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ORIGINAL ARTICLE

Alterations in biomarkers of cardiovascular disease (CVD) in active acromegaly

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Summary

Objectives In acromegalic patients, cardiovascular and metabolic comorbidities contribute to enhance mortality. Available data on the lipoprotein profile of these patients are controversial. Our aim was to characterize the lipoprotein profile and emergent biomarkers of cardiovascular disease in active acromegalic patients in comparison with sex- and age-matched healthy controls.

Patients Eighteen patients with active acromegaly and 18 controls were studied.

Measurements Glucose levels, hormonal status, lipoprotein profile and C reactive protein (CRP) were evaluated by standardized methods. Cholesteryl ester transfer protein (CETP) and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) were measured by radiometric techniques, endothelin-1 and vascular cell adhesion molecule (VCAM)-1 by enzyme-linked immunosorbent assay, and leucocytes CD18, CD49d and CD54 by flow cytometry.

Results After adjusting for body mass index (BMI), acromegalic patients presented a more atherogenic lipoprotein profile, consisting of higher levels of triglycerides and apolipoprotein B and alterations in the ratios which estimate insulin resistance and atherogenic risk. CETP activity was significantly increased in acromegalic patients as compared to controls (168 ± 17 *vs.* $141 \pm 30\%$ per ml h, respectively; $P < 0{\cdot}05$). Endothelin-1 levels evidenced an increase in the patients' group ($0{\cdot}9 \pm 0{\cdot}2$ *vs.* $0{\cdot}7 \pm 0{\cdot}2$ ng/l, respectively; $P < 0{\cdot}01$) and showed positive and significant correlations with GH, IGF-1 and IGFBP-3 ($r = 0{\cdot}45$, $0{\cdot}42$ and $0{\cdot}44$, respectively; $P < 0{\cdot}01$ for all of them; with BMI as a fixed variable). Lymphocytes from acromegalic patients showed increased CD49d content (282 ± 59 *vs.* 246 ± 48 arbitrary units, respectively; $P < 0{\cdot}05$).

Conclusions Taken together, the alterations described seem to contribute to constituting a state of higher propensity for the development of atherosclerotic cardiovascular disease, which adds to the presence of specific cardiomyopathy.

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Introduction

In acromegalic patients, cardiovascular, respiratory and metabolic comorbidities contribute to significantly enhance mortality, which doubles the death rates in comparison with healthy population. In fact, average life expectancy in patients with active acromegaly is reduced by approximately 10 years.[1] Accordingly, the integrated evaluation of the atherogenic risk, analysed through the Framingham score, and the coronary artery calcium quantification has shown that 41% of acromegalic patients have an increased risk of coronary atherosclerosis, which is not influenced by the control of acromegaly.[2]

The systemic complications observed in relation to acromegaly seem to be linked to permanently elevated GH and IGF-I levels.[3] The harmful effect of GH and IGF-I excess on cardiac structure and function has been widely demonstrated by *in vivo* and *in vitro* studies.[4] Nevertheless, the poor prognosis of acromegalic patients could be not only attributed to the presence of specific cardiomyopathy,[5] but also to atherosclerotic cardiovascular disease. Actually, this high cardiovascular risk could be attributed to the increased incidence of diabetes mellitus, hypertension and lipid disorders in acromegalic patients.

Although several studies agree on reporting the presence of an abnormal lipid and lipoprotein profile in acromegalic patients, controversial data arise when trying to identify the modified specific lipid parameters or when analysing the extent of those alterations. Accordingly, plasma triglyceride and total cholesterol levels have been found to be unchanged[6,7] or increased,[8] and high density lipoprotein-cholesterol (HDL-C) and apolipoprotein (apo) A-I either unchanged[7,9] or low.[10,11]

Regarding emergent biomarkers of cardiovascular disease, most studies have shown elevated lipoprotein (Lp) (a),[9,12] small and dense low density lipoprotein (LDL) particles,[7,11] and fibrinogen[13] in acromegaly. In contrast, Sesmilo *et al.*[14] showed reduced C reactive protein (CRP) and unchanged homocysteine levels. To our knowledge, no studies have been carried out to evaluate other atherogenic or inflammatory markers such as lipoprotein-associated phospholipase A_2 (Lp-PLA_2), endothelin-1, and soluble or leucocyte cell adhesion molecules in acromegalic patients in comparison to healthy controls.

Table 1. Clinical characteristics, biomarkers of insulin resistance and hormonal parameters from acromegalic patients and control subjects

	Acromegalic patients	Control subjects
N	18	18
Women/Men	15/3	15/3
Age (years)	44 (27–79)	43 (28–76)
BMI (kg/m^2)	28·0 (23·3–42·7)	23·4 (19·0–27·6)*
Waist (cm)	94 (77–112)	88 (70–99)
Glucose (mmol/l)	5·7 (4·2–17·7)	4·9 (4·0–5·5)**
Insulin (pmol/l)	121 (40–400)	37 (14–72)***
Glucose/Insulin	0·06 (0·03–0·11)	0·14 (0·08–0·40)***
HOMA	4·4 (1·1–44·1)	1·1 (0·5–2·2)***
GH (μg/l)	9·0 (2·2–308·0)	1·4 (0·2–9·3)*
IGF-1 (nmol/l)	90 (36–142)	19 (10–27)*
IGFBP-3 (mg/l)	7·6 (5·0–7·4)	4·5 (2·8–5·7)*

BMI, body mass index; HOMA, homeostasis model assessment; GH, growth hormone; IGF-1, insulin-like growth factor I; IGFBP-3, IGF binding protein-3. Results are expressed as median (range). *$P < 0·0001$; **$P < 0·005$, ***$P < 0·001$ *vs.* acromegalic patients.

The aim of the present study was to further characterize lipid, lipoprotein and apolipoprotein profile, and emergent biomarkers of cardiovascular disease, such as CRP, Lp-PLA$_2$, endothelin-1, vascular cell adhesion molecule-1 (VCAM-1) and leucocyte CD18, CD49d and CD54, in acromegalic patients in comparison with sex- and age-matched healthy controls.

Materials and methods

Subjects

Eighteen adult patients with diagnosis of active acromegaly were consecutively recruited from the Endocrinology Service, 'José de San Martín' Clinical Hospital, Buenos Aires, Argentina, during a period of 1 year (Table 1). Patients were included in the present study when presenting typical clinical features and increased GH and IGF-1 levels for corresponding sex and age.[15] Disease duration ranged between 2 and 10 years. Three acromegalic patients had type 2 diabetes, seven had hypertension and 10 macroadenomas. Eighteen healthy subjects, sex- and age-matched with the patients, agreed to participate in this study and were employed as controls. Both patients and controls had normal renal, hepatic and thyroid functions, none of them presented history of any cardiovascular event, and they were not under any treatment known to affect carbohydrates, lipids or biomarkers of cardiovascular disease. Informed consent was obtained from all participants and the protocol of this open transversal study was approved by the Ethical Committees from School of Pharmacy and Biochemistry and from 'José de San Martín' Clinical Hospital, University of Buenos Aires.

Study protocol and samples

After a 12-h overnight fast, venous blood was drawn from the antecubital vein. Aliquots were collected in clean and EDTANa$_2$-containing tubes. Samples were centrifuged at 1500 ***g***, for 15 min, at 4 °C. Serum was immediately employed for glucose determination and stored at 4 °C for lipid and lipoprotein characterization within 24 h. Serum aliquots were also stored at –70 °C for determination of insulin, GH, IGF-I, IGFBP-3 and VCAM-1 levels, and for cholesteryl ester transfer protein (CETP) and LpPLA$_2$ activities. Whole blood was stored at 4 °C and employed for the evaluation of leucocyte cell adhesion molecules by flow cytometry within 24 h.

Analytical procedures

Glucose, triglycerides and total cholesterol were quantified by standardized methods (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 autoanalyser. Within-run precision (CV) was 1·3% and 1·1%, respectively, for triglycerides and total cholesterol. Between-day precision (CV) was 2·4% and 1·5%, respectively. Laboratory bias was 1·1% and –1·7%, respectively. LDL-C level was determined as the difference between total cholesterol and the cholesterol contained in the supernatant obtained after selective precipitation of LDL with 10 g/l polyvinylsulphate in polyethylenglycol (M.W. 600; 2·5% w/v; pH = 6·7).[16] Within-run and between-day precisions (CV) were 4·7% and 5·0%, respectively. HDL was isolated in the supernatant obtained following precipitation of apo B-containing lipoproteins with 40 g/l phosphotungstic acid in the presence of magnesium ions.[17] Within-run and between-day precisions (CV) for HDL-C were 3·2% and 3·8%, respectively. Laboratory bias was –2·0%. VLDL-C was calculated as the difference between the cholesterol contained in the supernatants obtained for LDL and HDL measurements, while non-HDL-C was estimated as the difference between total cholesterol and HDL-C. Apo B and apo A-I were evaluated by immunoturbidimetry (Roche Diagnostics) in a Hitachi 917 autoanalyser. Within-run and between-day precisions (CV) were 1·2% and 2·1% for apo B, and 1·9% and 2·4% for apo A-I, respectively. The following ratios were calculated: triglycerides : HDL-C, total cholesterol : HDL-C, LDL-C : HDL-C and apo B : apo A-I. CRP concentration was determined by Tina-quant CRP (Latex) high sensitive immunoturbidimetric assay (Roche Diagnostics) in a Hitachi 917 autoanalyser. Within-run and between-day precisions (CV) were 0·4% and 3·4%, respectively. White blood cell count was determined by automated particle counter (Coulter MAXM).

Hormonal parameters

Insulin concentration was measured by microparticle enzyme immunoassay (MEIA) (ABBOTT, Japan). Within-run and between-day precisions (CV) were 2·9% and 4·4%, respectively. Homeostasis model assessment (HOMA) was calculated as [Glucose (mmol/l). Insulin (μU/ml)]/22·5. Serum GH was measured by the ultrasensitive immunometric assay (Access®, Beckman Coulter™) with analytical sensitivity of 0·003 μg/l. Within-run and between-day precisions (CV) were 12·3% and 15·5%, respectively. Serum IGF-I and IGFBP-3 levels were measured by Immulite 2000, solid phase chemiluminiscent enzyme immunoassay (Diagnostics Products Corp., Los Angeles, CA) with analytical sensitivity of 2·6 nmol/l and 0·1 mg/l. Within-run and between-day precisions (CV) for IGF-I were below

5·4% and 11·9%, respectively. Measurements of IGFBP-3 were all carried out within the same assay. Within-run precision (CV) was 4·8% for IGFBP-3.

CETP activity

CETP activity was determined in serum samples following the general procedure previously described[18] with a few modifications. Briefly, the ability of serum to promote the transfer of tritiated cholesteryl esters from a tracer amount of biosynthetically labelled HDL_3 (^{3}H-CE-HDL_3) (NEN Life Science Products, Boston, MA) to serum apo B-containing lipoproteins was evaluated. Samples were incubated with ^{3}H-CE-HDL_3 (50 μmol/l cholesterol) and 1·5 mmol/l iodoacetate for 3 h, at 37 °C. After incubation, lipoproteins were separated by a selective precipitation method employing 40 g/l phosphotungstic acid in the presence of magnesium ions.[16] Radioactivity was measured both in the incubation mixture and in the supernatant containing the HDL fraction in a Liquid Scintillation Analyser (Packard 210TR; Packard Instruments, Meridian, CT). Results were expressed as percentage of ^{3}H-cholesteryl esters transferred from HDL_3 to apo B-containing lipoproteins, per millilitres per hour. Measurements were all carried out in duplicate within the same assay. Within-run precision (CV) was 4·9%.

Lipoprotein-associated phospholipids A_2

$LpPLA_2$ activity was measured following the radiometric assay described by Blank *et al.*[19] with a few modifications. The separation of the released radiolabelled acetate from the lipid substrate was carried out by phase–phase partitioning and measurement of the radioactivity in the aqueous phase. Briefly, each incubation mixture contained 50 μl of 1/50 diluted serum and 10 μmol/l 1-hexadecyl-2-[^{3}H]acetyl-glycero-3-phosphocholine (Specific Activity = 25 μCi/μmol) in a total volume of 0·5 ml of phosphate-buffered saline (PBS) (pH = 7·4). The tritiated substrate 1-hexadecyl-2-[3H] acetyl-glycero-3-phosphocholine (13·5 Ci/mmol) was obtained from New England Nucleotides and the nontritiated one was obtained from Cayman Chemical. Once the substrates were mixed, the solvents were evaporated under a stream of nitrogen and redissolved in PBS. There was a sonication step consisting of one cycle of 5 min. Incubation was carried out for 5 min, at 37 °C and the enzymatic reaction was stopped by placing the tubes in an ice bath and by the addition of 1·5 ml of chloroform. Then, 0·5 ml of saturated sodium bicarbonate solution was added. After centrifugation, the aqueous phase was washed twice with 1·5 ml of chloroform. The radioactivity of the aqueous phase of each sample and sample-blanks was measured by liquid scintillation using a Packard autoanalyser. Radioactivity of the substrate-buffer was also measured. Results were expressed as μmol/ml h. Measurements were all carried out within the same assay. Within-run precision (CV) was 5·1%.

Endothelin-1 and VCAM-1

Endothelin-1 and VCAM-1 plasma levels were determined by the monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions, with a few modifications (R&D Systems, USA). Sample levels were calculated by analysing standards with known concentrations of recombinant molecules coincident with samples and plotting of signal *vs.* concentration. Within-run and between-day precisions (CV) were 4·5% and 5·5% for endothelin-1, and 3·5% and 7·7% for VCAM-1, respectively.

Leucocyte cell adhesion molecules

Expression of the following adhesion molecules in monocytes, polymorphonuclear cells, and lymphocytes was measured by flow cytometry (FACS Sort, Becton-Dickinson, San Jose, CA): CD18, CD49d and CD54·[20] Staining was performed in whole blood with commercial fluorescein-isothiocyanate (FITC)-conjugated anti-CD54 and anti-CD18, and phycoerythrin (PE)-conjugated anti-CD49d monoclonal antibodies (MAbs, BD Biosciences Pharmingen, Ontario, Canada). Whole blood was incubated with saturating concentrations of conjugated MAbs for 30 min at room temperature. Erythrocytes were lysed with commercially available solution (FACS Lysing Solution, BD Biosciences Pharmingen). The cells were then washed with PBS and immediately subjected to flow cytometry equipped with a 488 nm argon laser. To analyse monocytes, polymorphonuclear cells and lymphocytes, a gate was defined by forward (FSC) and right angle light scattering (SSC), using the Cell Quest™ software (Becton-Dickinson). Moreover, monocyte identity was confirmed by employing FITC or PE-conjugated anti CD14. The fluorescence intensity of 10 000 events was recorded as the mean channel number over a logarithmic scale of 1–1026 channels. Results were expressed as the mean channel of fluorescence intensity. Fluorescent-conjugated isotype control antibodies were used to detect nonspecific binding to cells.

Data and statistical analysis

Data distribution was tested employing the Shapiro-Wilk test. Results are expressed as mean ± standard deviation (SD) for normally distributed data and as median (range) for skewed data. In this last case, data were normalized by log-transformation. Then, analysis of covariance was used including body mass index (BMI) as a covariate. Partial correlations were performed between different parameters including BMI as a fixed variable. Differences were considered significant at $P < 0·05$ in the bilateral situation. For statistical analysis, INFOSTAT software was used.

Results

In the present study, 18 patients with active acromegaly were studied in comparison with 18 sex- and age-well-matched control subjects. Clinical characteristics, biomarkers of insulin resistance and hormonal parameters are shown in Table 1. In accordance with the well-known physical features of subjects with acromegaly, BMI was significantly increased in the patients' group. Given this difference between both studied groups, all the results obtained were compared performing analysis of covariance, including BMI as a covariate. Then, glucose and insulin levels, as well as glucose : insulin ratio and HOMA were higher in patients than in control individuals. Moreover, deriving

Table 2. Lipids, lipoproteins and apolipoproteins from acromegalic patients and control subjects

	Acromegalic patients ($n = 18$)	Control subjects ($n = 18$)
TG (mmol/l)	1·40 ± 0·43	0·91 ± 0·28*
TC (mmol/l)	5·50 ± 0·88	5·37 ± 1·03
VLDL-C (mmol/l)	0·59 ± 0·21	0·46 ± 0·18
LDL-C (mmol/l)	3·64 ± 0·75	3·20 ± 0·70
HDL-C (mmol/l)	1·27 ± 0·31	1·55 ± 0·39
Non-HDL-C (mmol/l)	4·23 ± 0·77	3·67 ± 0·80
Apo B (g/l)	1·09 ± 0·19	0·82 ± 0·18*
Apo A-I (g/l)	1·48 ± 0·29	1·52 ± 0·20
TG/HDL-C	2·7 ± 1·0	1·4 ± 0·4*
TC/HDL-C	4·5 ± 0·9	3·4 ± 0·6*
LDL-C/HDL-C	3·0 ± 0·8	2·2 ± 0·5*
APO B/APO A-I	0·8 ± 0·2	0·6 ± 0·1**

TG, triglycerides; TC, total cholesterol; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein. Results are expressed as mean ± S.D.
*$P < 0·005$; **$P < 0·01$ *vs.* acromegalic patients.

Table 3. Correlations of GH, IGF-1 and IGFBP-3 with different parameters in acromegalic patients and controls subjects ($n = 36$)

	GH r (P)	IGF-1 r (P)	IGFBP-3 r (P)
Glucose	0·48 (< 0·005)	0·40 (< 0·05)	0·49 (< 0·005)
Insulin	0·63 (< 0·0001)	0·74 (< 0·0001)	0·79 (< 0·0001)
Glucose/Insulin	–0·51 (< 0·005)	–0·70 (< 0·0001)	–0·70 (< 0·0001)
HOMA	0·63 (< 0·0001)	0·68 (< 0·0001)	0·75 (< 0·0001)
TG	0·58 (< 0·0005)	0·53 (< 0·005)	0·64 (< 0·0001)
VLDL-C	0·52 (< 0·005)	0·35 (< 0·05)	0·51 (< 0·05)
LDL-C	0·29 (= 0·094)	0·20 (NS)	0·27 (NS)
HDL-C	–0·04 (NS)	–0·31 (= 0·078)	–0·31 (= 0·078)
Non-HDL-C	0·40 (< 0·05)	0·25 (NS)	0·35 (< 0·05)
Apo B	0·37 (< 0·05)	0·37 (< 0·05)	0·35 (< 0·05)
Apo A-I	0·07 (NS)	–0·17 (NS)	–0·14 (NS)
TG/HDL-C	0·50 (< 0·005)	0·59 (< 0·0005)	0·68 (< 0·0001)
TC/HDL-C	0·38 (< 0·05)	0·50 (< 0·005)	0·59 (< 0·0005)
LDL-C/HDL-C	0·26 (NS)	0·43 (< 0·05)	0·49 (< 0·05)
APO B/APO A-I	0·40 (< 0·05)	0·41 (< 0·05)	0·39 (< 0·05)
CETP	0·20 (NS)	0·32 (= 0·066)	0·35 (< 0·05)

GH, growth hormone; IGF-1, insulin-like growth factor I; IGFBP-3, IGF binding protein-3; TG, triglycerides; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; CETP, cholesteryl ester transfer protein.

from the inclusion criteria, GH, IGF-I and IGFBP-3 concentrations were also elevated in patients with active acromegaly.

Table 2 shows lipid, lipoprotein and apolipoprotein levels. Acromegalic patients presented a more atherogenic lipoprotein profile than control subjects, consisting of higher triglyceride (53%) and apo B (33%) levels. Ratios indicating insulin resistance (TG : HDL-C) and atherogenic risk (TC : HDL-C, LDL-C : HDL-C and apo B : apo A-I) were also higher in acromegalic patients. Furthermore, CETP activity was found to be 19% higher in patients than in controls (Mean ± SD; 168 ± 17 *vs.* 141 ± 30% per ml h, respectively; $P < 0·05$).

When analysing the association between hormones, which define the acromegalic state, and different biochemical parameters evaluated in the present study, significant correlations were observed between GH, IGF-1 and/or IGFBP-3, and most indicators of insulin resistance and lipid risk factors for atherosclerosis (Table 3).

Regarding inflammatory and atherogenic biomarkers, endothelin-1 levels evidenced a significant increase of 29% in the patients' group. Moreover, positive and significant correlations were observed between endothelin-1 and GH, IGF-1 and IGFBP-3 ($r = 0·45$, 0·42 and 0·44, respectively; $P < 0·01$ for all of them; $n = 36$). Besides, endothelin-1 also showed positive associations with other biomarkers of cardiovascular disease, such as VCAM-1 and lymphocyte CD49d ($r = 0·41$ and $r = 0·40$, respectively; $P < 0·05$ for both of them). No statistically significant differences were detected in CRP and VCAM-1 concentrations, $LpPLA_2$ activity and white blood cell count (Table 4). On the other hand, CRP showed a negative correlation with GH ($r = –0·37$, $P < 0·05$) and a positive one with lymphocyte CD49d ($r = 0·47$, $P < 0·005$).

Cell adhesion molecules were also evaluated in circulating leucocytes and then, lymphocytes from acromegalic patients showed increased CD49d content (21%) in comparison to control subjects (Fig. 1, Panel C).

Table 4. Inflammatory and atherogenic markers from acromegalic patients and control subjects

	Acromegalic patients ($n = 18$)	Control subjects ($n = 18$)
CRP (mg/l)	0·2 (0·1–22·7)	0·9 (0·1–13·7)
Lp-PLA_2 (μmol/ml h)	8·1 ± 2·0	8·0 ± 1·7
Endothelin-1 (ng/l)	0·9 ± 0·2	0·7 ± 0·2*
VCAM-1 (μg/l)	41 ± 12	37 ± 7
White blood cell count (10^3/mm^3)	6·1 ± 1·2	6·3 ± 1·8

CRP, C-reactive protein; VCAM-1, vascular cell adhesion molecule-1; Lp-PLA_2, lipoprotein-associated phospholipase A_2. Results are expressed as mean ± SD, except for CRP which is expressed as median (range).
*$P < 0·01$ *vs.* acromegalic patients.

Discussion

In the present study, patients with active acromegaly, defined by typical clinical features and increased GH and IGF-1 levels, showed a more atherogenic lipid and lipoprotein profile than healthy control subjects matched for sex and age and analysed fixing BMI as a covariate. Moreover, among the different biomarkers of cardiovascular disease, we evaluated CRP, $LpPLA_2$, endothelin-1, VCAM-1, white blood cell count and leucocyte cell adhesion molecules. Acromegaly was associated with significantly higher levels of endothelin-1, the most potent constrictor of human vessels known up to date, and with an increased CD49d content in circulating lymphocytes, which are

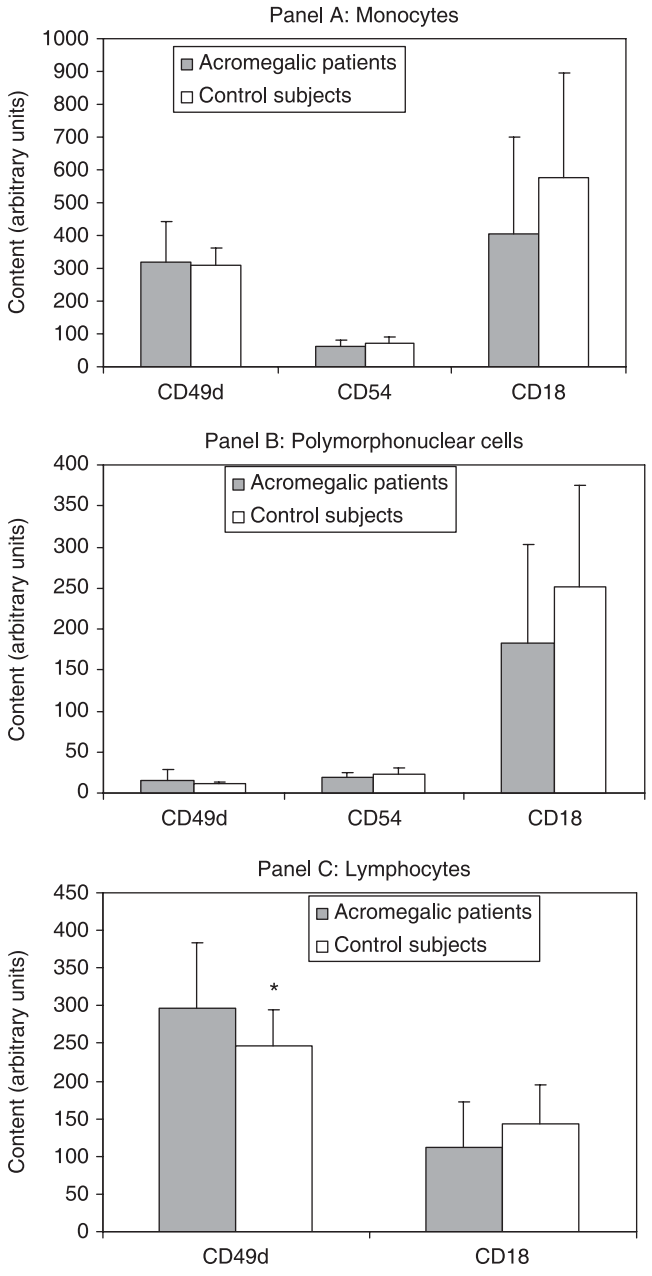

Fig. 1 Cell adhesion molecule content (CD18, CD49d, CD54) in monocytes (Panel A), polymorphonuclear cells (Panel B) and lymphocytes (Panel C) from acromegalic patients ($n = 18$) and control subjects ($n = 18$) measured by flow cytometry. $^*P < 0{\cdot}05$ *vs.* acromegalic patients.

partially responsible for the inflammatory process linked to atherosclerosis.

Evaluation of carbohydrate metabolism showed an increase in insulin resistance, evidenced by alterations in glucose and insulin plasma levels, glucose : insulin and HOMA ratios, as well as in triglyceride : HDL-C ratio. The latter has been proposed as an interesting marker to identify insulin-resistant individuals at high risk of cardiovascular disease.[21] Three of the acromegalic patients studied in this work suffered from type 2 diabetes. When they were discarded, no changes were detected in any of the evaluated parameters (data not shown). It is worthy to note that insulin resistance is a very common metabolic abnormality present in most, although not all, patients with acromegaly.[22,23] In the fasting state, acromegalic patients show an impaired glucose utilization by adipose tissue, while in the postprandial condition, this insulin resistance is further amplified and antilipolysis also appears.[23] An increased activity of GH axis could amplify the insulin-resistant state. In fact, several studies carried out in experimental animals have documented numerous changes in the insulin signalling pathway that are induced by GH excess.[24]

It is well-known that insulin resistance is responsible for different disturbances in lipid and lipoprotein metabolism.[25] In the present study, acromegalic patients showed higher triglyceride levels probably due to the accumulation of VLDL particles, and increased apo B concentration. This reflects the presence of a more atherogenic lipid and lipoprotein profile in acromegalic patients than in control subjects, which is also confirmed by higher values observed in different ratios evaluating atherosclerotic risk. Among them, apo B : apo A-I has been proposed as the marker with the highest predictive value for cardiovascular disease.[26] Moreover, acromegalic patients can also present an increment in the proportion of small and dense LDL particles, given that Mc Laughlin *et al.*[27] have recently proposed that the above mentioned triglycerides : HDL-C ratio can be employed as a predictor of insulin resistance and also of the proportion of this highly atherogenic LDL subpopulation. Arosio *et al.*[11] have also evaluated the physical properties of LDL by ultracentrifugation and proved that acromegalic individuals have smaller and/or denser LDL particles, coincident both with the results reported by Tan *et al.*[7] and with our conclusions. Matching with the alterations described in plasma lipoproteins, CETP activity was found to be higher in patients than in controls. As it has also been shown by Tan *et al.*,[7] this lipid transfer protein might be partially responsible for generating small and dense LDL particles. These results differ from another study that was carried out employing a different method which evaluates the transfer of cholesteryl esters form LDL to HDL particles.[9]

Although insulin resistance itself could be crucial for modifying the lipoprotein profile, it must be noted that GH has a direct effect on lipoprotein metabolism.[28,29] This relationship could explain the presence of an altered lipid profile in acromegalic patients in the absence of insulin resistance. Actually, GH inhibits lipoprotein lipase from adipose tissue and stimulates hepatic lipase and hormone-sensitive lipase, the latter being responsible for free fatty acid release from adipose tissue, which are then taken by the liver and used for triglyceride synthesis. Accordingly, in the present work, GH, IGF-1 and IGFBP-3 were positively associated with plasma levels of glucose, insulin, glucose : insulin, HOMA, triglycerides, VLDL-C, and apo B.

In the search of biomarkers which provide an independent diagnostic or prognostic value of the atherosclerotic process and/or of its sequels, we evaluated CRP, $LpPLA_2$, endothelin-1, VCAM-1, white blood cell count and leucocyte cell adhesion molecules. These factors reflect the underlying biology of the vessel wall, which could also be influenced by a direct effect of GH and IGF-1. Some authors have postulated that GH and its tissue effector, IGF-1, would exert a protective effect on the cardiovascular system.[30] Nevertheless, this hypothesis may be hardly extrapolated to acromegalic patients, as

most of those studies have been carried out administering exogenous GH or IGF-1, thus creating a short-term hormonal excess, in contrast to acromegaly which represents an *in vivo* model with a chronic increment in GH and IGF-1 levels. Moreover, most of these studies measured parameters dealing with cardiac contractility and not biomarkers of endothelial dysfunction.

To our knowledge, this is the first time that higher endothelin-1 levels are reported in acromegalic patients in comparison to healthy controls. Furthermore, endothelin-1 was positively related to GH, IGF-1 and IGFBP-3, thus suggesting an association between endothelin-1 and the extent of acromegaly activity. These are interesting findings given that endothelin-1 has several atherogenic and/or inflammatory properties such as the regulation of the release of vasoactive substances and the stimulation of smooth muscle mitogenesis and cardiac contraction.[31] Elevated plasma levels of endothelin-1 have been associated with coronary artery disease, essential hypertension and heart failure.[32] In acromegalic patients, endothelin-1 can be increased in response to GH excess, which has been reported to induce tumour necrosis factor (TNF)-α and interleukin (IL)-1 secretion by mononuclear blood cells.[33] Both TNF-α and IL-1, together with IGF-1, which is also increased in acromegaly, are able to regulate endothelin-1 expression.[34] This regulation seems to primarily occur at the level of mRNA, which is achieved by controlling the activity of the promoter and the stability of mRNA.[35] Our data also evidenced a positive association between endothelin-1 and VCAM-1, a cell adhesion molecule of endothelial location that belongs to the immunoglobulin superfamily and that actively participates in the firm adhesion and extravasation of circulating leucocytes into the artery wall.[36] The observed correlation is in agreement with the mechanism described by Li *et al.*[37] by which endothelin-1 seems to directly stimulate arterial VCAM-1 expression through its receptor-mediated activation of NADPH oxidase and superoxide formation. Moreover, endothelin-1 also correlated with lymphocyte CD49d, which was, in turn, significantly higher in acromegalic patients than in healthy controls. This increment in lymphocyte CD49d seems to reflect the presence of activated lymphocytes given that this cell adhesion molecule is part of the VLA-4 complex, which interacts with endothelial VCAM-1 and, consequently, mediates leucocyte migration into the intima.[36] Then, lymphocytes in the artery wall trigger the inflammatory process by producing diverse cytokines, such as interferon γ, which has been shown to play different roles in mediating foam cell formation, smooth muscle cell proliferation and regulating the production of matrix metalloproteinases, thereby influencing plaque stability.[38] Among the different CDs evaluated in monocytes, lymphocytes and polymorphonuclear cells in the present study, only lymphocyte CD49d varied in relation with acromegaly. Diverse CDs located on the surface of different cellular types seem to respond to differential regulatory mechanisms, which have not been fully elucidated up to date.

Regarding CRP, which has been implicated in multiple aspects of atherogenesis and plaque vulnerability, similar values were detected in both groups. Nevertheless, it must be noted that CRP was positively associated with lymphocyte CD49d, which was increased in acromegalic patients. Sesmilo *et al.*[14] attributed the lack of higher CRP levels in acromegalic patients to low-IL-6 concentration, a decrease that was, in turn, assigned to the decrease in fat proportion. In this and other studies, CRP was negatively associated with GH, consistent with a negative regulation of the acute-phase response by GH.[39] The mechanisms underlying this negative association are unclear, but there is strong evidence of a complex crosstalk between GH and acute-phase cytokine signalling at the molecular level.[40]

Other two candidate biomarkers of cardiovascular disease, $LpPLA_2$ and white blood cell count, failed to evidence any difference between acromegalic patients and healthy controls. Although different epidemiological studies have reported that $LpPLA_2$ may be a predictor of coronary artery disease, the topic is highly controversial. Some authors postulate that $LpPLA_2$ would only vary in parallel to variations in LDL levels, whereas others attribute an antioxidant antiatherogenic capacity to $LpPLA_2$.[41] Increases in white blood cell count have been fundamentally observed in acute myocardial infarction, which is known to trigger a systemic response to a necrotic insult characterized not only by leucocytosis but also by the synthesis of acute-phase proteins such as CRP.[42] In our study, the synthesis of this protein was not modified in this group of acromegalic patients. Further studies are necessary to disclose the precise factors that regulate these atherogenic biomarkers or risk factors in acromegaly.

In conclusion, active acromegalic patients presented subtle modifications in the lipid and lipoprotein profile, higher endothelin-1 levels and elevated lymphocyte CD49d content, all of which were independent of BMI. Taken together, the alterations here described seem to contribute to constituting a state of higher propensity for the development of atherosclerotic cardiovascular disease, which adds to the presence of specific cardiomyopathy in acromegaly.

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