

RESEARCH ARTICLE

Peripheral activation of inflammatory intracellular signaling pathways and their correlation with IL6, IL10 and TNF α in obesity and type 2 diabetes mellitus

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Background: We investigated the activation of intracellular signaling pathways in individuals with and without T2D/obesity and correlated them with clinical characteristics and IL6, IL10 and TNF α serum levels. **Methods:** This study included 42 patients with T2D and 42 healthy controls matched by BMI. Intracellular signaling pathways Akt, p38 and ERK in peripheral blood mononuclear cells (PBMCs); IL6, IL10 and TNF α serum levels were evaluated. **Results:** Western blot analysis revealed that phosphorylation values of Akt, p38 and ERK were not significant different between the groups; but, once individuals were stratified according to BMI, activation of Akt and ERK was associated with obesity and T2D while the activation of p38 with obesity. The phosphorylation values of these proteins were also positively correlated with BMI and waist circumference. IL6 levels correlated with P-p38 in control group, while IL10 and TNF α did not correlated with these pathways. **Conclusions:** These results suggest that there is a link among obesity, T2D and intracellular signaling pathways in peripheral circulating immune cells, which may be associated with systemic low grade inflammation observed in T2D and obese patients.

Keywords: type 2 diabetes; obesity; intracellular pathways; cytokines; inflammation

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Introduction

Type 2 diabetes mellitus (T2D) is the most common form of diabetes, affecting 90-95% of patients with diabetes [1, 2]. The T2D leads to the development of long term macrovascular complications, including atherosclerosis, and microvascular complications, such as nephropathy, retinopathy and peripheral neuropathy [3].

Several studies have demonstrated that low-grade inflammation is a condition associated with T2D. Furthermore, it has been accepted the hypothesis that a chronic subclinical inflammation is part of the metabolic syndrome and insulin resistance [4, 5, 6, 7]. Indeed, pro-inflammatory cytokines levels such as IL6 and TNF α are increased in the serum of T2D patients, which corroborates

the conclusion that inflammation is involved in T2D pathogenesis [3, 8, 9].

Obesity is the major environmental factor that influences the T2D development [10]. Obesity is directly correlated with insulin resistance in peripheral tissues and consequently with elevated insulin levels [10, 11]. Insulin resistance attributed to obesity is associated with an increased release of IL6 and TNF α , specially produced by the adipose tissue, and decrease of anti-inflammatory cytokine such as IL10 [12]. Thereby, these two metabolic diseases are closely associated, and inflammation is the major link between obesity and T2D, especially in insulin-dependents tissues as muscle, adipose and liver [13].

Mitogen-activated protein kinase (MAPK) pathway constitutes a complex kinase network that controls a variety of physiological processes, such as apoptotic cell death, cell growth and differentiation [14, 15]. Deregulation of MAPK activity has been implicated in pathological situations, including inflammation. To date, three MAPK pathways have been well characterized: ERK, JNK and p38. MAPKs are serine/threonine kinases, which are regulated by phosphorylation cascades and are activated mainly in response to mitogens, growth factors, environmental stress and inflammatory cytokines [14, 15]. Activation of JNK and p38 pathways has been linked to the pathogenesis of diabetic neuropathy [16]. The p38 MAPK is also involved in downregulation of glucose transporter expression and is significantly increased in adipocytes from T2D patients [14, 17]. Endothelial cells from patients with diabetes presented increased activation of ERK when compared with health controls [18].

The insulin dependent regulation of systemic and cellular metabolism is controlled by the PI3K/Akt pathway. This pathway is activated in response to insulin, growth factors, cytokines and environmental stress. Further, PI3K/Akt can be regarded as the major pathway of metabolic action of insulin [19] and is thought to contribute to numerous cellular functions, such as cell growth and survival, nutrient metabolism, and transcriptional regulation [20]. Studies focusing on Akt and diabetes showed both increase and decrease of its activity, indicating a controversial hypothesis about Akt role in diabetes [21, 22, 23].

Few studies have evaluated systemic inflammation in T2D patients through activation of MAPKs and Akt pathways in peripheral cells. Furthermore, there are no studies evaluating these intracellular proteins according to the body mass index (BMI) stratification. As obesity can be also regarded as an inflammatory disease, in this study we investigated the status of the intracellular signaling pathways Akt, p38 and ERK in peripheral blood mononuclear cells (PBMCs) of individuals with and without T2D in order to correlate with clinical characteristics and BMI.

MATERIAL AND METHODS

Subjects

This observational study included 42 patients with clinical and laboratory diagnosis of T2D (8 men and 34 women) aged from 32 to 70 years, and 42 healthy controls matched by age, gender and BMI. Controls did not present diabetes, impaired fasting glucose, impaired glucose tolerance, or insulin resistance. Patients were consecutively recruited from the Clinic of Endocrinology, Hospital Santa Casa (Belo Horizonte, Minas Gerais, Brazil) from June 2012 to February 2013. T2D diagnosis was based on the American Diabetes Association (ADA). Clinical and laboratory data were obtained after interviews with the individuals. Laboratory parameters were measured in the morning after overnight fasting. Subjects were requested to refrain from physical activity 24 hours before the tests.

The project was approved by the Ethics Committee of the Universidade Federal de Minas Gerais and Santa Casa de Belo Horizonte and from all subjects was obtained the term of consent. The exclusion criteria were subjects who presented more than 70 years old, infectious process current or recent, cancer or autoimmune disease, history of cardiovascular disease, and treatment with anti-inflammatory drugs.

Patients and controls were classified according to BMI, with 30 patients and 27 controls as lean or overweight (BMI<30kg/m²); 12 patients and 15 controls as obese (BMI \geq 30kg/m²).

Blood collection

Serum and heparin plasma were collected by venipuncture after resting for at least 30 minutes, during the same time of the clinical interviews. The tubes for serum samples were centrifuged at 3.000g for 10 minutes. Aliquots were stored at -80 °C until further analysis of cytokines. The heparin samples were destined to peripheral mononuclear blood cells (PBMC) obtainment as described below.

PBMCs and protein isolation (Cytosolic extracts)

PBMC were immediately separated by standard Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. Briefly, 10 mL of heparinized blood were diluted with Phosphate-buffered saline solution (PBS; 1:1 dilution) and submitted to a Ficoll gradient. After 40 min of centrifugation (600g, at room temperature), the PBMCs were collected between the plasma and the Ficoll interface and washed three times by centrifugation with PBS. The PBMCs were lysed with

Table 1. Clinical and biochemical characteristics of T2D patients and controls

	Patients (T2D) (n=42)	Controls (n=42)	p value
Male	8	8	-
Female	34	34	-
Age	57.5 (11.0) ^a	54.0 (22.0) ^a	0.202
BMI	28.1 (6.2) ^a	28.0 (6.6) ^a	0.383
FPG	133.5 (98.0) ^a	83.3 (12.0) ^a	<0.001*
GHb	9.6 ± 2.1	...	-
PPG	232.3 ± 92.1	...	-
Waist circumference	98.1 ± 15.4	97.3 ± 16.2	0.844
WHR	0.96 (0.02) ^a	0.84 (0.11) ^a	0.002*

^a - value in median (interquartile range). For others - mean ± standard deviation. *p significant. BMI - body mass index; FPG - fasting peripheral glucose; GHb - glycohemoglobin; PPG - postprandial blood glucose; WHR - waist-to-hip ratio.

Table 2. Cytokine levels in T2D patients and controls

	Patients (T2D) (n=42)	Controls (n=42)	p value
IL-6	2.5 (3.7)	1.0 (1.1)	<0.001*
IL-10	209.5 (78.0)	181.0 (46.0)	0.006*
TNF-α	97.5 (40.5)	88.5 (67.75)	0.420

IL-6: pg/mL; IL-10 and TNFα: fg/mL. *p significant.

ice-cold hypotonic lysis buffer (0.5mM DTT, NaF 25mM, 10mM NaCl, 3mM MgCl₂, 0.002% NaN₃, 10mM Tris pH 7.4, 1mM PMSF, 20μM leupeptin, 0.1mM EDTA, 10μM aprotinin) ice-cold for 15 min and added 5% NP-40 for 5 minutes. The lysate was centrifuged and the cytosolic fraction on the supernatant was removed and stored at -20°C. Protein concentration was determined by Bradford method using the "Bio-Rad Assay kit" (Bio-Rad Laboratories USA).

Western blot analysis

Cytosolic extracts (30 μg) were separated on a denaturing 10% polyacrylamide-sodium dodecyl sulfate gel electrophoresis and transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked for one hour at room temperature with PBS (5% w/v nonfat dry milk and 0.1% Tween-20), washed three times with PBS (0.1% Tween-20), and incubated at dilution of 1:1000 with specific anti-phospho antibodies (P-Akt, P-ERK1/2 and P-p38) (Cell signaling Technology, Beverly MA, USA) or β-actin (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (5% w/v BSA and 0.1% Tween-20). Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3000, Santa Cruz Biotechnology, USA). The phospho proteins (immunoreactive bands) were visualized by chemiluminescence detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ). The values of P-Akt, P-ERK1/2, and P-p38 were quantified of autoradiography films by a densitometry analysis software (ImageJ, Image Processing and Analysis in Java; NIH, Bethesda, MD), and the phosphor-proteins values were normalized with β-actin and expressed in arbitrary units. Variations in protein levels were estimated, and the results

were presented as P-Akt, P-ERK1/2 or P-p38/β-actin ratio, measured in arbitrary units. Western blot for each protein was performed in batches to minimize the inter-assay variation for samples run on different gels.

Serum cytokine determination

Serum IL6 levels were measured by ELISA assay by Quantikine HS High sensitivity human IL6 kit (R&D Systems), IL10 and TNFα were measured by flow cytometry using CBA kit (BD) according to manufacturer's instructions.

Statistical analysis

Statistical analyzes were performed with SPSS version 13.0. All variables were tested for normality by Shapiro-Wilk test. P-p38 was normally distributed (values expressed as mean and standard deviation) and Akt, ERK, IL10, TNF-α and IL6 were non-normally distributed (values expressed as median and interquartile range). Differences between two groups were compared with T test or Mann-Whitney U test for parametric and non-parametric variables, and ANOVA or Kruskal-Wallis for more than two groups, respectively. Spearman's correlation analyses were performed to examine the relationship between the variables.

We primarily performed a univariate analysis considering as independent variables: P-Akt, P-p38 and P-ERK values and IL6, IL10 and TNFα levels. We selected those with p <0.2 to enter in the multivariate logistic regression analysis, where the final model followed the adequacy according to Hosmen and Lemeshow test. P-values <0.05 were considered significant.

Results

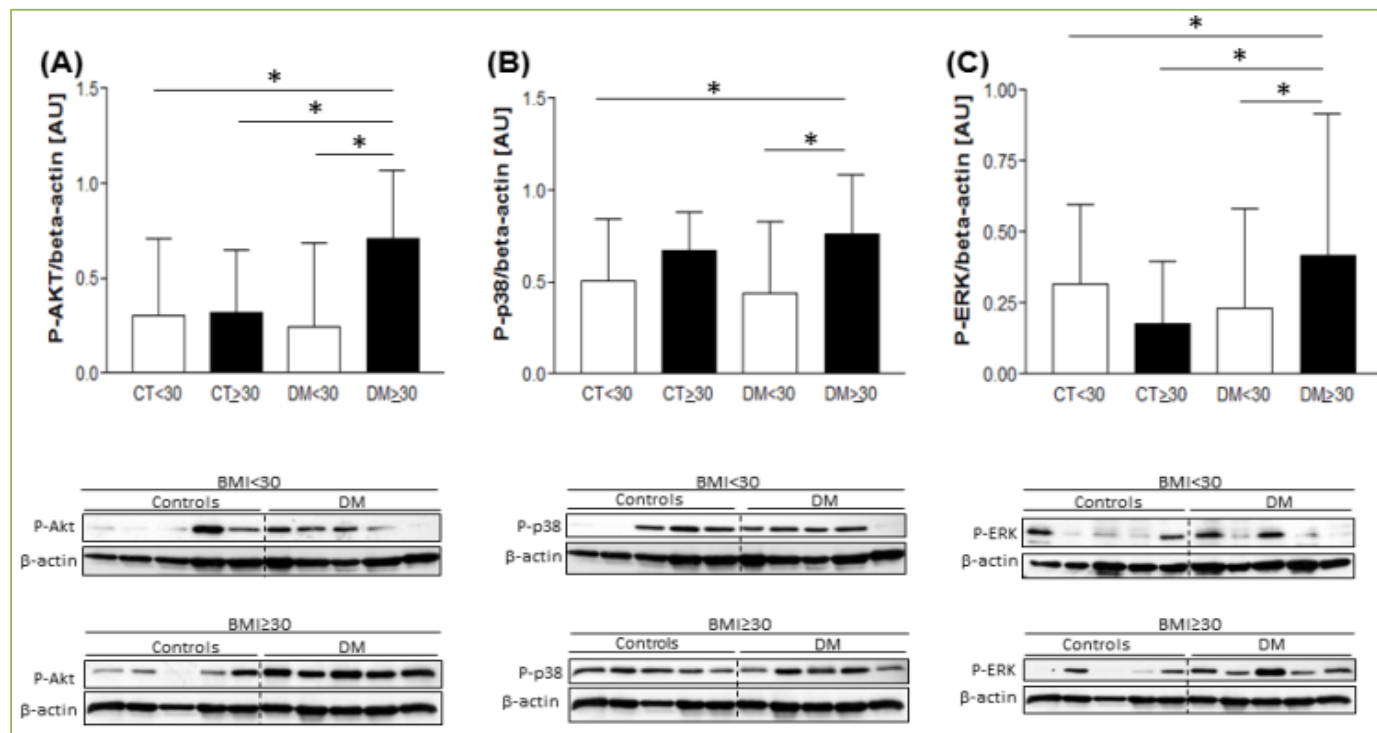


Figure 1. Western blot analysis of phosphorylated levels of Akt, and MAPKs p38 and ERK1/2 in cytoplasmic cell extracts of PBMCs of diabetic patients (T2D) versus controls in each group, according to BMI (Body Mass Index). Phosphorylation values of Akt (A), p38 (B) and ERK (C) in each group were obtained by densitometry analysis of autoradiography films using ImageJ software. The values were normalized to the values of β -actin in the same sample and expressed in arbitrary units. Representative autoradiographies of the Western blot membranes were showed below the graphics. Data are presented as arbitrary units (CT<30= controls with BMI<30 kg/m²; CT≥30 = controls with BMI≥30 kg/m²; T2D<30= patients with BMI<30 kg/m²; T2D≥30 = patients with BMI≥30 kg/m²). *Significant p<0.05.

Subjects characteristics

Clinical and biochemical characteristics, as well as cytokine levels of the both groups are shown in Table 1 and 2. Glycohemoglobin (GHb) and postprandial blood glucose (PPG) were obtained only from patients. Fasting blood glucose, IL6 and IL10 levels were significantly higher in patients (T2D) than controls. The waist-to-hip ratio (WHR) was also significantly higher in patients than in controls. The other parameters did not presented significant differences between patients and controls (p>0.05).

Phosphorylation values of Akt, p38 and ERK in TD2 patients and controls

Western blot analysis of PBMC showed that phosphorylation values of Akt, p38 and ERK were not significantly different between patients (0.27 (0.56); 0.53 ± 0.30; 0.24 (0.36); respectively) and controls groups (0.29 (0.24); 0.54 ± 0.24; 0.27 (0.28); respectively).

When patients and controls were divided in groups according to BMI, we observed differences in the phosphorylation values. Values of phosphorylated Akt were higher in obese (0.85 (0.51)) than in lean/overweight (0.14

(0.35)) patients, and also when compared with obese (0.37 (0.28)) and lean/overweight (0.28 (0.25)) controls (Figure 1A). P38 phosphorylation values were higher in obese patients (0.77 ± 0.27) than lean/overweight (0.40 ± 0.23; 0.51± 0.23) patients and controls, respectively, but not when compared to obese controls (0.67 ± 0.28) (Figure 1B). Finally, values of P-ERK were significantly higher in obese patients (0.40 (0.30)) when compared with other groups (lean/overweight patients and control: 0.17 (0.15) and 0.28 (0.32); obese control: 0.19 (0.25), respectively) (Figure 1C).

Correlation analysis

For all participants, significant correlation was found between BMI and phosphorylation values of Akt and p38 (r= 0.345, p=0.004 and r= 0.409, p<0.001, respectively) (Figure 2). Accordingly, we found a significant positive correlation between waist circumference and P-Akt and also P-p38 (r=0.295, p=0.014 and r=0.242, p=0.046, respectively), but not P-ERK (p=0.179) (Figure 3).

The correlation between the cytokines levels and phosphorylation values was also evaluated. There was a positive correlation between IL6 levels and P-p38 values for all subjects (r=0.294, p=0.016). No significant correlation

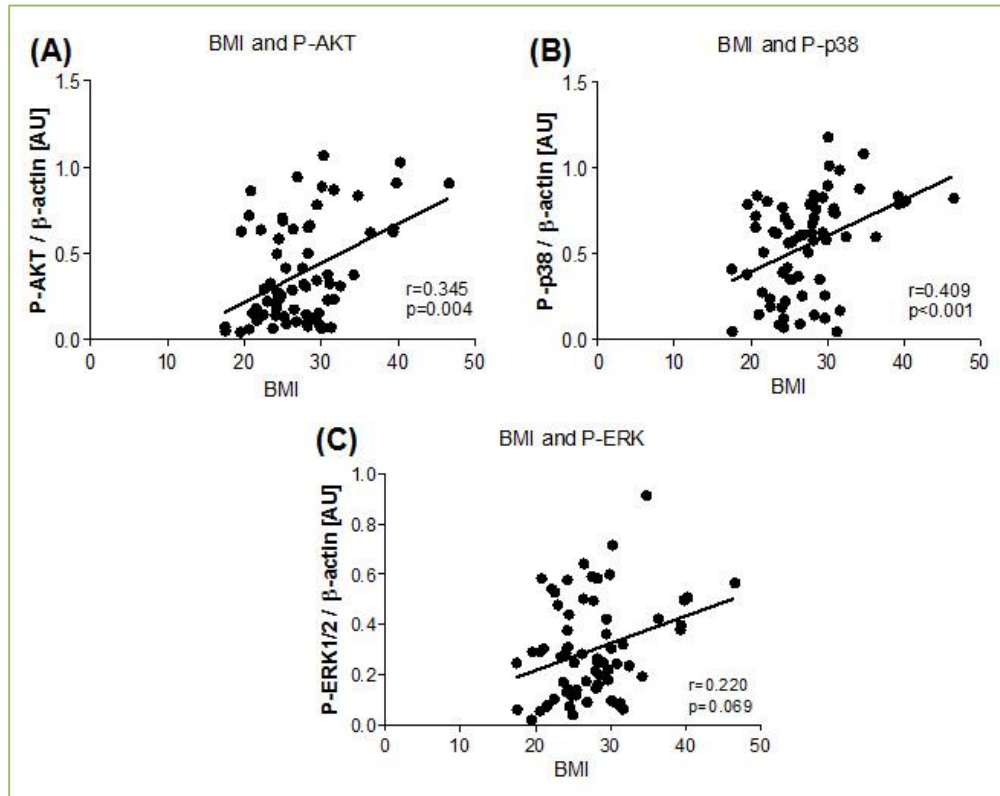


Figure 2. Correlation Analysis. (A) BMI and Akt phosphorylation values. (B) BMI and p38 phosphorylation values. (C) BMI and Erk1/2 phosphorylation values. Significant: $p < 0.05$.

between IL6 and P-p38 was found for patients, but a positive correlation was observed for controls ($r=0.478$, $p=0.007$). The other correlations were not significant for IL10 and TNF α ($p > 0.05$).

Multivariate logistic regression was performed, considering 3 models according to dependent variable: T2D, obesity for both groups and obesity only for T2D group ($BMI \geq 30 \text{ kg/m}^2$). For the first model, only IL6 was independently associated to T2D ($p=0.008$, $OR=0.7427$, $IC=0.595-0.925$). For the second model, AKT was the only variable independently associated to obesity considering diabetic and non-diabetic group ($p=0.040$, $OR=40.09$, $IC=0.9794-1.5272$). Finally, no parameter was independently associated to the occurrence of obesity in T2D group (all $p > 0.05$).

Discussion

In this study, we investigated the activation of inflammatory intracellular signaling pathways Akt, p38 and ERK in mononuclear cells obtained of peripheral blood from individuals with and without T2D. No difference was found in phosphorylation values of Akt, p38 and ERK between T2D patients and matched controls. Although these results could suggest that the phosphorylation values of these proteins are

not associated to the disease, we should consider that T2D is a low-grade chronic inflammatory disease [3] and perhaps the activation of inflammatory pathways occurs mainly in adipose, liver and muscle. Indeed, IL6 levels were evaluated in these groups and we observed higher values independently associated with the T2D occurrence, indicating a sub-clinical inflammation status in this metabolic disorder. In agreement, we observed higher levels of TNF α in T2D group, although not significant, when compared to controls. Moreover, IL10 levels were also higher in T2D, which suggest a regulatory and anti-inflammatory effect of this cytokine in this group.

When the individuals were classified in groups based on BMI, our data showed that cytosolic values of P-Akt were more elevated in obese patients when compared to the other group of patients (lean/overweight) and controls. This data indicate that higher peripheral immune cellular activation, measured by Akt phosphorylation, is associated to T2D only in obese individuals, but not independently. According to our results, a previous study also demonstrated an increase phosphorylation of Akt in endometrium of obese patients [24].

In the present study, the difference observed between obese subjects with and without diabetes shows that beyond the adipose tissue, the presence of T2D also increases the phosphorylation of Akt systemically. However, these data

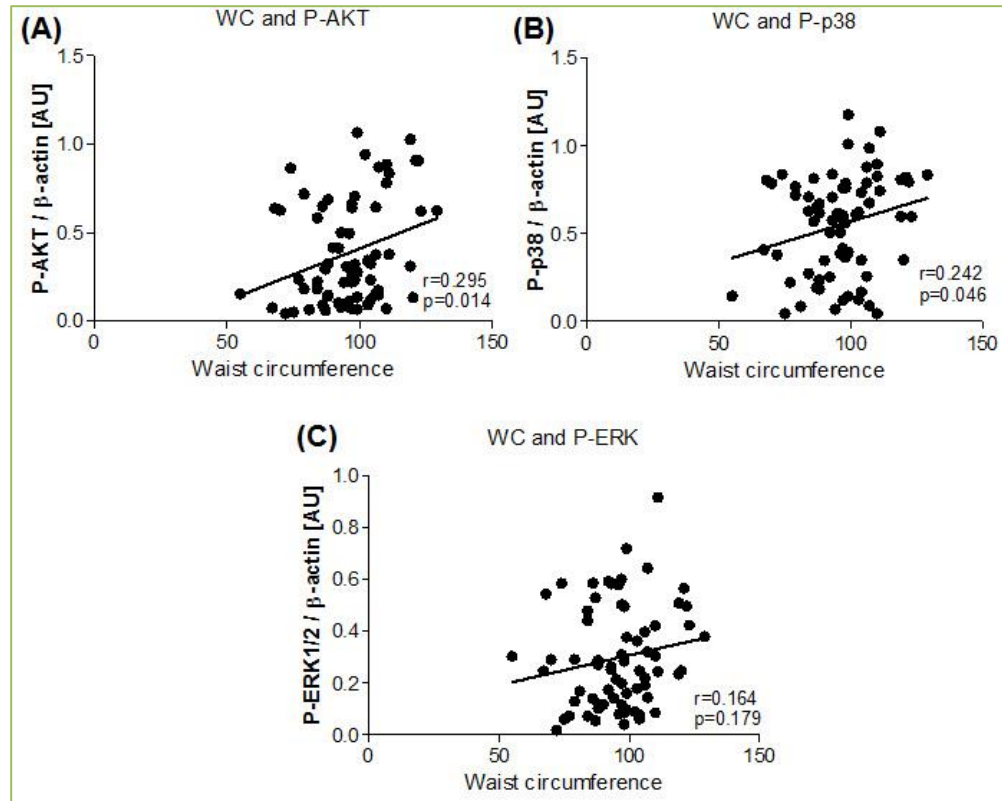


Figure 3. Correlation Analysis. (A) Waist circumference and Akt phosphorylation values. (B) Waist circumference and p38 phosphorylation values. (C) Waist circumference and Erk1/2 phosphorylation values. Significant: $p<0.05$.

differ from other studies that have used other cells source. Kim *et al.* [25] analyzed skeletal muscle of obese humans with and without T2D and found that the insulin effect on Akt phosphorylation was similar in obese nondiabetic and diabetic subjects. Another study involving obese diabetic patients and lean and obese nondiabetic subjects found decreased activity of PI3K and Akt induced by insulin in skeletal muscle of obese diabetic patients, which indicates that insulin resistance was associated with reduced PI3K/AKT pathway activity [26]. These findings suggest that Akt pathway activation depends on the insulin responsive tissue.

We also observed that P-ERK values were higher in obese patients when compared to other patients and control groups, which indicates that increased P-ERK values are associated to obesity in T2D patients. Analysis of P-p38 showed increased values in obese patients when compared to lean/overweight patients and controls, but not with obese controls, suggesting that this pathway is only associated with obesity.

A study analyzed the p38, ERK1/2 and JNK activation in adipocytes isolated from healthy and T2D individuals and found fivefold increase values of p38 phosphorylation in patient's adipocytes when compared to controls [17]. However, this study did not consider the distinction of BMI and used

different cells type. But in agreement with our results, Carlson *et al.* (2003) [17] also showed that ERK phosphorylation was twofold higher in adipocytes from T2D patients when compared with healthy control subjects and Gogg *et al.* (2009) [18] observed an increase of P-ERK in endothelial cells from T2D patients when compared to controls. It is noteworthy that in our work we used PBMC and none of the previous has used these cells to demonstrate the activation of phosphokinases. Therefore, besides the adipocytes, our findings indicate that the activation of these MAPKs pathways occurs in circulating cells, suggesting that the activation of these pathways is associated to the maintenance of systemic inflammation in obese T2D patients.

Since PBMCs has been known to express insulin receptors, these signaling pathways could be activated by insulin [27]. As hyperinsulinemia is a condition frequently observed in obese and T2D individuals, we cannot rule out the hypothesis that this condition helps in maintaining the phosphokinases activated state, favoring the exacerbation of the inflammatory state.

The relationship between the phosphorylation values of phosphokinases (Akt, p38 and ERK) and obesity could be confirmed by the correlation with BMI. Also, P-Akt and

P-p38 were positively correlated with waist circumference. These results indicate that there is a link between the body mass and activation of these pathways.

IL6 levels were only correlated with P-p38 in the control group. It is known that activation of p38 pathway plays essential roles in the production of pro-inflammatory cytokines, including IL6, which could represent a hypothesis for our results [28]. No correlation with the phosphokinases was observed in T2D group, suggesting that the higher IL6 levels observed in this group could be a result of the stimulation of other intracellular pathways, such as NF- κ B. Similarly, the increased levels of IL10 and TNF α are not associated with peripheral phosphorylation of Akt, p38 and ERK in T2D group.

Limitations in our study, as the small sample size and the use of cytoplasmic extract instead whole cell extracts, can be suggested. Indeed, activated Akt and ERK may localize to some extent in nuclei and p38 to the cytoskeleton. Thus, the use of cytoplasmic extracts could miss a small amount of protein and underestimate phosphorylation. Moreover, all patients were under therapeutic treatment and we cannot exclude the potential effect of medications on the activation of MAPK pathways, as insulin and statins [29, 30].

In conclusion, our study showed that obesity and T2D are associated with systemic changes in intracellular signaling pathways involved with the production of inflammatory mediators. This may be one the mechanisms underlying the low grade inflammation in T2D and obesity.

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Conflict of interest

The authors declare that they have no Conflicting interests.

List of abbreviations

ADA: American Diabetes Association; AKT: Protein Kinase B; BMI: Body mass index; IL: Interleukin; JNK: c-Jun N-terminal kinases; MAPK: Mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; T2D: Type 2 diabetes mellitus; TNF: Tumor necrosis factor.

Author contributions

NTP, KBG and LPS: conception, acquisition data, analysis and interpretation, draft the manuscript. LOP, KFR, MCO,

ALT, AVF: acquisition data. AAB, CMAFV: patient selection.

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