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Ghrelin and C-peptide as biomarkers for hypercholesterolemia

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SUMMARY

Inflammation and hypercholesterolemia have been reported as risk factors involved in the pathogenesis of cardiovascular diseases. Ghrelin and its receptors are present in the myocardium and vasculature, were they exert cardio-protector and anti-inflammatory functions. C-peptide is a product of proinsulin that plays a physiological role in different types of cells and has pro and anti-inflammatory properties. However, the mechanism that involves these peptides in conditions of low-grade inflammation is complex and not yet well understood. In the present study, we assessed plasma levels of ghrelin, C-peptide, inflammatory mediators, and C-reactive protein (CRP) in a group of hypercholesterolemic and overweight Venezuelan voluntary subjects. They were divided into two groups according to their total cholesterol: control and hypercholesterolemic. After nocturnal fasting, clinical parameters were determined in venous blood samples: fasting plasma glucose, total cholesterol, HDLc, triglycerides, and CRP. Cytokines, chemokines, and growth factor levels were assessed by microsphere multiplex analysis. Our findings demonstrated that hypercholesterolemic patients show significantly higher levels of CRP, ghrelin, and C-peptide. Cholesterol correlated positively with IL-2, IL-8, IFN-γ, eotaxin, ghrelin, VEGF, MCP-1, TNF-α, bFGF, G-CSF, CRP, and C-peptide. Ghrelin correlated negatively with IL-8, IL-4, IL-6, IL-17, and RANTES. C-peptide correlated positively with eotaxin and INF-γ, and diabetes markers as insulin, leptin, PAI-1, resistin, ghrelin, visfatin, adiponectin, and adipsin. Meanwhile, C-peptide correlated negatively with IL-4, IL-5, IL-7, IL-10, and IL-13. These data indicate that in the Venezuelan population studied, hypercholesterolemic patients underlie alterations in ghrelin, C-peptide, inflammatory cytokines, adhesion molecules, chemokines, and diabetes markers.

Key words: *Ghrelin, C-peptide, cholesterol, inflammation, C reactive protein, biomarkers, hypercholesterolemia.*

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RESUMEN

La inflamación y la hipercolesterolemia son considerados factores de riesgo involucrados en la patogénesis de las enfermedades cardiovasculares. La grelina y sus receptores están presentes en el

miocardio y la vasculatura, donde ejercen funciones cardioprotectoras y antiinflamatorias. El péptido C es un producto de la proinsulina que juega un papel fisiológico en diferentes tipos de células y tiene propiedades pro y antiinflamatorias. Sin embargo, el mecanismo que involucra a estos péptidos en condiciones de inflamación de bajo grado es complejo y aún no se conoce bien. En el presente estudio, evaluamos los niveles plasmáticos de grelina, péptido C, mediadores inflamatorios y proteína C reactiva (PCR) en un grupo de sujetos voluntarios venezolanos hipercolesterolémicos y con sobrepeso. Los sujetos se dividieron en dos grupos según su colesterol total: control e hipercolesterolémicos. Después del ayuno nocturno, se determinaron parámetros clínicos en muestras de sangre venosa: glicemia en ayunas, colesterol total, HDLc, triglicéridos y PCR. Los niveles de citocinas, quimiocinas y factores de crecimiento se evaluaron mediante análisis multiplex de microesferas. Nuestros hallazgos demostraron que los pacientes hipercolesterolémicos muestran niveles significativamente más altos de PCR, grelina y péptido C. El colesterol se correlacionó positivamente con IL-2, IL-8, IFN-γ, eotaxina, grelina, VEGF, MCP-1, TNF-α, bFGF, G-CSF, PCR y péptido C. La grelina se correlacionó negativamente con IL-8, IL-4, IL-6, IL-17 y RANTES. El péptido C se correlacionó positivamente con eotaxina, y INF-γ, y marcadores de diabetes como insulina, leptina, PAI-1, resistina, grelina, visfatina, adiponectina y adipsina. Mientras, el péptido C se correlacionó negativamente con IL-4, IL-5, IL-7, IL-10, e IL-13. Estos datos indican que en la población venezolana estudiada, en los pacientes hipercolesterolémicos subyacen alteraciones en grelina, péptido C, citocinas inflamatorias, moléculas de adhesión, quimiocinas y marcadores de diabetes.

Palabras clave: *Grelina, péptido C, colesterol, inflamación, proteína C reactiva, biomarcadores, hipercolesterolemia.*

INTRODUCTION

Hypercholesterolemia and inflammation have been reported as important risk factors in the pathogenesis of cardiovascular diseases (1,2). Hypercholesterolemia is a key factor involved in endothelial dysfunction and the pathogenesis of cardiovascular diseases (1). Indeed, oxidized low-density lipoproteins (LDLox) have been shown to inhibit nitric oxide (NO) release and endothelium-dependent vasodilation, and decrease endothelial nitric oxide synthase (eNOS) expression (3,4); also are key components in the activation of endothelial cells and macrophages, and are associated with hypercoagulability (1). Furthermore, the evidence indicates the presence of an inflammatory phenotype in animal models with hypercholesterolemia, even before the appearance of fatty streak in large arteries (5-8). These changes are associated with impaired basal NO release and progressive endothelial surface expression of endothelial cell adhesion molecules (9), with the consequent rolling, adhesion, and migration of leukocytes in the endothelial wall (5,6).

One of the most widely used inflammation markers for cardiovascular risk stratification is C-reactive protein (CRP), which is an acute-phase reactant, synthesized mainly by the liver under the influence of inflammatory agents stimuli such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor–α (TNF-α). Also, CRP is a regulator of signaling pathways associated with thrombosis, angiogenesis, and inflammation (10). This protein is involved in the pathogenesis of cardiovascular diseases and the progression of atherosclerosis, exerting its pro-inflammatory effects, modulating the innate immune response, activating the complement system, promoting platelet activation, thrombus formation, vascular remodeling and angiogenesis (11). Evidence indicates that elevated plasma CRP levels are a risk factor as well as a marker of cardiovascular disease since CRP is a predictor of the risk of myocardial infarction, peripheral arterial disease even in healthy individuals (12).

Ghrelin is a 28 amino acid residue peptide produced and secreted mainly by the stomach, which was identified as an endogenous ligand for the orphan growth hormone secretagogue receptor (GHSR); therefore it exerts a strong releasing activity of growth hormone (GH) (13). Likewise, ghrelin has multiple functions, since it participates in the regulation of the energy balance; inducing body weight gain by stimulating food intake and reducing fat utilization (14,15). Also, ghrelin and its receptors are present in the cardiovascular system (16), where it exerts various actions independent of those of GH, exhibiting cardioprotective properties, as it is involved in the regulation of blood pressure (17), inhibits endothelial apoptosis, inflammation, increases left ventricular function and is a vasodilator (18,19). Therefore, ghrelin plays an

important protective role against atherosclerosis and cardiovascular diseases (20), possibly due to its anti-inflammatory effects, inhibiting the activation of nuclear transcription factor κ-B (NFκ-B) and the production of inflammatory cytokines in human endothelial cells (21). Even though the cardioprotective properties of ghrelin are known (27,18,19), the mechanism that involves ghrelin in conditions of low-grade inflammation is complex and has not yet been clarified.

C-peptide is a 31-residue peptide that originates from the middle portion of proinsulin, corresponding to the segment between the insulin A and B chains (22). Both C-peptide and insulin are stored within the secretory granules of pancreatic beta cells and are released into the bloodstream in equimolar amounts in response to glucose stimulation (23,24). Deficiency of C-peptide, together with that of insulin, is the main characteristic of type 1 diabetes mellitus (T1DM), and of the late phase of type 2 diabetes mellitus (T2DM) as a consequent to the progressive loss of beta cells (25). Therefore, C-peptide quantification in clinical practice is a useful and widely used method to monitor pancreatic beta-cell function, discrimination between T1DM and T2DM, detection of absolute insulin deficiency, and patient identification with adult-onset diabetes (26).

Initially, C-peptide was considered as an inactive molecule; however, this peptide is now known to exert a physiological role in different cell types (27). Studies in animal models of T1DM reveal that C-peptide has positive effects on the early phase of nephropathy, retinopathy, and neuropathy (28-30), also in patients with T1DM and T2DM, C-peptide, have shown a beneficial impact on the kidney, retina, and nerves (31-33). Also, C-peptide has been shown to have antithrombotic or thrombotic properties, depending on the physiological environment and disease conditions. In this sense, it has been postulated that C-peptide can stimulate adenosine triphosphate (ATP) production by erythrocytes, which induces NO production in both endothelium and platelets, and inhibition of atherogenic cytokine release (34). On the other hand, various studies have shown that C-peptide has proinflammatory properties, since the deposition of this peptide in the intima layer promotes the infiltration of monocytes/macrophages and lymphocytes CD4+, favoring the atherosclerotic process (35,36). Furthermore, C-peptide is related to increased lipid deposits, proliferation, and migration of vascular smooth muscle cells (VSMC) in the vessel wall, promoting the development of atherosclerosis during diabetes along with neo-intima formation. However, incubation of C-peptide with rat aortic smooth muscle cells reduces VSMC proliferation and migration, under hyperglycemic conditions (37). This evidence suggests that C-peptide can be used as a serum marker of cardiovascular diseases. Despite these findings, the role that C-peptide plays in conditions of inflammation and hypercholesterolemia is not completely understood.

Given the aforementioned, in the present study, we assessed by multiplex microsphere analysis (Bio-Plex), the plasma levels of inflammatory mediators, CRP, ghrelin, and C-peptide in a group of overweight hypercholesterolemic Venezuelan adults.

MATERIALS AND METHODS

Experimental subjects

200 subjects of both genders, aged between 18 and 65 years old, selected from those who voluntarily attended the Internal Medicine Department of the Hospital Vargas de Caracas and the Neuropeptides Unit of the School of Pharmacy, Universidad Central de Venezuela (UCV) (Caracas, Venezuela), were studied. They were subdivided into two groups according to their total cholesterol values: Group 1: cholesterol <200 mg/dL (N = 160) and Group 2: cholesterol> 200 mg/dL (N = 40). A questionnaire was applied to find out about their medical, personal, family, and lifestyle habits. Subjects were informed about the characteristics and importance of the study and written informed consent was obtained. Exclusion criteria included smoking habit greater than 20 pack/year, presence of proteinuria and/or previous diagnosis of kidney disease regardless of their etiology, ischemic heart disease, heart failure, lupus erythematosus, multiple myeloma and patients with grade 2 or 3 hypertension. Physical examination was

performed to determine the height, weight, body mass index (BMI), waist circumference (WC), and blood pressure (BP). The last was determined, in the morning, after 10 minutes of rest, using a mercury sphygmomanometer. The pre-analytical conditions were the ones recommended worldwide for this type of assessment: fasting for 8-12 hours and without a regular diet, avoiding strenuous exercises, and stress, among others. Blood was collected into EDTA-containing tubes by direct venipuncture in the antecubital region with multiple needles (Venojet®). Samples were stored at 4 °C during the collection period, after which plasma was stored at -80 °C until the assessment of plasma concentrations of CRP, lipid profile, and glucose, cytokines, chemokines, and diabetes markers. Glucose, total cholesterol, high-density lipoprotein cholesterol (HDLc), and triglycerides were determined by Trinder (Stanbio) enzymatic methods, measurements were made with a spectrophotometer, and the results expressed in mg/dL. CRP determination was carried out by an immunoturbidimetric method (Alpco, EE.UU). Bioethics Committee of the Hospital Vargas de Caracas approved all procedures and protocols which complied with the Declaration of Helsinki for experimentation with human beings (1975 and revised in 1983).

Determination of cytokines, adhesion molecules, and chemokines

All plasma samples were evaluated in duplicate by multiplex microsphere analysis (Bio-Plex Pro Assays Cytokine, Chemokine, and Growth Factors, Life Science Group, BIORAD). Briefly, the Bio-Plex® system is based on three technological cores. The first constitutes a novel technology that uses up to 100 polystyrenes (5.6 μm) or magnetic (8 μm) microspheres, fluorescently stained encoded with a spectral code (xMAP Technology), which allows the simultaneous detection of 100 different molecules in one of the wells of the 96-well microplate. The second is a flow cytometer with two laser beams associated with an optical system that allows the different molecules attached to the surface of the microspheres to be quantified. The third is made up of a high-speed digital signal processor that handles fluorescence data with high efficiency. This technique allowed us to simultaneously study the circulating concentrations of ghrelin, the interleukin 1 receptor antagonist (IL1-ra), interleukins (IL): -2, IL-4, IL-6, IL-8, IL-10, cytokines as eotaxin, IL-12, IL-13, IL-15 and IL-17, basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophages - colony stimulating factor (GM-CSF), interferon-gamma (INF-γ), chemokine-10 motif CXC (IP-10/ CXCL10), monocyte chemotactic protein type 1 (MCP-1), inflammatory protein macrophage-1 alpha/ beta (MIP-1a/b), platelet-derived growth factor (PDGF), ligand 5 of CC chemokine (CCL5/ RANTES), TNF-α and vascular endothelial growth factor (VEGF). The data were expressed as pg/mL.

Statistical analysis

The data were analyzed using the GraphPad Instat program. Data are presented as the mean ± standard error of the mean (S.E.M.). The Mann Whitney U test was used to compare the values of the variables of the groups under study. The correlations between the variables were made by the Spearman correlation test. A value of $P<0.05$ was considered significant.

RESULTS

Clinical and biochemical characteristics of the subjects

The clinical characteristics of the experimental groups are summarized in Table 1. The sex distribution of the subjects was 110 women (Group 1 = 84 and Group 2 = 26) and 90 men (Group 1 = 76 and Group 2 = 14). No significant differences in age, weight, height, BMI, WC, systolic arterial pressure (SAP), diastolic arterial pressure (DAP), and HDLc were observed between the two experimental groups. A significant increase in blood glucose, triglyceride, and total cholesterol values was observed in Group 2 patients when compared to Group 1 ($P<0.0001$).

Table 1

Subjects clinical and biochemical characteristics

Parameter	Cholesterol <200 mg/dL	Cholesterol >200 mg/dL
N=200 F/M	N= 160 84/76	N= 40 26/14
Age (years)	37,41 ± 1,01	44,07 ± 0,94
Weight (Kg)	80,08 ± 1,63	79,54 ± 1,5
Size (cm)	1,66 ± 0,01	1,63 ± 0,01
BMI (Kg/m^2)	28,8 ± 0,55	29,60 ± 0,51
WC (cm)	94,69 ±1,30	96,14 ± 1,16
SAP (mmHg)	123,71 ± 1,24	126,48 ± 1,35
DAP (mmHg)	81,48 ± 0,9	83,67 ± 0,95
Glycemia (mg/dL)	85,89 ± 1,30	91,19 ± 0,75***
Tryglicerides (mg/dL)	108,04 ± 4,7	161,15 ± 8,63***
HDLc (mg/dL)	39,71 ± 0,82	41,01± 1,03
Cholesterol t (mg/dL)	144,5 ± 2,99	234,39 ± 3,07***

***P<0,0001 compared with cholesterol <200 mg/dL.
** P=0,0067 compared with cholesterol <200 mg/dL.

Plasma levels of CRP, ghrelin, and C-peptide in adult subjects

As shown in Figure 1, plasma levels of CRP, ghrelin, and C-peptide were significantly higher in Group 2 when compared to Group 1 (*P=0.047 and **P<0.001) (Figure 1).

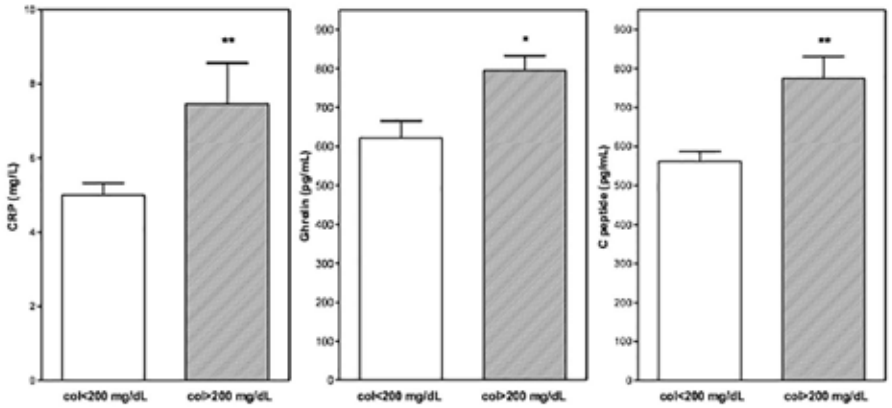

Figure 1. Plasma levels of CRP, ghrelin and C-peptide in adult subjects subdivided into two groups according to total cholesterol levels: Group 1: cholesterol <200 mg/dL (N= 160) and Group 2: cholesterol >200 mg/dL (N=40), *P=0.0407 and **P<0,001 compared to cholesterol <200 mg/dL.

Spearman's correlations analysis between total cholesterol and clinical characteristics, CRP levels, plasma levels of pro-inflammatory cytokines, adhesion molecules, chemokines, C-peptide, and ghrelin, for all subjects, is shown in Table 2. The results demonstrate that total cholesterol was positively and significantly correlated with age, glycemia, triglycerides, CRP, IL-2, IL-8, IFN-γ, eotaxin, VEGF, MCP-1, TNF-α, bFGF and G-CSF, C-peptide and ghrelin.

Table 2

Spearman correlation analysis between total cholesterol and the clinical and biochemical characteristics and cytokines

	r	P
CHOLt & Age	0.1869	0.0074
CHOLt &Glycemia	0.2619	0.0002
CHOLt &Trygliceride s	0.2319	0.0009
CHOLt &CRP	0.3859	0.0001
CHOLt & IL-2	0.2426	0.0772
CHOLt & IL-8	0.2267	0.0011
CHOLt & IFN-γ	0.2882	0.0001
CHOLt &eotaxin	0.2743	0.0001
CHOLt &bFGF	0.1407	0.0450
CHOLt & VEGF	0.1190	0.0942
CHOLt & MCP-1	0.1177	0.0969
CHOLt & TNF-α	0.1946	0.0054
CHOLt & G-CSF	0.3459	0.0001
CHOLt & C-peptide	0.4400	0.0001
CHOLt & ghrelin	0.3524	0.0001

Correlations between C-peptide and plasma levels of interleukins, cytokines, and diabetes markers

In Table 3 is shown Spearman's correlation analysis between C-peptide and plasma levels of interleukins, cytokines, and diabetes markers for all subjects. The results demonstrate that C-peptide was inversely correlated with IL-4, IL-5, IL-7, IL-10 and IL-13, and positively and significantly with eotaxin, and IFN-γ, and with the diabetes markers such as insulin, leptin, PAI-1, resistin, ghrelin, visfatin, adiponectin, and adipsin.

Table 3

Spearman correlation analysis between C-peptide with interleukins, cytokines, and diabetes markers

	r	P
C-peptide & IL-4	-0.2025	0.0121
C-peptide & IL-5	-0.2764	0.0012
C-peptide & IL-7	-0.1764	0.0338
C-peptide C & IL-10	-0.3347	0.0001
C peptide & IL-13	-0.2577	0.0013
C-peptide & eotaxin	0.2649	0.0010
C-peptide & IFN-γ	0.3867	0.0001
C-peptide & Insulin	0.5807	0.0001
C-peptide & Leptin	0.3675	0.0001
C-peptide & PAI-1	0.2768	0.0005
C-peptide & Resistin	0.3278	0.0001
C-peptide & Ghrelin	0.3506	0.0001
C-peptide&Visfatin	0.4157	0.0001
C-peptide &Adiponectin	0.3479	0.0001
C-peptide &Adipsin	0.2039	0.0112

DISCUSSION

The role of hypercholesterolemia and inflammation in the pathogenesis of cardiovascular diseases such as atherosclerosis is universally accepted (9). Inflammation and hypercholesterolemia are related in a vicious circle in which the excess of cholesterol that accumulates in the arterial walls induces an inflammatory response that accelerates the deposit of more cholesterol and amplifies inflammation (38). Accumulation of excess lipids within the artery due to the presence of increased circulating LDL promotes endothelial dysfunction and activation, which results in increased production of pro-inflammatory cytokines and reactive oxygen species, overexpression of adhesion molecules, chemokines, CRP, and decreased NO bioavailability (39,40). These processes contribute to the recruitment and infiltration of monocytes, which differentiate into macrophages and following the uptake of modified-LDL via scavenger receptors, become foam cells, which are essential steps in atherogenesis (39,41,42).

CRP is a non-specific inflammatory serum marker of atherosclerotic vascular disease (43), that in addition to the liver (44) is also synthesized by other cells such as those found in human atherosclerotic plaque since it has been demonstrated colocalization between CRP and LDLox and macrophages in atheromatous plaques of patients with stable or unstable angina and acute myocardial infarction (45). The role of CRP produced by the atheromatous plaque is not entirely known; however, it has been proposed that it plays a relevant role in atherosclerosis and cardiovascular disease (46).

In the present study, high levels of CRP were found in overweight hypercholesterolemic patients, when compared with overweight normocholesterolemic, suggesting the presence of an inflammatory state, possibly induced by high cholesterol levels. It has been demonstrated that CRP shows a positive association with cardiovascular events independently of other cardiovascular risk factors (47). The involvement of CRP in the pathogenesis of cardiovascular diseases is controversial; however, CRP has been shown to exhibit prothrombotic properties, to inhibit eNOS expression, to promote on the endothelial surface increased expression of cellular adhesion molecules such as type 1 vascular adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1) and selectin-E. Also, CRP increases monocyte adhesion and migration determines the synthesis of chemotactic factors such as MCP-1 and induces endothelial secretion of other pro-inflammatory factors such as NFκ-B, IL-6, IL-8 (48-50). Therefore, our results showing increased CRP levels in overweight hypercholesterolemic individuals suggests the presence of a low-grade chronic inflammatory state in these patients. In addition, our results show the existence of a positive association between cholesterol concentrations and levels of IL-2, IFN-γ, TNF-α, IL-8, eotaxin, MCP-1, VEGF, bFGF, G-CSF, CRP, and ghrelin, further supporting the concept that hypercholesterolemia promotes systemic and vascular inflammation characterized by an increase in proinflammatory cytokines, chemokines, growth factors, and acute phase reactants. Some of these molecules could contribute to the amplification of the inflammatory response.

In this respect, hypercholesterolemia, in particular the increase or modification of LDL, has been described as a key component in the activation of endothelial cells and macrophages, favoring the expression of different cell adhesion molecules on the surface of endothelial cells and proinflammatory cytokine production such as TNF-α, IL-1β, and IL-6 (51-53), promoting the change of the endothelial phenotype from one non-adhesive, non-thrombogenic and non-proliferative, to another that expresses and secretes various adhesion molecules and chemoattractants capable of promoting leukocyte recruitment through the endothelium (1).

Likewise, the influence that some inflammatory mediators have on lipid metabolism has been described. TNF-α is a pro-inflammatory cytokine that contributes to the development of atherosclerosis by inducing endothelial dysfunction and initiating the inflammatory cascade within the arterial wall (41). TNF-α may interfere with cholesterol metabolism by decreasing apolipoproteins secretion and reducing cholesterol catabolism and excretion which results in decreased LDLc concentrations, and by reducing HDLc levels and altering its

composition (54). Additionally, inflammatory mediators such as IL-6, IL-1β, and TNF-α may alter lipid metabolism (55), by increasing the production and secretion of very-low-density lipoproteins (VLDL) by the liver and decreasing the clearance of lipoproteins enriched with triglycerides, with the consequent increase in serum triglyceride concentrations (55).

There is evidence that biomarkers such as ghrelin and C-peptide could play an important role in conditions of inflammation and hypercholesterolemia.

Concerning ghrelin, the evidence indicates that both the peptide and its receptors are present in the cardiovascular system, in human cardiomyocytes (56,57), and the endothelial cells of arteries and veins (58). Also, plasma ghrelin concentration is positively associated with the degree of carotid atherosclerosis, suggesting a possible involvement or a compensatory effect during this disease. Ghrelin plays a beneficial role in the cardiovascular system both under normal and pathological conditions. In effect, *in vitro,* and *in vivo* studies indicate that the peptide show hemodynamic and vasoactive effects (59-61); improves endothelial dysfunction, and increases eNOS expression in GH deficient rats (62), indicating that ghrelin cardiovascular effects are independent of those of GH.

Support of the possible functional role of ghrelin in cardiovascular and metabolic diseases and atherosclerosis are our present findings which demonstrate that patients with hypercholesterolemia and overweight had higher ghrelin plasma levels than those overweight normocholesterolemic subjects, suggesting that increased ghrelin levels could represent a compensatory and protective response of this hormone to the condition of chronic low-grade inflammation induced by overweight and hypercholesterolemia, as reflected by the increase in plasma levels of CRP. In agreement with our results are several studies in which they report alterations in the concentration and/or expression of this peptide in cardiovascular diseases (63-66). In effect, the evidence indicates that high plasma ghrelin concentration could protect from coronary heart disease events or deaths in healthy people (65). Furthermore, it was shown a negative correlation between ghrelin levels and the severity of coronary artery disease, with a close correlation between serum ghrelin levels and the extent and severity of coronary atherosclerosis lesions in patients with diabetes (64,66). These results point to a prognostic value of serum ghrelin in coronary atherosclerosis and cardiovascular risk. On the other hand, ghrelin concentration was positively correlated with the degree of carotid atherosclerosis in men (67) suggesting that ghrelin has a different role in atherosclerosis depending on the phase of the disease (68). Indeed, the density of ghrelin receptors is increased in atherosclerotic lesions (56), which could reflect the beneficial role of this peptide in human atherosclerosis. However, some studies show decreased levels of ghrelin in patients with acute myocardial infarction (69) and with metabolic disorders such as insulin resistance, T2DM, and metabolic syndrome (70).

Ghrelin appears to exert immunoregulatory effects in relation to three essential processes of atherosclerotic activity, namely as an antioxidant, anti-inflammatory, and vasodilator (71,72). Indeed, ghrelin inhibits *in vitro* eotaxin and TNF-α induced cytokine production in human endothelial cells, and activation of NFκ-B (21), and *in vivo* inhibits the release of cytokine IL-1β and IL-6 in obese rats (73,74). Likewise, ghrelin is capable of reversing endothelial dysfunction in patients with metabolic syndrome by increasing the production and NO bioavailability (16). Our findings show a positive association between ghrelin and cholesterol levels, which is in line with the finding that ghrelin interacts with triglyceride-rich lipoproteins, HDL, VLDL, and LDL (75,76). Indeed, it was reported that ghrelin binds to HDL particles and is concentrated in HDL-containing lipid fractions from human plasma, with a positive association between ghrelin levels and HDLc, supporting the possible role of HDL particles as circulating transporters of ghrelin (11).

C-peptide has been proposed as an important new risk factor for cardiovascular disease or general deaths in non-diabetic adults (77). Indeed, it was shown that C-peptide can be deposited in the blood vessel wall from the initial stages of atherosclerosis and promote the recruitment of monocytes and lymphocytes (35,36). Results show that C-peptide is a marker of insulin resistance and obesity, specifically in patients with T2DM, since its levels have

been found elevated in patients with metabolic syndrome and diabetes (78,79), and has been positively associated with myocardial infarction, independently of insulin levels in patients with and without diabetes, suggesting that elevated C-peptide levels due to insulin resistance contribute to atherosclerotic vascular disease (80-82). Accordingly, our present results show an increase in plasma C-peptide levels in subjects with hypercholesterolemia compared to normo-cholesterolemics; this associated with a positive correlation of C-peptide with cholesterol, and with markers of diabetes, obesity, and inflammation such as insulin, CRP, leptin, ghrelin, resistin, PAI-1, visfatin, adiponectin, and adipsin. The increased C-peptide levels could be the consequence of the hypercholesterolemia observed in patients due to the pro-inflammatory effects described for this peptide; alternatively, it could constitute a compensatory response to the increase in plasma cholesterol levels, due to the anti-inflammatory effects of this molecule. Therefore, C-peptide could be a marker of cardiovascular risk. In this regard, C-peptide has been postulated as a better predictor of general mortality and deaths related to cardiovascular diseases than serum insulin levels in non-diabetic individuals; however, it is not clear what the mechanism of such association is (77). Evidence-based on clinical, epidemiological, and experimental studies have shown that hyperinsulinemia is associated with different cardiovascular risk factors, such as hypertension, obesity, high triglyceride levels, and low HDLc levels (83), which can promote atherogenesis through its effects on lipid metabolism and blood pressure, with obesity being one of the most important factors associated with hyperinsulinemia (84). Since high levels of insulin and C-peptide coexist, C-peptide may exhibit an insulin-like mechanism promoting atherogenesis (22). Furthermore, serum C-peptide levels were more strongly associated with total and body regional fat distribution in nondiabetic subjects than were serum insulin levels (80). Abdullah et al (85), in a study of 80 young, overweight/obese Arab women found higher values of BMI and WC, and markers such as leptin, fasting insulin, uric acid, insulin resistance (HOMA-IR), C-peptide, CRP, HDL-C, SAP, DAP in women with overweight and obesity compared to women with normal weight. Likewise, they observed that C-peptide was significantly correlated with WC, leptin, uric acid, and HDLc, suggesting the association between metabolic syndrome and cardiovascular disease, as well as its role as an additional biomarker in predicting the early development of the cardiovascular disease. Similarly, Li et al (86) proposed serum C-peptide as a risk factor for the cardiovascular disease since it was significantly and negatively associated with serum HDLc levels in individuals without diabetes, suggesting that serum C-peptide levels associated with cardiovascular death can be caused, at least in part, by the low serum HDLc level, indicating that serum C-peptide may increase the risk of cardiovascular events via a pathway that reduces HDLc. Similarly, Wang et al (87) found higher levels of C-peptide and insulin, total cholesterol, triglycerides, and LDLc and lower of HDLc in patients with insulin resistance compared to normoglycemic controls, indicating that dyslipidemia prevails in states of insulin resistance; which is in agreement with previous studies that showed that insulin resistance and dyslipidemia occur in combination or profoundly interact. However, it is not entirely clear whether dyslipidemia leads to insulin resistance or vice versa since it is thought that high triglyceride levels and low plasma HDLc levels are consequences of insulin resistance (88); whereas other studies indicate that insulin resistance is caused by dyslipidemia (89).

The effects of C-peptide on inflammation, diabetes, and cell proliferation are controversial. On one hand, it has been described that C-peptide inhibits the interaction of leukocytes with the endothelium since it can inhibit the transendothelial rolling, adherence, and migration of leukocytes to the endothelium, by reducing the expression of cell adhesion molecules, such as selectin-P and ICAM-1 in the microvascular endothelium and increasing eNOS expression (90). Conversely, different studies point to this peptide as a pro-inflammatory molecule; since it promotes leukocyte migration (35,36) and induces proliferation of different cells such as VSMC and endothelial cells (91), being able to participate in various inflammatory processes. Indeed, expression of C-peptide deposits in the subendothelial space

in the thoracic aorta of diabetic patients show 77 % infiltration of monocytes/macrophages and 57 % of CD4+ lymphocytes (35). Moreover, *in vitro* studies report that C-peptide induces the migration of CD4+ lymphocytes and monocytes/ macrophages; these effects being similar to those induced by chemokines such as MCP-1 or RANTES (35, 36).

Similarly to these findings, the present study demonstrated the existence of a positive association between C-peptide and pro-inflammatory markers such as CRP, IFN-γ, and eotaxin; and a negative correlation with anti-inflammatory markers such as IL-4, 5, 7, 10 and 13. Likewise, a positive correlation was shown between cholesterol and C-peptide and pro-inflammatory markers; suggesting that hypercholesterolemia leads to a low-grade inflammatory state. Thus, serum C-peptide levels could be a non-invasive plasma marker of inflammation and cardiovascular risk. Various studies have shown a chemotactic effect of C-peptide, therefore correlating with its involvement in the early stages of atherogenesis. Indeed, immunohistochemical studies of arteries obtained from individuals who died of external causes, have shown deposits of C-peptide in the intima and subendothelium of diabetic patients, colocalizing with CD68+ monocytes and macrophages, which are responsible for the phagocytosis of LDLox, originating the foam cells. Likewise, subcutaneous administration of C-peptide to apolipoprotein E (ApoE) deficient mouse, fed with a diet enriched with cholesterol, caused an increase in monocyte chemotaxis, lipid and C-peptide deposits in the aorta wall and early atherosclerotic lesions, as well as elevated VSMC recruitment and proliferation within the lesion, compared to untreated control mice (Apo E deficient fed a diet enriched with cholesterol). The C-peptide deposition was accompanied by an increased local inflammatory infiltrate and lipid deposits (79,92); therefore, increased C-peptide expression in the blood vessel wall of Apo E deficient mice induces local inflammation leading to lipid deposition and increased proliferation of VSMC, crucial processes in the pathogenesis of atherosclerosis (79). Furthermore, it has been shown that C-peptide, in physiological concentrations, is capable of causing an increase in monocyte migration *in vitro*, which was not exerted by insulin, suggesting a role for C-peptide in the early stages of atherogenesis and inflammation (35).

In conclusion, our results suggest that hypercholesterolemia and overweight can alter inflammation and metabolic markers such as CRP, ghrelin, and C-peptide in a Venezuelan population, highlighting the role of these molecules in the physiopathology of cardiovascular diseases, thus constituting possible biomarkers of risk for cardiovascular diseases.

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CONFLICT OF INTEREST

The authors have no conflict of interest

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