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Research Article

Pro-Inflammatory Cytokines, Leptin and HGF in Obese and Type 2 Diabetic Patients

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Abstract

Aim: The aim was to compare the serums level of a panel of T2D-related inflammatory cytokines, adipokines and vascular growth factors in urban Ecuadorian and Dutch T2D outpatients and non-diabetic controls.

Methods: Commercially available test panel was used to measure cytokines and adipokines in the serum of 33 Quitonian and 28 Rotterdam T2D ambulatory patients and 28 Quitonian and 22 Dutch non-diabetic controls.

Results: Overweight/obesity was more prevalent in the Ecuadorian non-diabetic than in the Dutch controls, with IL-8 and IL-6 raised. Ecuadorian had shorter duration and less severe T2D than the Dutch patients. In the less severe Quito patients, hyperglycemia and overweight were primary drivers of pro-inflammatory cytokine levels (IL-8 and TNF-α significantly higher than in non-diabetic controls). In the more sever Dutch patients, microvascular complications were strong drivers of pro-inflammatory cytokine levels with the highest levels of IL-8, IL-6 and TNF-α. Leptin levels correlated with BMI (but not with hyperglycemia), and this correlation was stronger in the Dutch group than in the Ecuadorian people. Notably, leptin levels were in the normal range in Ecuadorian T2D patients and controls. Raised HGF levels in the Dutch T2D patients were associated with reduced levels of HDL and correlated with disease duration.

Conclusion: Overweight/obesity is more prevalent in Ecuadorian than Western European cities. Obesity, hyperglycemia and microvascular kidney complications were key drivers of pro-inflammatory cytokine production. Leptin levels were not raised in the overweight Ecuadorian T2D and non-diabetic controls; the Amerindian background may play a role in this phenomenon.

Keywords: *Diabetes; Inflammation; Cytokines; Adipokines*

Abbreviations

Abs: Antibodies; BMI: Body Mass Index; CCL2: Chemokine Ligand 2; CCL4: Chemokine Ligand 4; GAD65: Glutamic Acid Decarboxylase 65 kDa Isoform; HDL: High Density Lipoprotein; HGF: Hepatocyte Growth Factor; IKKβ: Inhibitor of Kappa B Kinase Subunit Beta; IL-1β: Interleukin 1 Beta; IL-6: Interleukin 6; IL-8: Interleukin 8; JNK: Jun Kinase; LDL: Low Density Lipoprotein; MCP-1: Monocyte Chemoattractant Protein 1; MIP1β: Macrophage Inflammatory Protein-1β; NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cell; NDC: Non-Diabetic Controls; PAI-1: Plasminogen Activator Inhibitor 1; T2D: Type 2 Diabetes; TNF-α: Tumor Necrosis Factor Alpha

Introduction

The link between inflammation, obesity and type 2 diabetes (T2D) is currently well established. Research from Hotamisligil., *et al.* in the early 1990s pointed to a pathogenic role of inflammatory cytokines in the development of insulin resistance and diabetes. The authors showed that TNF-α level was proportional to insulin resistance in obese people and animals [1]. Further research, confirmed this hypothesis, demonstrating that TNF-α was capable to induce insulin resistance in lean animals [1-4]. Nowadays, it is known that several pro-inflammatory cytokines are capable to inhibit the insulin signaling pathway through the activation of molecular pathways such as Nuclear Factor Kappa-light-chain-enhancer of activated B cell (NF-κB), IB kinase-β (IKKβ) and Jun kinase (JNK) [5-9].

Pro-inflammatory cytokines and adipokines are raised due to the activation of macrophages and adipocytes in obese adipose tissue, which leads to a so-called "chronically inflamed adipose tissue". A disturbed lipid metabolism in overweight/obesity is also capable of inducing a chronic pro-inflammatory state [10,11]. In monocytes and macrophages, the Nlrp3 inflammasome senses lipotoxicity-associated increases in intracellular ceramide, thereby inducing caspase-1 cleavage and facilitating interleukin-1β (IL-1 β) secretion [12]. Additionally, lipid alterations like high levels of Ox-LDL and low levels of HDL are related to inflammatory activation [9,13-15], while free fatty acids enhance the secretion of TNF-α, IL-6 and PAI-1 from macrophages. Activated macrophages secrete ever more inflammatory cytokines and chemokines creating a feed-forward loop of inflammation [3,14,16]. In sum, the literature contains numerous reports on increased levels of pro-inflammatory cytokines in the metabolic syndrome (MetS) and T2D, including excellent reviews on this topic [17-22].

There is a remarkable epidemiologic transition from infectious diseases to non-communicable diseases in recent decades in Latin America [23,24]. A plausible explanation for this phenomenon is the increased prevalence of overweight/obesity particularly in urban populations [25]. This increase is associated with an increased prevalence of the metabolic syndrome (MetS), cardiovascular diseases (CVD), and T2D - currently the leading causes of morbidity and death in Latin America [23,25-27]. Recently, in Ecuadorian population wide study we found a high prevalence of the MetS particularly in urban areas (e.g. 33.7% vs. rural: 27.0%) and at high socioeconomic status (high income 22.7% vs. low income 16.3%) [28]. This high prevalence of metabolic disorders represents an extreme risk to develop T2D in a broad percentage of the Ecuadorian population.

In another recently published study we reported on the serum levels of a panel of T2D-related cytokines, chemokines and growth factors, namely IL-8, TNFα, IL-6, IL-1β, CCL4, CCL2, Resistin, Leptin, Adiponectin, hepatocyte growth factor and PAI-1 [29]. We measured the levels of these 11 factors in the serum of 96 individuals living in the Ecuadorian city of Quito, of whom 56 were randomly selected T2D outpatients and 40 were non-diabetic controls (mainly caretakers). Of the factors that we tested only IL-8 and hepatocyte growth factor (HGF) were significantly higher in the T2D patients than in the non-diabetic controls. There were no differences between the two groups for the classical pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6. We concluded that while the T2D patients had signs of an increased inflammatory state in serum (due to the increase of IL-8), the increase was only mild.

In this study, we determined whether the clinical profiles of T2D outpatients and non-diabetic controls from Quito, Ecuador and from Rotterdam, the Netherlands differ in terms of serum levels of pro-inflammatory cytokines, chemokines, and growth factors measured by the test kit.

Patients and Methods

Patients and controls

In 2012 we recruited 28 Dutch patients with T2D participating in the Diabetes Pearl. The Diabetes Pearl is an observational cohort study, in which all eight Dutch academic medical centers participate, one of the tertiary medical centers is Erasmus MC in Rotterdam. Patients were included if they had been diagnosed with T2D and were attending the outpatient department. Patients were excluded if their ability to understand and write the Dutch language enabled them to provide written informed consent.

In 2012, we also recruited 33 Ecuadorian patients T2D, according to the criteria of The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [30], from 4 medical centers in Quito, Ecuador (Eugenio Espejo Hospital, Club de Leones Sur, Fundación Oftalmológica del Valle and Fundación de la Psoriasis). We excluded any patients who had immune disorders, serious medical illness, recent infections (last 2 weeks), obvious vascular complications, fever, pregnancy/postpartum, also LADA patients (positive GAD65 Abs) were excluded.

Regarding the non-diabetic Dutch and Ecuadorian control groups, we selected 22 Dutch and 28 Ecuadorian subjects by asking hospital staff and accompanying care takers to volunteer to donate blood at the same time as the patients were donating blood. These subjects came from the same ethnic and societal background and did not have serious medical disorders (including acute infection). Controls were over 30 years of age, and we matched for gender where possible.

The Medical Ethical Review Committee of the Erasmus MC approved the Dutch study. The Medical Ethical Review Committee of the Ecuadorian Corporation of Biotechnology in Quito, Ecuador approved the Ecuadorian study, and this was validated by the Ethic Committee of the Central University. Written informed consent was obtained from patients and controls. The Ecuadorian Ministry of Health (MSP) issued the permit necessary to export and process the samples at the Erasmus MC, Rotterdam, The Netherlands.

Serum cytokines and growth factors

In the same year that we collected and tested sera from the individuals living in the urban area in Ecuador, we also used the same series of assays and the same test (a commercially available test kit, the CBA, Milliplex® Map, U.S.A.) to analyze sera collected from 50 individuals living in the Dutch city of Rotterdam, of whom 28 were T2D outpatients and 22 were non-diabetic controls (all hospital and laboratory staff). The Dutch T2D outpatients were randomly and consecutively recruited from the outpatient diabetes clinic of the Erasmus Medical Center in Rotterdam, the Netherlands.

In the morning, fasting venous peripheral blood (10 mL) was collected in a clotting tube and processed within 4 hours. Serum was frozen and stored at minus 80°C for approximately 12 months before testing. Levels of TNFα, IL-1β, IL-6, HGF, PAI, resistin, CCL2 (MCP-1), adiponectin, leptin, IL-8, and MIP1β (CCL4) were measured by flow cytometry (BD LSR II Biosciences, California, and EE.UU.) using a commercially available multi-analyte cytometric bead array system (Milliplex® Map, U.S.A.). The data were analyzed using a 5-parameter logistic method for calculating analyte concentrations in samples (MAGPIX® with xPONENT software, Luminex, Austin, USA). Undetectable serum analyte levels were recorded as 0 pg/ml and included in the statistical analysis.

Data analysis

Statistical analysis was performed using SPSS 20 (IBM, Inc.) package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The Grubbs' test for outlier detection was applied (http://graphpad.com/support/faqid/1598/). Since the distribution pattern was different in the several cytokines, adipokines and growth factors measured; when necessary the data was log transformed to normalize the distribution. Thus, a parametric group comparison was applied (Independent T test). Correlations were determined by Spearman correlation. Graphs were designed with GraphPad Prism 5.04 (GraphPad Software, Inc) for Windows. Levels of significance were set at p= 0.05 (two tailed).

Results

Patients and controls characteristics

The characteristics of the Dutch and Ecuadorian T2D patients and healthy controls are shown in table 1. As can be seen from the table, there were considerable differences between the Dutch and Ecuadorian patients and controls.

	Ecuador T2D				Rotterdam T2D				p- Value
Group size n	33				28				
Age mean (range)	60 (40-85)				60 (37-77)				ns
Gender									
Female n (%)	18 (55%)				11 (39%)				ns
Male n (%)	15 (45%)				17 (61%)				ns
BMI mean (range); %	28 (22-39)	Normal 17%	Ow 45%	Ob 36%	31 (23-43)	Normal 4%	Ow 42%	Ob 54%	0.01*
Comorbidities									
HBP %	48.5%				71.4%				0.01
Systolic Blood Pressure (mean)	132 mmHg				141.5 mmHg				ns
Diastolic Blood Pressure (mean)	82 mmHg				83.6 mmHg				ns
Glucose state									
HbA1C mean (range); %	7.3 (4.8 - 12.5)	Normal 30%	High 70%		8.4 (6.1 - 13)	Normal 7.1%	High 92.9%		0.02*
Disease time mean (range)	5.6 (1-20)				14.2 (5-24)				0.00
Lipid Profile									
Chol. mg/dL mean (range); %	243 (165-436)	Normal 39%	High 61%		78 (58- 106)	Normal 100%	High 0%		0.00*
TGD mg/dL mean (range); %	202 (80 -409)	Normal 67%	High 33%		38 (16 - 92)	Normal 100%	High 0%		0.00*
HDL mg/dL mean (range); %	43 (18 -67)	Normal 58%	Low 42%		22 (10 -35)	Normal 3.6%	Low 96%		0.00*
LDL mg/dL mean (range); %	168 (77 - 361)	Normal 55%	High 45%		44 (24- 76)	Normal 100%	High 0%		0.00*
Hepatic Profile									
ASAT mg/dL mean (range); %	33.1 (6-78)	Normal 58%	High 42%		29.5 (17- 81)	Normal 89%	High 11%		0.00*
ALAT mg/dL mean (range); %	45.0 (7 -131)	Normal 52%	High 49%		27.2 (12 - 64)	Normal 89%	High 11%		0.00*
GGT mg/dL mean (range); %	58 (17 -360)	Normal 70%	High 30%		52 (11 -332)	Normal 68%	High 32%		ns
Medication									
Oral Anti-diabetic treatment %	73%				61%				ns
Insulin treatment %	21%				93%				0.00
Aspirin %	12%				32%				ns
Statins %	21%				82%				0.00
	Ecuador NDC				**Rotterdam NDC**				
Group size n	28				22				
Age mean (range)	52 (32-87)				39 (22-54)				ns
Gender									
Female n (%)	19 (68%)				12 (54.5%)				ns
Male n (%)	9 (32%)				10 (45.5%)				ns
BMI mean (range); %	28.5 (23-35)	Normal 14.8%	Ow 48.1%	Ob 37%	25 (21-32)	Normal 60%	Ow 26.7%	Ob 13.3%	0.01†

Table 1: *General characteristics of T2D and NDC patients from Ecuador and Rotterdam.*

**p value < 0.05, Ecuador T2D normal vs Rotterdam T2D normal. † p value < 0.05, Ecuador NDC normal vs Rotterdam NDC normal. NDC, non-diabetic controls. T2D, type 2 diabetes.*

T2D patients

In the Dutch patients, disease duration was considerably longer (14,2 vs 5,6 years, mean), and the prevalence of obese individuals was higher (54 vs 36%).

Dutch patients also had a higher prevalence of hypertension (71,4% vs 48,5%), while their albumin creatinine ratio was disturbed in 46,6% (unfortunately this was not determined in the Ecuadorian patients).

The Dutch patients had a slightly poorer control of their hyperglycemia, while the large majority (90%) were on insulin. In both groups, 60 - 70% were taking oral anti-diabetics.

82% of Dutch patients used statins, while only 21% of Ecuadorian patients were on this drug. Not surprisingly, the Dutch patients had a better lipid profile and a lower prevalence of non-alcoholic fatty liver disease (NAFLD).

In summary, the Dutch patients had a longstanding and more severe T2D with more microvascular kidney damage, yet had a better lipid profile, most likely due to the degree of statin use in this group.

Non-diabetic controls

The Ecuadorian non-diabetic controls (for 90% caretakers) had an obesity and overweight prevalence of 48% and 37% respectively, while the Dutch non-diabetic controls (all hospital staff) were much slimmer, with an obesity and overweight prevalence of 27% and 13% respectively. In fact, the Ecuadorian non-diabetic controls were just as overweight as the Ecuadorian T2D patients (It must be noted that in the Ecuadorian group, there was no difference in BMI between the non-diabetic hospital staff and the non-diabetic care-takers, but the hospital staff only comprised 10% of this control group).

Although average age did not differ between the non-diabetic control groups, patients in the Dutch T2D group were significantly older than their respective controls. We therefore corrected for age in the statistical comparisons between patients and controls.

Levels of cytokines and growth factors in serum

Table 2 shows the mean and standard deviation (SD) for the two groups of patients and their respective controls.

	Ecuador Cohort			Rotterdam Cohort		
	NDC	T2D	p-Value	NDC	T2D	p-Value
	Mean (SD)	Mean (SD)	T test Ecu-NDC vs. Ecu-T2D	Mean (SD)	Mean (SD)	T test Rott-NDC vs. Rott- T2D
IL8	8.4 (6.2)*	15.5 (18.7)	***0.04***	5.5 (1.9)	22.1 (26.0)	0.005
TNF-α	3.9 (2.0)	5.2 (2.5)	***0.03***	4.8 (1.8)	7.4 (3.1)	0.000
+IL6	4.5 (2.6)*	7.9 (9.8)	0.08	1.3 (1.3)	8.0 (7.1)	0.000
IL1-β	0.5 (1.5)	0.8 (1.3)	0.54	0.9 (0,5)	1.7 (2.2)	0.09
CCL4	114 (95)	92 (95)	0.39	82 (59)	111 (97)	0.21
CCL2	305 (131)	330 (176)	0.53	263 (113)	318 (148)	0.14
Resistin	389 (131)*	448 (254)	0.25	694 (558)	629 (391)	0.65
Leptin	1100 (964)	892 (1055)	0.42	712 (592)	2285 (3503)	0.04
Adiponectin	2711 (1747)	3054 (2881)	0.57	2785 1892)	1930 (1053)	0.07
HGF	764 (495)	1018 (1652)	0.08	874 (461)	1437 (938)	0.01
PAI1	935 (303)	830 (278)	0.17	954 (219)	1161 (483)	0.07

Table 2: *Cytokines, adipokines and growth factors statistical analysis between each study cohort, otherwise indicated. NDC: Non-Diabetic Controls. * p value < 0.05 Ecuador non-diabetic controls versus Rotterdam non-diabetic controls.*

The pro-inflammatory cytokines and chemokines IL-6, TNF-α, IL-8, IL-1, CCL4 and CCL2

Non-diabetic controls: As the table shows, there was a difference between the two non-diabetic control groups. The Ecuadorian non-diabetic controls had significantly higher levels of IL-8 and IL-6 and lower levels of resistin than the Dutch non-diabetic controls. The IL-6, IL-8 and resistin levels of the Ecuadorian non-diabetic controls correlated strongly and positively to the increased BMI ($r = 0.532$, $p = 0.004$; $r = 0.512$, $p = 0.006$ and $r = 0.476$, $p = 0.01$ respectively).

T2D patients: In the T2D patients, IL-8 and TNF-α levels were significantly higher in both the Ecuadorian and Dutch patient groups than the levels in their respective controls. Of the T2D patients, the Dutch group had the highest levels of pro-inflammatory cytokines, but when comparing the two patient groups statistical significance was only reached for IL-8. The Dutch patients also had significantly higher IL-6 levels than those in the Dutch controls.

To determine any relationships with clinical signs and symptoms, we performed correlation analyses between clinical variables and the level of the cytokines/chemokines/growth factors. Table 3 gives the main results. The pro-inflammatory cytokines IL-8, TNF-α and IL-6 correlated with disease duration (particularly in the Dutch group), and with Hb1Ac levels (particularly in the Ecuadorian group); the strongest correlation was observed with hypertension (in both groups); and in the Dutch group we also observed a strong correlation with the albumin/creatinine ratio (this parameter was only measured in the Dutch disease group).

Leptin and adiponectin

Leptin levels were not raised in the T2D patients from Ecuador (in fact they were the lowest in this group of the 4 tested groups) (Table 2 and Figure 1). Leptin levels were clearly and significantly raised in the Dutch T2D patients (and were the highest of the 4 tested groups). Adiponectin levels had an opposite pattern, being low in Dutch patients and high in the Ecuadorian patients (Table 2). In both the Dutch and Ecuadorian groups, leptin levels were correlated the strongest with BMI, although to a lesser extent in the Ecuadorian group (See figure 2). In the Dutch group, leptin levels were also correlated with disease duration (Table 3).

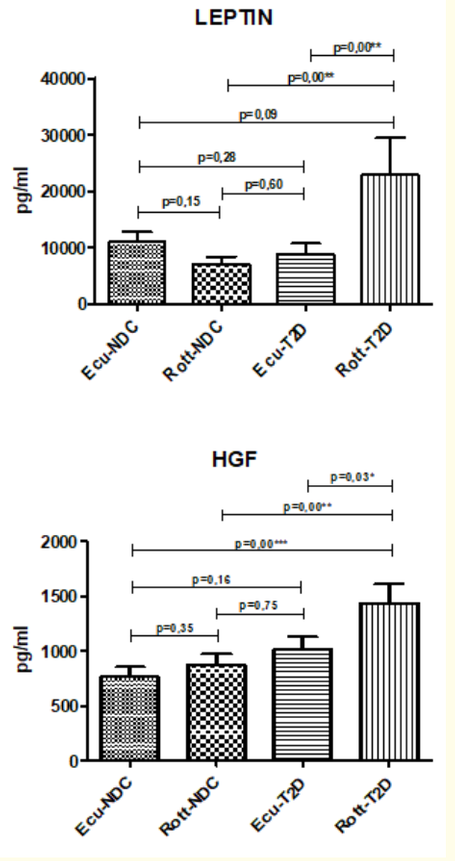

Figure 1: *Comparison of Leptin and HGF measurements between the four distinct groups; Ecuadorian non-diabetic controls (Ecu-NDC), Ecuadorian T2D (Ecu-T2D), Rotterdam non-diabetic controls (Rott-NDC), and Rotterdam T2D (Rott-T2D). **p value < 0,001; *p value < 0,05. HGF, hepatocyte growth factor.*

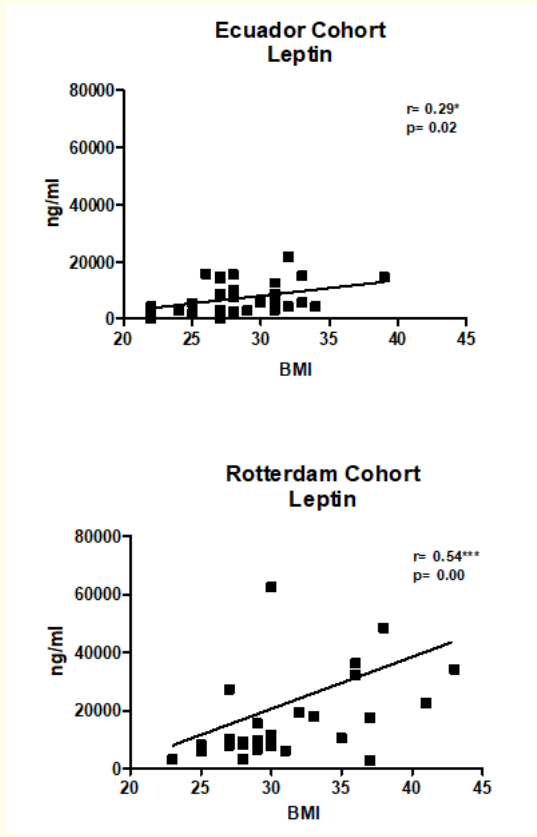

Figure 2: *Correlations of leptin with BMI (body mass index) in Ecuadorian T2D and Rotterdam T2D. *** strong correlation; *moderate correlation.*

HGF and PAI-1

HGF levels were significantly higher in the Dutch T2D patients than in the Dutch non-diabetic controls. The Ecuadorian T2D patients did not have elevated HGF levels (Table 2). PAI-1 levels were equal in all tested groups (Table 2).

In the Dutch group, HGF levels correlated with disease duration and BMI, while correlating strongly, but negatively with HDL levels (Table 3). In the Ecuadorian group, HGF correlated neither with disease duration, nor BMI. It did correlate positively with hypertension and negatively with triglyceride levels (Table 3).

		Disease time		Hyperglycemia		Hypertension		ACR		BMI		HDL		TGD	
		Dutch	Ecuador	Dutch	Ecuador	Dutch	Ecuador	Dutch	Ecuador	Dutch	Ecuador	Dutch	Ecuador	Dutch	Ecuador
Pro-Inflammatory	IL-8	0.60	0.42	ns	0.34	0.56	0.30	0.39	x	ns	ns	ns	ns	ns	ns
	TNF-α	0.35	ns	ns	ns	0.29	0.33	0.44	x	ns	ns	ns	ns	ns	ns
	IL-6	0.78	ns	ns	0.30	ns	ns	0.42	x	ns	ns	ns	ns	ns	ns
Adipokine	Leptin	0.47	ns	ns	ns	ns	ns	ns	x	0.54	0.30	ns	ns	ns	ns
Vascular Growth	HGF	0.39	ns	ns	ns	ns	0.26	ns	x	0.44	ns	-0.44	ns	ns	-0.33

Table 3: *Correlations of cytokines, adipokines and growth factors with clinical parameters.*
R values are shown only where there was significant difference (p value < 0.05), otherwise no significant (ns) legend was placed. X, no data available. Minus sign (-); negative correlation. ACR, albumin-to-creatinine ratio. BMI: Body Mass Index; HDL: High Density Lipoprotein Cholesterol; TGD: Triglycerides.

Discussion

This study shows that there are considerable differences in clinical profiles between the two cohorts of T2D outpatients and non-diabetic controls from Quito, Ecuador and from Rotterdam, the Netherlands. The Quitonian non-diabetic controls (mainly caretakers of the patients) had a much higher prevalence of obesity than the Rotterdam nondiabetic controls. This finding is in line with the high prevalence of obesity in the urban general population of Ecuador reported in a 2012 epidemiological survey [28]. Particularly urban-dwelling men of middle and high socio-economic class are at risk in Ecuador, while in Western Europe this group is more aware of a healthy life style. Indeed, the in-general-leaner Rotterdam control group consisted of hospital personnel, likely more aware of healthy life style. Compared with the Dutch non-diabetic controls, the Ecuadorian non-diabetic controls were not only more overweighed, but also had higher serum levels of the pro-inflammatory cytokines IL-8 and IL-6, which correlated significantly with the prevalence of obesity in this group. This is in accordance with the large body of literature providing evidence for pro-inflammatory cytokine production by adipocytes and macrophages in adipose tissue (see introduction).

Regarding the clinical profile of the T2D ambulatory patients of both countries, mean disease duration was longer in the Dutch patients than in the Ecuadorian patients, i.e. 14 versus 6 years. The T2D in the Ecuadorian patients was also milder and reasonably well controlled, with lower Hb1Ac levels than those in the Dutch patients. The Dutch T2D patients were also more obese and reported more vascular complications, such as high blood pressure. The albumin-to-creatinine ratio was disturbed in 46,6% of the Dutch patients. Although, we have no data on the albumin-to-creatinine in the here studied Ecuadorian T2D patients, a recent study of ours in a similar Ecuadorian T2D outpatient cohort from the same hospital detected a low prevalence of renal failure (2.8%). We therefore assume that microvascular kidney disease was also less prevalent in the Ecuadorian T2D patient group that took part in the current study.

Another notable difference between the Ecuadorian and Dutch T2D patients was in the use of medication: most of the Dutch patients were on insulin and used statins, while only a minority of Ecuadorian patients used this medication. The higher use of statins in Rotterdam resulted in a much more beneficial blood lipid profile and patients in this group had hardly any abnormal liver function values.

The differences in clinical profiles between the T2D groups were reflected in the serum profiles regarding pro-inflammatory cytokine, leptin/adiponectin and vascular growth factors.

Inflammatory cytokines

The Ecuadorian patients had mildly increased signs of inflammation, with only increased levels of IL-8 and TNF-α as compared to the Ecuadorian non-diabetic controls (who were equally obese). On average these higher cytokine levels correlated with the hyperglycemia in the T2D patients. Collectively this suggest that in ambulatory Ecuadorian patients with a mild form of T2D, levels of pro-inflammatory cytokines are driven by adipose tissue and the hyperglycemic state.

The serum of Dutch T2D patients had high (in fact the highest) levels of pro-inflammatory cytokines, which correlated strongly with the presence of hypertension and the albumin-to-creatinine ratio. In the Ecuadorian patients, levels of pro-inflammatory cytokines, although lower, also correlated with hypertension. We thus assume that the kidney's microvascular state plays another important role in elevating the levels of pro-inflammatory cytokines in T2D patients. It is known that dysfunctional endothelium can be an important source of inflammatory cytokines, particularly in older age [31,32].

In sum, in the T2D patients in this study, serum levels of the classical pro-inflammatory cytokines IL-8, TNF-α and IL-6 were determined by obesity, hyperglycemia and micro-vascular disease, as evidenced in the kidney.

Leptin: Leptin levels were clearly raised in the Dutch patients, while they were normal to low in the Ecuadorian patients. Similarly, while being obese in the Dutch patients was significantly associated with high levels of leptin, in the Ecuadorian patients, who were only slightly less overweight/obese than the Dutch T2D patients, this was not the case (although leptin levels correlated weakly with BMI in Ecuadorian patients). Apparently being overweight/obese had no clear and significant effect on leptin levels in the Ecuadorian T2D patients.

This raises the question of what factors might be responsible for the low leptin levels in the Ecuadorian T2D patients as compared with the Dutch T2D patients?

Bribiescas., *et al.* also reported differences in mean leptin levels between Ache Amerindians of Paraguay and long-distance runners from the US. Although the Ache had a higher mean adiposity (fat %, Ache 17.9 ± 1.8 SD; US runners 9.7 ± 3.2, $p < 0.0001$), leptin levels were nonetheless significantly higher in the runners (Ache 1.13 ng/ml ± 0.38 SD; runners 2.19 ± 1.15; $p < 0.007$) [33]. These results were taken as showing that there is an important ethnic variation in leptin levels independent of adiposity. Further support for an ethnic background causing differences in leptin levels comes from a study by Mente., *et al.* who investigated the ethnic differences in adiponectin and leptin concentration and their relationship with insulin resistance. They found serum leptin to be significantly higher in South Asians and Aboriginal people than in Europeans and Chinese people, while adiponectin concentrations were significantly higher in Europeans and

Aboriginal people than in South Asians and Chinese [34]. Similarly, Delgadillo-Guzman., *et al*. compared nutritional status and circulating leptin levels between a group of Amerindian Tepehuán people and Mestizo populations of Durango City, Mexico. Both normal and overweigh Amerindian Tepehuán subjects had lower leptin concentrations than the comparable Mestizo subjects [35].

In sum, there is certainly evidence in the literature for the notion that Amerindians have lower leptin production, irrespective of obesity.

HGF: HGF, an important vascular repair and neogenesis factor [36], was only raised in the Dutch T2D patients. These levels correlated negatively to HDL, and it can thus be considered a marker of a high atherosclerosis risk. Konya., *et al.* (2014) have reported serum HGF levels to be positively associated with carotid atherosclerosis in diabetes [37]. Nowak., *et al.* (2008) have found that HGF levels in diabetics are determined by the presence or absence of retinopathy [38], while Nishimura., *et al.* (1998) have reported HGF levels to be the highest in patients with proliferative retinopathy [39]. It is thus likely that in longstanding and severe patients with T2D, raised HGF levels are determined by the micro- and macro-vascular disease state of the patients.

In the Ecuadorian patients with relatively mild T2D of short duration, HGF correlated with the presence of hypertension. These data agree with a report of Rajpathak., *et al.* (2010), who found that HGF concentrations were associated with systolic blood pressure in postmenopausal women at risk for stroke [40]. These investigators also found that HGF was a marker for atherosclerosis, being positively correlated with LDL cholesterol and negatively correlated with HDL. In our study, HGF was negatively correlated with levels of triglycerides in the Ecuadorian patients, which is at odds with the general view that HGF is a marker of atherosclerosis risk.

Study Strengths and Limitations

A strength of this study is that it is to our knowledge the first report (as far as we know) comparing cytokines and growth factors in Ecuadorian and Dutch patients with T2D. This makes it possible to determine putative associations between ethnicity and serum levels of leptin. Furthermore, the serum was collected in the same year and analyzed by the same group using the exact same kit, thereby allowing for a direct comparison between the study groups.

A weakness of the study is that we did not collect more clinical determinants to look for possible links with the cytokine and growth factor levels. Particularly, the vascular state of the kidneys, the carotid artery and the eyes were not systematically studied in our patient groups. In addition, a better picture of the patient's disease state would have been obtained by also measuring levels of other vascular growth factors and endothelial factors. Furthermore, detailed studies in follow-up (progression of T2D) are indicated. Additionally, the numbers of patients and controls included in this study are too small to draw solid epidemiological conclusions and there is a need for a control group without any metabolic disorder.

Conclusion

The results of our study agree with evidence in the literature that overweight/obesity is more prevalent in Ecuadorian cities than in West-European cities, particularly in groups of middle and high socio-economic class. Overweight/obesity, hyperglycemia and microvascular kidney complications were key drivers of pro-inflammatory cytokine production in T2D, the first two playing a major role in relatively mild and recent forms of the Ecuadorian T2D patients, while the last factor played a key role in the longstanding, more severe disease of the Dutch patients.

In the overweight Ecuadorian T2D and non-diabetic controls, leptin levels were not raised. Further studies need to prove reports in the literature that an Amerindian background may play a role in this low production of leptin by adipose tissue.

Declarations

Ethics Approval and Consent to Participate

The Medical Ethical Review Committee of the Erasmus MC approved the Dutch study. The Medical Ethical Review Committee of the Ecuadorian Corporation of Biotechnology in Quito, Ecuador approved the Ecuadorian study, and this was validated by the Ethic Committee of the Central University. Written informed consent was obtained from patients and controls. The Ecuadorian Ministry of Health (MSP) issued the permit necessary to export and process the samples at the Erasmus MC, Rotterdam, The Netherlands.

Consent for Publication

Not applicable.

Availability of Data and Material

The datasets generated and/or analyzed during the current study are not publicly available because the datasets belong to two different institutions, one from the Netherlands and one from Ecuador and the author will have to ask to the respective institution on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Autor's Contributions

JPG: Data acquisition, carried out the experimental analysis and interpretation of results. Manuscript preparation, and critical evaluation of the manuscript. BO: Data acquisition and contribution to the Analysis and interpretation of results and drafting of the manuscript and critical evaluation of the manuscript. LBR: Study design, data acquisition, carried out the experimental studies, analysis and interpretation of results. Critical evaluation of the manuscript. HdW: Study design, data acquisition, analysis and interpretation of results, critical evaluation of the manuscript, and carried out the experimental studies. DA: Data acquisition, analysis and interpretation of results, critically evaluated the manuscript, and carried out the experimental studies. VA: Data acquisition, analysis and interpretation of results, critically evaluated the manuscript, and carried out the experimental studies. ES: Study design, analysis and interpretation of results, manuscript preparation, and drafting of the manuscript and critically evaluated the manuscript. HD: Study design, analysis and interpretation of results, manuscript preparation, drafting of the manuscript, and critical evaluation of the manuscript and supervision of experimental studies. All authors read and approved the final manuscript.

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