, the lower APPL1 content in atherosclerotic plaque was associated with higher oxidative stress, pro-inflammatory cytokine TNF-α, nitrotyrosine, and MMP-9 levels along with higher interstitial vimentin content, thus suggesting that the beneficial anti-atherosclerotic effect of adiponectin might be mediated by APPL1 signaling. Previous studies have reported that APPL1 is an important mediator of insulin secretion and actions in the liver (Cheng et al., 2009), skeletal muscles (Cleasby

et al., 2011), adipocytes (Saito, Jones, Huang, Czech, & Pilch, 2007), and endothelium (Wang et al., 2011). Increasing evidence demonstrates that APPL1 is a positive regulator of insulin sensitivity by acting as a common relay of both adiponectin and insulin signaling pathways. The expression of APPL1 is dramatically decreased in pancreatic islets and vascular endothelium in several rodent models of diabetes and obesity (Cheng et al., 2012). Furthermore, recent findings linking APPL1 mutations to familial forms of diabetes reaffirm the critical role of APPL1 in glucose homeostasis (Prudente et al., 2015). Accordingly, in our study atherosclerotic plaques from diabetic subjects have significantly lower content of APPL1 compared with atherosclerotic plaques from non diabetic subjects. Interestingly, in vitro experiments on cultured EC, demonstrated that short-term exposure to high-glucose down-regulates APPL1 protein, suggesting that, a detrimental vicious circle in which hyperglycemia alters APPL1 signaling may occur. This, in turn, could affect the anti-inflammatory and cytoprotective effects of APPL1 on EC favoring the instability of the atherosclerotic plaques.

More intriguing, among diabetes subjects, current incretin-users showed higher levels of adiponectin and APPL1 and reduced plaque inflammation compared to never incretin-users. It has been previously demonstrated that incretin therapy reduces atherosclerosis progression in patient with T2DM, probably through the reduction of over daily inflammation and oxidative stress (Barbieri et al., 2013; Burgmaier et al., 2013; Kubota et al., 2012; Li et al., 2011). A more recent study have demonstrated that incretin treatment is associated with morphological and compositional characteristics of a potential
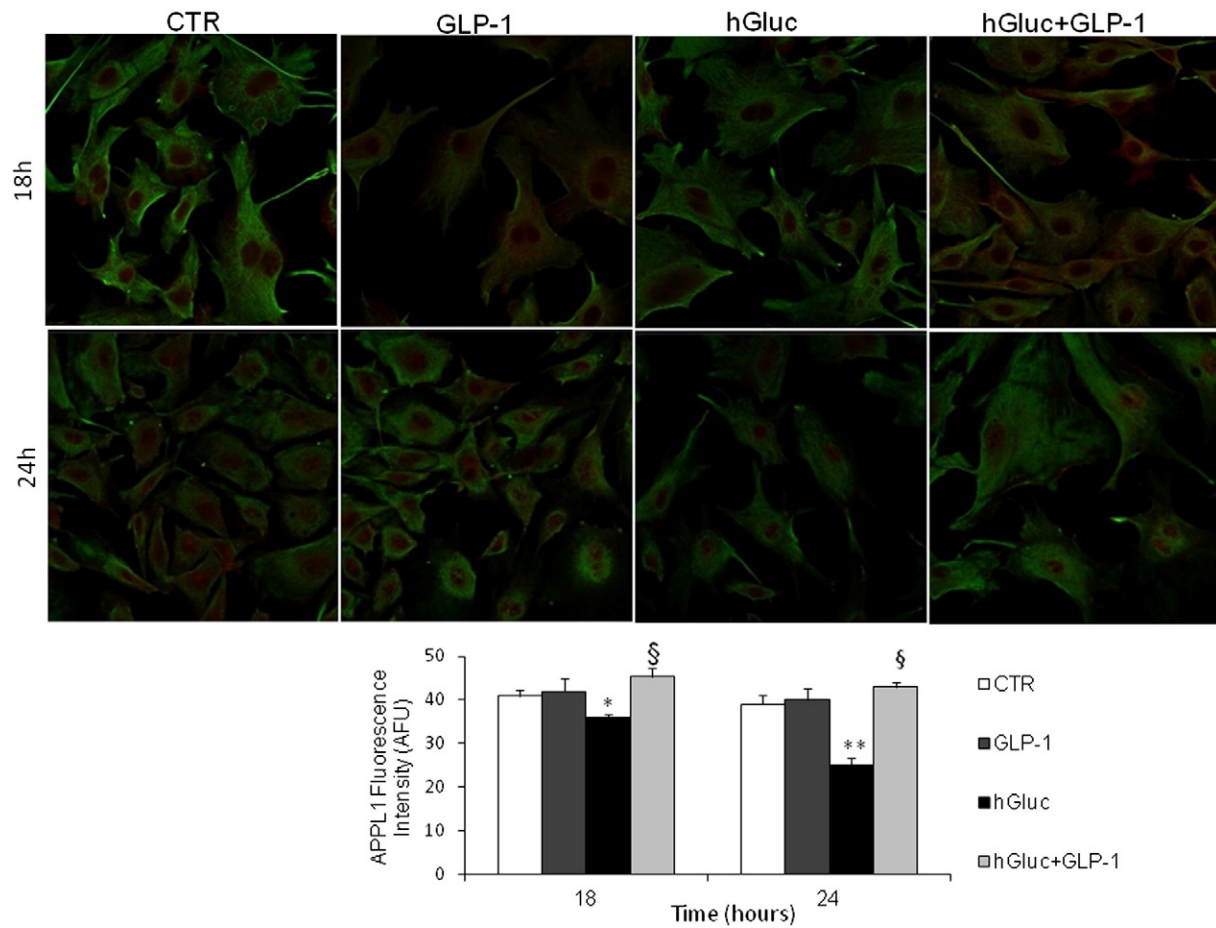
Fig. 4. Confocal laser scanning microscope analysis of APPL1 expression in endothelial cells after short-term exposure to high-glucose and GLP-1. Representative confocal images of APPL1 (red) and vimentin (green) of control (CTR) cells and cells treated for 18 h and 24 h with hGluc, GLP-1 (GLP-1), and high-glucose in the presence of GLP-1 (hGluc + GLP-1). Bar graph of APPL1 AFU. Data are mean ± SD (n = 6) with $^{*}P < 0.05$ vs. CTR, $^{**}P < 0.01$ vs. CTR, and $^{§}P < 0.05$ vs. hGluc.

stable plaque phenotype (Balestrieri et al., 2015). Although, previous studies have demonstrated that incretin therapy promotes adiponectin secretion (Li et al., 2008), increases its circulating levels (Quan et al., 2014) and attenuates hypoadiponectinemia-induced alterations in key regulatory factors implicated in glucose and lipid metabolism (Li et al., 2011), our results are the first demonstration of a potential role of APPL1 signaling in mediating the effect of incretin in the prevention of atherosclerosis progression or plaque vulnerability in T2DM. Such hypothesis is strengthened by the in vitro results indicating that EC co-treated with high-glucose and GLP-1 receptor agonist show higher APPL1 protein levels compared to high-glucose-treated EC and firstly suggesting a potential role of APPL1 as a target for GLP-1 signaling. However, before drawing a firm conclusion, further in vitro and/or in vitro studies are needed to define whether blocking APPL1 could prevent the effects of incretin on high-glucose damages. Potential limitations in our study are the relatively low number of current incretin users which precluded the inclusion of GLP1-R agonist users and DPPIV users as separate groups, the lack of in vitro experiment linking incretin therapy with adiponectin levels, and the need of replicating our finding in an independent larger population group.

5. Conclusions

In conclusion, the evidence of the involvement of APPL1 signaling in the inflammatory process occurring in the atherosclerotic plaques of T2DM patients, and in the complex framework of the signaling pathways altered by hyperglycemia in EC opens new perspectives in defining the mechanism of action of incretin as an effective strategy for preventing atherosclerosis progression in T2DM patients.

Ethics approval and consent to participate

The study was approved by the ethics committee of Cardarelli Hospital, and informed written consent was obtained for each patient. The study has been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jdiacomp.2016.10.001.

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