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# The Inflammation Spectrum of Monocytes in Relation to Obesity and Severity of Type 2 Diabetes (T2D), A Case-Control Study

**Jorge Pérez-Galarza[1*], Lucy Baldeón R[2,3], Fernando Sempértegui O[3], Behiye Özcan[4], Eric JG Sijbrands[4], Francisco Barrera-Guarderas[5], Harm de Wit[6], Anne Marie Wijkhuijs[6], and Hemmo A. Drexhage[6]**

[1]Central University of Ecuador, Department of Molecular Biology, Faculty of Medicine, Quito, Ecuador

[2]Central University of Ecuador, Research Institute of Biomedicine, Quito, Ecuador

[3]Central University of Ecuador, Department of Immunology, Faculty of Medicine, Quito, Ecuador

[4]Erasmus University Medical Center, Department of Internal Medicine, Rotterdam, The Netherlands

[5]Pontifical Catholic University of Ecuador, Faculty of Medicine, Quito, Ecuador. Julio Endara Hospital, Internal Medicine Department, Ministry of Health, Quito, Ecuador

[6]Erasmus University Medical Center, Department of Immunology, Rotterdam, The Netherlands

**ABSTRACT**

**Introduction.** Increased monocyte and macrophage inflammatory state and pro-inflammatory cytokine production are linked to type 2 diabetes (T2D).

**Research design and Methods.** This is a case-control study aimed to examine the expression of 23 monocyte genes related to inflammation, adhesion, and repair in individuals with mild (mean HbA1c 7.3%, illness duration 5.6 years) and severe type 2 diabetes (mean HbA1c 8.4%, disease duration 14.2 years) compared also with lean and obese controls. In addition, we determined a set of serum inflammatory cytokines and growth factors.

**Results.** The monocytes of mild T2D patients (who were in general overweight/obese) showed overexpression of a subset of genes related to adhesion (CD9), vascular repair and growth (HGF). The monocytes of the severe T2D patients showed in contrast an upregulation of many of the pro-inflammatory genes, without a significantly increased expression of the repair gene HGF and the adhesion gene CD9. Serum cytokine expression in the severe T2D patients supported the increased inflammatory state of the patients showing high levels of IL-6, IL-1β, and TNF-α.

**Conclusions.** This study, therefore, shows a pro-inflammatory gene expression profile of monocytes of severe T2D patients, while patients with mild T2D did not show such monocyte profile.

**KEYWORDS:** Monocytes; genes; T2D; inflammation; cytokines.

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## 1. INTRODUCTION

Overweight and obesity, the upbeat to type 2 diabetes (T2D), are huge problems of global public health. In adipose tissue, particularly of individuals with severely overweight, there is an increased production of pro-inflammatory cytokines and chemokines leading to a state of systemic low-grade inflammation [1]–[3]. This state induces insulin resistance [1], [4], ultimately leading to T2D [1].

Vascular complications constitute the major source of morbidity and mortality among patients with T2D. This concerns both microvascular problems in conditions such as retinopathy and nephropathy, as well as macrovascular problems in athero-sclerosis of the larger vessels [5]. These vascular problems are caused by increased endothelial damage induced by hyperglycemic (metabolic) conditions and a decreased ability to repair damage by re- and neo-vascularization. In the process of re- and neo-vascularization, bone marrow (BM)-derived anti-inflammatory cells, also called circulating angiogenic cells (CACs) play an essential role in repair [6]–[8].

In previous studies, members of our group reported that anti-inflammatory and inflammatory cells were derived from the same monocyte-like precursor fraction in the bone marrow [9]. This notion led to the concept of a skewing of the

myelomonocytic differentiation at the precursor level of the monocyte, to a spectrum of cells starting from anti-inflammatory endothelial cells, via anti-inflammatory macrophages/dendritic cells to pro-inflammatory macrophage/mature dendritic cells (see Figure 1). A rostrum of stimuli governs these various forms of differentiated cells, amongst which various anti- and pro-inflammatory factors, growth factors, but also hyperglycemic conditions play a role. In general, in experimental hyperglycemic and pro-inflammatory conditions, the development and function of myelomonocytic anti-inflammatory cells are hampered, while the number and function of pro-inflammatory macrophages are increased [10], [11].

We previously reported that the circulating monocytes of a group of well-controlled, moderately severe, but hyperlipidemic diabetic patients, showed an upregulated gene expression of genes involved in cell adhesion, cell differentiation, growth, and vascular repair, such as HGF, while inflammatory genes, such as TNFAIP3, were down-regulated [12]. Since HGF is considered a marker of CACs [13], this observation was taken as a sign that the circulating monocytes of these well-controlled, moderately severe T2D patients had differentiated into anti-inflammatory-like CACs instrumental in the repair of endothelium damaged by the diabetic process. Since it is generally accepted that monocytes/macrophages are pro-inflammatory activated, our data on anti-inflammatory monocytes were counterintuitive. We hypothesized that the relatively mild character and the well controlled glycemic state of the patients might have played a role.

This study aims to investigate the inflammatory and repair gene expression profile of monocytes, in a group of T2D patients with more severe (i.e., less controlled and more signs of hyperglycemia) using the same techniques as in the previously reported T2D patients. We found these patients in the Dutch Pearl Consortium [14]. The monocyte gene profiles of the mild and the severe patients were compared to those of healthy controls from the general population consisting of hospital personnel and caretakers of the patients. In addition, we measured the serum concentration of various pro-inflammatory cytokines and growth factors to be compared to the inflammatory/repair gene status of the monocytes.

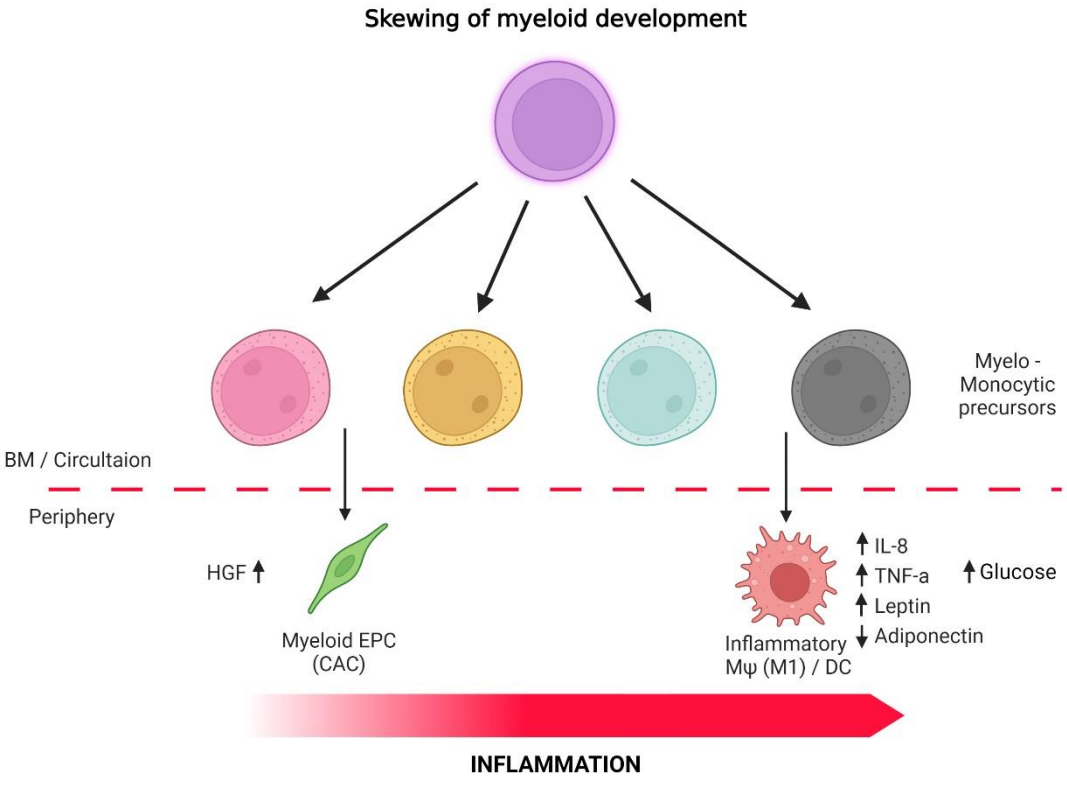


**Figure 1. Skewing of myeloid development. Inflammation and hyperglycemia induce the development of a proinflammatory macrophage profile in severe T2D, hampering the myeloid EPC/CAC development. IL: interleukins. TNF: Tumor necrosis factor. HGF: hepatocyte growth factor. EPC: endothelial progenitor cells. CAC: circulating angiogenic cells. DC: dendritic cells.**

## 2. MATERIALS AND METHODS

### 2.1. Subjects

We used cells and serum of 28 patients with T2D participating in the Diabetes Pearl Consortium and respective controls (n=22). 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 Medical Center (Erasmus MC) in Rotterdam. The T2D

outpatients had been randomly and consecutively recruited from the outpatient diabetes clinic of the EramsusMC and had in general, severe, and longstanding T2D. Inclusion criteria for the Pearl Consortium study have been given in E. Van't Riet, et al. [14]. Data were compared to previously reported outcomes [15] of test results obtained with the cells and serum of 33 patients with mild T2D and respective controls (n=28). Patients came from 4 medical centers in Quito, Ecuador (Eugenio Espejo Hospital, Club de Leones Sur, Fundación Oftalmológica del Valle, and Fundación de la Psoriasis) and had been selected based on the criteria of The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [16] (figure 2).

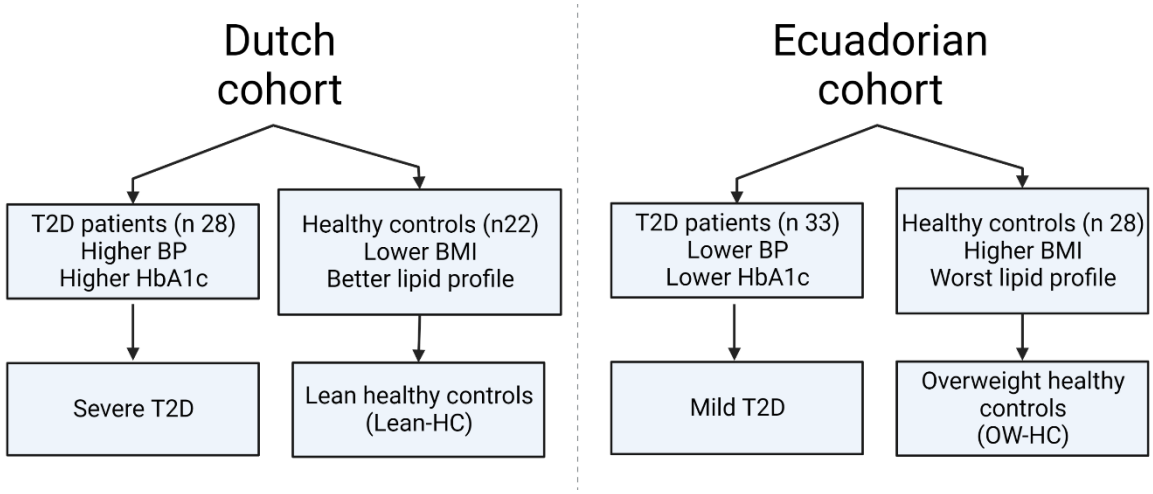


**Figure 2. Flow chart of patient groups. BP: blood pressure. BMI: body mass index. HbA1c: glycosylated hemoglobin.**

We excluded patients who had immune disorders, serious systemic medical illness, recent infections (last 2 weeks), obvious vascular complications, fever, pregnancy/postpartum, and positive GAD65 Abs (LADA). In addition, patients were not included if they were unable to provide written informed consent due to their inability to interpret and write.

Regarding the non-diabetic control groups, we selected subjects by asking hospital staff and accompanying caretakers to volunteer to donate blood at the same time as the patients were donating blood. The subjects were over 30 years of age and matched to patients for gender as much as possible, came from the same regional, ethnic, and societal background as the patients, and were free of any serious medical conditions.

We assigned the Ecuadorian T2D patients to the group denominated Mild T2D, due to a lower average of HbA1c of 7.3%, mean disease time of 5.6 years, and fewer comorbidities compared to Dutch patients which have an average HbA1c of 8.4%, mean disease time 14.2 years and more comorbidities (Severe T2D) (see table 1). The control group from Ecuador was considered as overweight healthy control (OW-HC), and the Dutch control group was described as lean healthy control (Lean-HC) because of their mean BMI of 28, 5, and 25, 0 respectively.

### 2.2. Blood collection and preparation

For the collection of serum, fasting venous peripheral blood (10 mL) was collected in a clotting tube and processed within 4 hours. The serum was frozen and stored at minus 80°C for approximately 12 months before testing. Levels of TNFα, IL-1β, IL-6, HGF, PAI, resisting, 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 multianalyte cytometric bead array system (CBA, Milliplex® Map, U.S.A.). The data was analyzed using a 5-parameter logistic method for calculating analyte concentrations in samples (MAGPIX® with xPONENT software, Luminex, Austin, USA). Undetectable serum analytic levels were recorded as 0 pg/ml and included in the statistical analysis.

### 2.3. Isolation of monocytes

For the collection of PBMCs, fasting venous peripheral blood (10 mL) was collect-ed in tubes containing sodium heparin. From the heparinized blood, peripheral blood mononuclear cells (PBMC) suspensions were prepared in the afternoon by low-density gradient centrifugation, as previously described in detail [17] within 8 hours to avoid activation of the monocytes. PBMCs were frozen in 10% dimethyl sulfoxide and stored in liquid nitrogen. This enables us to test patients and control immune cells in the same series or experiments later. CD14-positive (CD14+) monocytes were isolated from fro-zen PBMCs by a magnetic cell sorting system (MACS; Miltenyi Biotec, Auburn, California). The purity of monocytes was >95% (determined by morphological screening after Trypan Blue staining and flow cytometry analysis). As previously reported, the positive vs. negative selection of immune cells did not influence gene expression profiles [18].

### 2.4. Real-time quantitative PCR (qRT PCR) for monocytes

#### 2.4.1. mRNA expression in monocytes via TaqMan Array Cards

For a previous report, we determined the expression of 24 mRNAs in the mono-cytes of the T2D cases and the non-diabetic controls. RNA had been isolated from monocytes using RNeasy columns (Qiagen, Hilden, Germany), and both this method and quantitative RT-PCR has been described in detail elsewhere [12]. 1 µg of RNA was reverse transcribed using the High-Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using custom TaqMan Arrays, format 48 (Applied Biosystems), according to the manufacturer's protocol and validated against the single RT-qPCR method. Per fill port, 400 ng of cDNA (converted from total RNA) was loaded. PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system with TaqMan Array block. Thermal cycler conditions were 2 min at 50°C, 10 min at 94.5°C, and then 30s at 97°C, and 1 min at 59.7°C for 40 cycles. Relative to the housekeeping gene ABL1, the expressions of ATF3, BCL2A1, CCL20, CCL2, CCL7, CD9, CDC42, CXCL2, DHRS3, DUSP2, EMP1, FABP5, HSPA1A/HSPA1B, IL-1B, IL-6, MAPK6, NAB2, PDE4B, PTGS2, PTPN7, PTX3, STX1A, TNF, and TNFAIP3 were determined, and values were calculated using the comparative threshold cycle (CT) method. ABL was chosen as a reference gene because it was previously shown that was the most consistently expressed reference gene in hematopoietic cells [19]. The quantitative value obtained from q-PCR is a cycle threshold (Ct). The fold change values between different groups were determined from normalized Ct values (Ct gene – Ct housekeeping gene), by the ΔΔCt method.

#### 2.4.2. Individual mRNA qRT–PCR assays for HGF and resistin.

We additionally determined the gene expression for HGF and resistin using the same cDNA used in the previous experiments [12]. To obtain cDNA for q-PCR, 1 µg RNA was reversed-transcribed using the cDNA high-capacity cDNA Reverse Tran-scription kit (Applied Biosystems, USA). Applied Biosystems provided TaqMan probes and consensus primers for HGF and Resistin. PCR amplification of the housekeeping gene ABL was performed for each sample to allow normalization between the samples. The quantitative value obtained from q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene − CT housekeeping gene).

### 2.5. Data analysis

The SDS software (ABI) was used to collect the data and the RQ Manager Program (ABI) was used to assign, check, and standardize CT values. The Data Assist software (ABI) was used to normalize the data (ABL for mRNA expression). For threshold cycles below 40, the corresponding mRNA was considered detected, higher cycle numbers were not included in calculations. The results were quantified using the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, ABI). Statistical analysis was performed using the SPSS (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/). Because of the non-parametric distribution pattern and the number of groups, a Kruskal-Wallis test with multiple comparisons test was applied. Graphics were created with BioRender.com.

## 3. RESULTS

### 3.1. General Characteristics

Table 1 shows the characteristics of the study participants. The hyperglycemic status was higher in the severe patient group with a mean HbA1c of 8,4 vs 7,3% in the mild group. Most of the severe group (93%) was on insulin as compared to 21% of the mild group. The severe patients also had almost three times longer disease duration (mean 14.2 vs 5.6 years) and a higher prevalence of overweight and obesity as compared to the mild patients (BMI 31 vs. 28). The use of oral antidiabetic treatments between groups was not significantly different. The prevalence of hypertension also differed between the severe and mild groups (71% vs 48%).

Remarkably, the use of statins was considerably higher in severe patients as compared to the mild patients (82% vs 21%). The reason is that in the Netherlands statins are more easily prescribed and financially available for patients. It is therefore not surprising that the severe (Dutch) diabetic patients showed a better lipid profile than the mild patients (Ecuadorian) (see Table 1).

The two non-diabetic control groups appeared to differ significantly. The Dutch non-diabetic controls (Lean Healthy Controls; Lean-HC) had a lower prevalence of obesity and overweight (13% and 27% respectively) as compared to the Ecuadorian non-diabetic controls (Overweight Healthy Controls; OW-HC) who had a higher prevalence of obesity and overweight (37% and 48% respectively). This is compatible with the observations that in Southern America overweight and obesity are frequent and linked to socioeconomic status [20]. The prevalence of overweight in elderly Ecuadorian men and women with low socioeconomic levels was 33% and 55%, respectively [21]. In our study, age did not differ significantly; comorbidities, blood glucose state, and lipid profile were not recorded in the non-diabetic general population controls.

**Table 1. Demographic details and Clinical Characteristics. This table shows the number of patients and controls used in this study and their ages, gender, BMI, comorbidities, glucose state, lipid profile, hepatic profile, and medication use.**

| | Mild T2D | Severe T2D | p- Value | OW-HC | Lean-HC | p- Value |
|---|---|---|---|---|---|---|
| Group size n | 33 | 28 | | 28 | 22 | |
| Age mean (range) | 60 (40-85) | 60 (37-77) | ns | 52 (32-87) | 39 (22-54) | ns |
| **Gender** | | | | | | |
| Female n (%) | 18 (55%) | 11 (39%) | ns | 19 (68%) | 12 (54.5%) | ns |
| Male n (%) | 15 (45%) | 17 (61%) | ns | 9 (32%) | 10 (45.5%) | ns |
| **BMI mean (range)** | 28 (22-39) | 31 (23-43) | ***0.01**** | 28.5 (23-35) | 25 (21-32) | ***0.01***† |
| Normal % | 17% | 4% | | 14,80% | 60% | |
| Overweight % | 45% | 42% | | 48,10% | 26,70% | |
| Obese % | 36% | 54% | | 37% | 13,30% | |
| **Comorbidities** | | | | | | |
| HBP % | 48.5% | 71.4% | ***0.01*** | NA | NA | |
| 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) | 8.4 (6.1 - 13) | ***0.02**** | NA | NA | |
| Normal % | 30% | 7,10% | | | | |
| High % | 70% | 92,90% | | | | |
| Disease time years mean (range) | 5.6 (1-20) | 14.2 (5-24) | ***0.00*** | | | |
| **Lipid Profile** | | | | | | |
| Chol. mg/dL mean (range) | 243 (165-436) | 78 (58- 106) | ***0.00**** | NA | NA | |
| Normal % | 39% | 100% | | | | |
| High % | 61% | 0% | | | | |
| TGD mg/dL mean (range) | 202 (80 -409) | 38 (16 - 92) | ***0.00**** | | | |
| Normal % | 67% | 100% | | | | |
| High % | 33% | 0% | | | | |
| HDL mg/dL mean (range) | 43 (18 -67) | 22 (10 -35) | ***0.00**** | | | |
| Normal % | 58% | 3,60% | | | | |
| Low % | 42% | 96% | | | | |
| LDL mg/dL mean (range) | 168 (77 - 361) | 44 (24- 76) | ***0.00**** | | | |
| Normal % | 55% | 100% | | | | |
| High % | 45% | 0% | | | | |
| **Hepatic Profile** | | | | | | |
| ASAT mg/dL mean (range) | 33.1 (6-78) | 29.5 (17- 81) | ***0.00**** | NA | NA | |
| Normal % | 58% | 89% | | | | |
| High % | 42% | 11% | | | | |
| ALAT mg/dL mean (range) | 45.0 (7 -131) | 27.2 (12 - 64) | ***0.00**** | | | |
| Normal % | 52% | 89% | | | | |
| High % | 49% | 11% | | | | |
| GGT mg/dL mean (range) | 58 (17 -360) | 52 (11 -332) | ns | | | |
| Normal % | 70% | 68% | | | | |
| High % | 30% | 32% | | | | |
| **Medication** | | | | | | |
| Oral Anti-diabetic treatment % | 73% | 61% | ns | NA | NA | |
| Insulin treatment % | 21% | 93% | ***0.00*** | | | |
| Aspirin % | 12% | 32% | ns | | | |
| Statins % | 21% | 82% | ***0.00*** | | | |

*p value < 0.05. OW-HC, overweight healthy controls. T2D, type 2 diabetes. HBP, high blood pressure. Chol, cholesterol. TGD, triglycerides. HDL, high density lipoproteins. LDL, low density lipoproteins. ASAT, aspartate transaminase. ALAT, alanine transaminase. GGT, gamma-glutamyl transferase.

### 3.2 Serum Cytokine Levels

In analyzing the data, it appeared that the two collected non-diabetic control groups differed regarding serum cytokine profile. The Ecuadorian non-diabetic group had a pro-inflammatory profile fitting their weight status as compared to the Dutch non-diabetic and relatively lean control group:

serum IL-6 was significantly increased (p=< 0.001) in the Ecuadorian non-diabetic controls. The risk to develop type 2 diabetes and the neurological consequences was indicated by the increased levels of resistin and decreased levels of nerve growth factor (NGF). We, therefore, have listed the two non-diabetic control groups separately in Table 2.

The mild T2D group (who was in general overweight/obese) showed a cytokine and growth factor profile similar to the overweight non-diabetic control group, with slightly higher levels of the pro-inflammatory factors IL-6, TNF, IL-8, and IL-1β, while resistin levels were not significantly increased.

The severe T2D group (also associated with overweight/obesity) showed an exaggerated pro-inflammatory profile showing a significant rise of IL-6, TNF-α, IL-8, and leptin. The anti-inflammatory adiponectin was decreased as compared to non-diabetic lean controls, although it did not reach significance (table 2). IL-6, TNF-α, IL-8, and IL-1β were also increased as compared to the overweight non-diabetic controls. Interestingly the anti-inflammatory vascular repair factor HGF was increased in the serum of severe T2D patients. NGF was decreased when compared with their respective lean healthy controls and resistin was increased when compared with OW-HC and mild T2D patients.

**Table 2. Cytokines, adipokines, and growth factors. Serum expression of mild and severe T2D compared to overweight healthy controls and lean healthy controls.**

| | Lean-HC mean (SEM) (n22) | OW-HC mean (SEM) (n28) | P value | Mild-T2D mean (SEM) (n33) | P value (Vs Lean-HC) | P value (Vs OW-HC) | Severe-T2D mean (SEM) (n28) | P value (Vs Lean-HC) | P value (Vs OW-HC) | P value (Vs Mild T2D |
|---|---|---|---|---|---|---|---|---|---|---|
| **Leptin (pg/ml)** | 7120 (1263) | 11002 (1823) | 0.138 | 8924 (1837) | 0.644 | 0.250 | **22851 (6621)** | ***0.001*** | 0.071 | ***0.002*** |
| **Adiponectin * (pg/ml)** | 27.85 (4.03) | 27.11 (3.30) | 0.147 | 30.54 (5.01) | 0.147 | 0.584 | 19.30 (1.99) | 0.147 | 0.147 | 0.147 |
| **IL-6 (pg/ml)** | 1.29 (0.28) | **4.50 (0.49)** | ***0.000*** | **7.85 (1.70)** | ***0.000*** | 0.645 | **7.95 (1.33)** | ***0.000*** | ***0.034*** | 0.081 |
| **TNF-α (pg/ml)** | 4.81 (0.39) | 3.89 (0.37) | 0.146 | 5.15 (0.43) | 0.736 | ***0.049*** | **7.44 (0.58)** | ***0.002*** | ***0.000*** | ***0.002*** |
| **IL-8 (pg/ml)** | 5.54 (0.40) | 8.36 (1.18) | 0.233 | **15.45 (3.25)** | ***0.000*** | ***0.001*** | **22.05 (4.92)** | ***0.000*** | ***0.000*** | 0.317 |
| **IL-1β (pg/ml)** | 0.93 (0.10) | **0.54 (0.29)** | ***0.000*** | **0.76 (0.23)** | ***0.004*** | 0.210 | **1.69 (0.42)** | 0.460 | ***0.000*** | ***0.000*** |
| **CCL4* (pg/ml)** | 82.32 (12.54) | 113.54 (18.04) | 0.505 | 92.33 (16.53) | 0.505 | 0.505 | 110.82 (18.37) | 0.505 | 0.505 | 0.505 |
| **CCL2* (pg/ml)** | 263.09 (24.11) | 304.79 (24.85) | 0.502 | 329.64 (30.69) | 0.502 | 0.502 | 317.75 (27.91) | 0.502 | 0.502 | 0.502 |
| **HGF (pg/ml)** | 874.32 (98.26) | 763.50 (93.64) | 0.416 | 1017.94 (113.47) | 0.652 | 0.116 | **1437.37 (177.27)** | ***0.014*** | ***0.000*** | ***0.025*** |
| **NGF (pg/ml)** | 4.47 (0.28) | **1.61 (0.42)** | ***0.000*** | **2.06 (0.92)** | ***0.000*** | 0.396 | **2.75 (0.29)** | ***0.021*** | ***0.002*** | ***0.000*** |
| **PAI1 (pg/ml)** | 95452 (4671) | 93526 (5734) | 0.562 | **82997 (4846)** | ***0.041*** | 0.122 | **116141 (9130)** | 0.332 | 0.098 | ***0.001*** |
| **Resistin (pg/ml)** | 69406 (11886) | **38878 (2481)** | ***0.003*** | **44759 (4422)** | ***0.016*** | 0.487 | 62923 (7389) | 0.862 | ***0.003*** | ***0.017*** |

The Kruskal-Wallis test was performed. P-values < 0.05 in italics and bold. *Multiple comparisons were not performed because the overall test does not show significant differences across samples, the shown p-value is the overall comparison for all. OW-HC, overweight healthy controls. T2D, type 2 diabetes. IL, interleukins. CCL, chemokine ligand. HGF, hepatocyte growth factor. NGF, nerve growth factor. Red color: cytokine overexpression and green color: cytokine downregulation when compared to mild Vs. severe T2D.

### 3.3 Monocyte Gene Expression

The severe and the mild diabetic patients were compared to each other and the two non-diabetic control groups using the fold change of expression (FC) versus the reference gene ABL. Table 3 gives the data. FC below one indicates reduced expression, while FC above 1 indicates higher expression as compared to the reference gene ABL. The FC and the p values between the tested groups are expressed. The order of the genes in Table 3 is given based on the gene cluster analysis

published by Baldeón et al., [22] using the gene expression of the monocytes of the mild T2D group. Three main clusters of mutually correlating genes were identified in that study; the gene clusters were arbitrarily called A, B, and C.

In sum, Cluster A is formed by a mixed group of genes, which are involved in ad-hesion/motility/fusion of membranes (EMP1, CDC42, STX1A), but also in inflammation (PTX3, CXCL2) and regulation of the MAP-kinase pathway (PTPN7 and NAB2). Cluster B consists predominantly of genes for inflammatory cytokines such as IL-6, IL-1β and TNF, the gene for the inflammatory compound PTGS/COX2, genes for the in-flammatory chemokines CCL20 and CCL2, and genes for PDE4B and DUSP2, which are regulators of the transcription of these inflammatory factors. Cluster C consists of genes related to adhesion (CD9), vascular repair (HGF), metabolism (DHRS3, FABP5), and the regulation of growth/differentiation/inflammation via the MAP-kinase path-way (MAPK6 or p38).

In Table 3, we first show that there is a difference between the lean and overweight/obese healthy control groups. The overweight/obese group showed overexpres-sion of half of the cluster C genes (including HGF), while the majority of the inflammatory cluster B genes were downregulated as compared to the lean HC group. This is compatible with an anti-inflammatory character of the cells.

Table 3 also shows the previously reported anti-inflammatory character of the monocytes in mild T2D cases, like the monocyte profile in overweight/obese healthy controls. It is of note that the mild T2D cases are similarly overweighed/obese than the overweight/obese healthy controls.

The pattern of monocyte gene expression was different in the severe T2D patient group. The severe T2D patients did not show an increase in the cluster C genes HGF, CD9, and DHRS3, but showed up-regulation of many of the cluster B and A genes for the expression of pro-inflammatory cytokines/factors IL-6, IL-1β, CCL20, CCL4, CXCL2, and PTX3 as compared to mild T2D cases (Table 3 and Figure 3).

**Table 3. Monocytes gene expression profile. Mild and Severe T2D compared to overweight healthy controls and lean healthy controls.**

| | Lean HC FC to ABL | OW-HC FC to ABL | P value (Vs Lean-HC) | Mild T2D FC to ABL | P value (Vs Lean-HC) | P value (Vs OW-HC) | Severe T2D FC to ABL | P value (Vs Lean-HC) | P value (Vs OW-HC) | P value (Vs Mild T2D) |
|---|---|---|---|---|---|---|---|---|---|---|
| **CLUSTER A** | | | | | | | | | | |
| **CXCL2** | 19.78 | 0.79 | ***0.000*** | 0.62 | ***0.000*** | 0.981 | 40.98 | 0.830 | ***0.000*** | ***0.000*** |
| **STX1A** | 0.01 | 0.01 | ***0.003*** | 0.01 | ***0.004*** | 0.928 | 0.02 | 0.654 | ***0.001*** | ***0.004*** |
| **NAB2*** | 0.42 | 0.27 | 0.239 | 0.19 | 0.239 | 0.239 | 0.18 | 0.239 | 0.239 | 0.239 |
| **EMP1** | 0.19 | 0.43 | ***0.000*** | 0.42 | ***0.000*** | 0.531 | 0.28 | 0.165 | ***0.002*** | ***0.000*** |
| **CDC42** | 0.75 | 1.21 | 0.103 | 1.04 | 0.233 | 0.535 | 0.86 | 0.522 | ***0.002*** | ***0.012*** |
| **PTX3** | 5.15 | 0.60 | ***0.000*** | 0.46 | ***0.000*** | 0.463 | 11.29 | 0.681 | ***0.000*** | ***0.000*** |
| **PTPN7** | 0.24 | 0.19 | 0.168 | 0.21 | 0.227 | 0.802 | 0.26 | 0.392 | ***0.002*** | ***0.004*** |
| **CLUSTER B** | | | | | | | | | | |
| **IL-6** | 1.08 | 0.01 | ***0.000*** | 0.01 | ***0.000*** | 0.835 | 20.97 | 0.999 | ***0.000*** | ***0.000*** |
| **TNF** | 143.35 | 2.59 | ***0.000*** | 2.63 | ***0.000*** | 0.972 | 107.22 | 0.568 | ***0.000*** | ***0.000*** |
| **IL-1β** | 626.74 | 11.55 | ***0.000*** | 6.90 | ***0.000*** | 0.568 | 1244.95 | 0.838 | ***0.000*** | ***0.000*** |
| **CCL2*** | 0.17 | 0.45 | 0.329 | 0.40 | 0.329 | 0.329 | 0.85 | 0.329 | 0.329 | 0.329 |
| **CCL20** | 5.99 | 0.06 | ***0.000*** | 0.04 | ***0.000*** | 0.927 | 15.32 | 0.924 | ***0.000*** | ***0.000*** |
| **TNFAIP3** | 45.84 | 2.26 | ***0.000*** | 2.44 | ***0.000*** | 0.412 | 40.27 | 0.613 | ***0.000*** | ***0.000*** |
| **CCL4** | 392.03 | 1.56 | ***0.000*** | 1.61 | ***0.000*** | 0.668 | 886.00 | 0.885 | ***0.000*** | ***0.000*** |
| **PDE4B** | 10.45 | 3.46 | ***0.001*** | 2.86 | ***0.000*** | 0.328 | 9.37 | 0.098 | ***0.028*** | ***0.002*** |
| **DUSP2** | 16.57 | 1.69 | ***0.000*** | 1.00 | ***0.000*** | 0.465 | 12.64 | 0.285 | ***0.000*** | ***0.000*** |
| **PTGS2** | 24.41 | 4.03 | ***0.000*** | 1.91 | ***0.000*** | 0.210 | 30.95 | 0.443 | ***0.000*** | ***0.000*** |
| **CLUSTER C** | | | | | | | | | | |
| **FABP5** | 0.28 | 1.97 | ***0.000*** | 1.61 | ***0.000*** | 0.890 | 0.30 | 0.829 | ***0.000*** | ***0.000*** |
| **MAPK6** | 0.74 | 1.81 | ***0.001*** | 1.60 | ***0.003*** | 0.567 | 1.24 | 0.587 | ***0.000*** | ***0.001*** |
| **DHRS3*** | 0.06 | 0.04 | 0.562 | 0.05 | 0.562 | 0.562 | 0.05 | 0.562 | 0.562 | 0.562 |
| **CD9** | 2.94 | 3.01 | 0.532 | 4.48 | ***0.029*** | ***0.028*** | 1.91 | 0.930 | 0.328 | ***0.002*** |
| **HGF** | 0.55 | 0.96 | ***0.000*** | 0.99 | ***0.000*** | 0.196 | 0.56 | 0.834 | ***0.000*** | ***0.000*** |
| **Resistin*** | 0.64 | 4.07 | 0.283 | 1.04 | 0.283 | 0.283 | 1.36 | 0.283 | 0.283 | 0.283 |

The Kruskal-Wallis test was performed. P-values < 0.05 in italics and bold denoted a significant difference between the groups described in each column. *Multiple comparisons were not performed because the overall test does not show significant differences across samples, the shown p-value is the overall comparison for all. OW-HC, overweight healthy controls. T2D, type 2 diabetes. IL, interleukins. CCL, chemokine ligand. HGF, hepatocyte growth factor. NGF, nerve growth factor. Red color: gene overexpression, green color: gene downregulation when compared mild Vs. severe T2D.

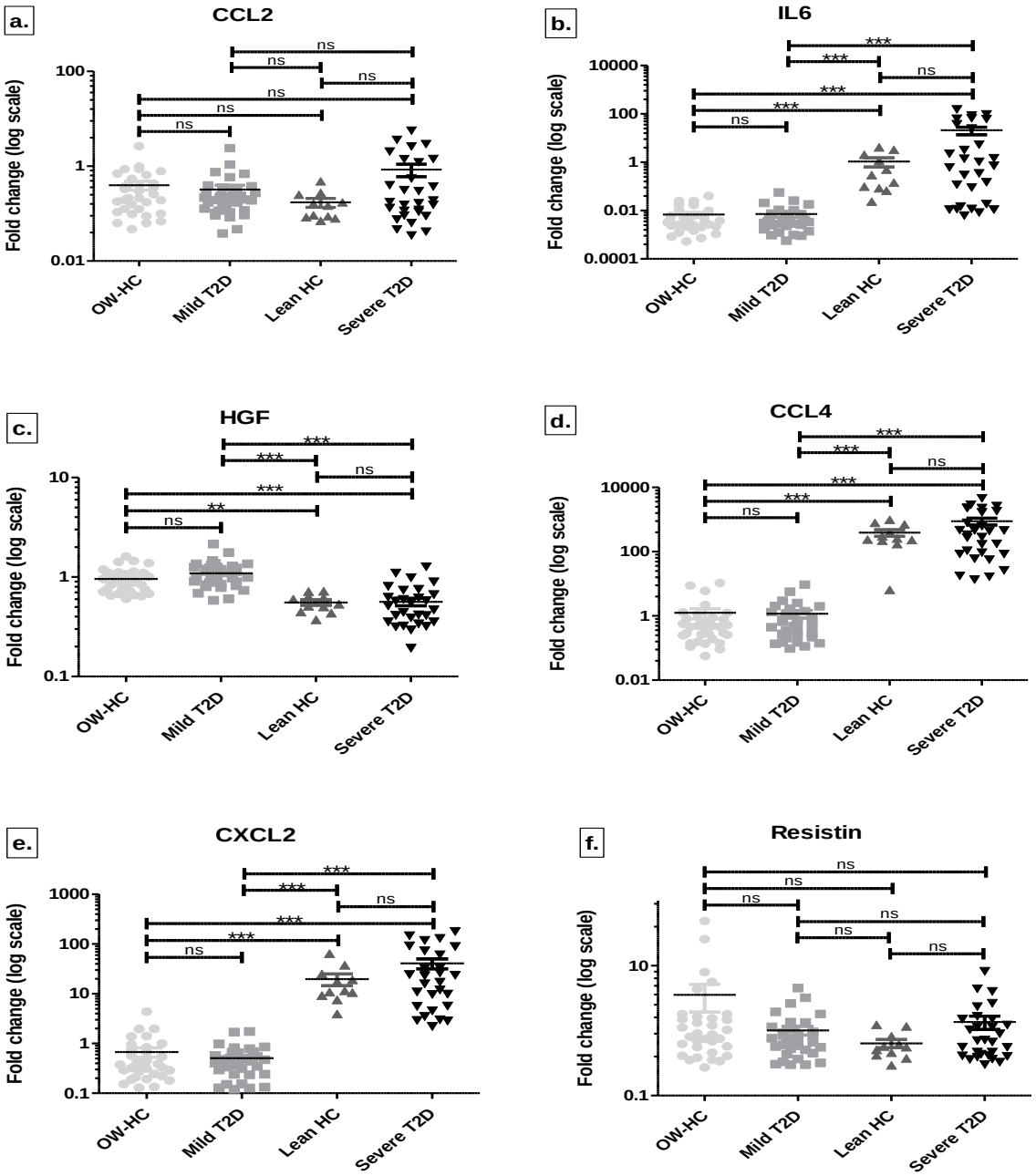


**Figure 3. Dot plots of the expression of certain genes. Comparison of gene expression of overweight healthy controls (n 28), lean healthy controls (n 22), mild (n 33), and severe T2D (n 22) a) CCL2; b) IL-6; c) HGF; d) CCL4; e) CXCL2; f) Resisitin. Fold change was calculated compared to ABL gene. Logarithmic scale was used in the y axis, to show a clear view of the expression; * p<0.05, ** p<0.001. Bars are SD. HGF: hepatocyte growth factor. TNF: tumor necrosis factor. IL: interleukin. CCL: chemokine ligand. CXCL: chemokine (C-X-C motif) ligand.**

## 4. DISCUSSION

This study shows that the inflammatory gene profile of monocytes of reasonably controlled and mild T2D cases differs from that of severe T2D cases. The monocyte of severe cases had a pro-inflammatory gene expression profile, while the mild cases did not. In contrast they earlier had an anti-

inflammatory profile. The serum cytokine profile supported the view of a pro-inflammatory state of the severe T2D cases showing the highest levels of TNF-α, IL-8, and leptin and the lowest levels of adiponectin.

It is worthy to note that also the gene expression of repair factor HGF was reduced in the pro-inflammatory monocytes of the severe T2D cases. This might suggest that the vascular repair capability of the cells is also reduced, but this needs further functional studies on the monocytes of severe T2D cases.

In experimental conditions, hyperglycemia act as a pro-inflammatory (in vitro and animal studies), and in such experimental condition the myelomonocytic anti-inflammatory cell formation is hampered [9]. Therefore, we assume that the higher glucose level as indicated by the higher Hb1Ac level of the more severe T2D cases could have played a significant role in the generation of pro-inflammatory monocytes/macrophages (figure 1). However, an additional role of dyslipidemia should not be neglected as well. In a previous article on mild T2D patients, we showed that high serum lipid levels correlated with the reduced expression of many of the inflammatory genes in circulating monocytes, reaching significance for ATF3, DUSP2, and PTGS2 [22]. Thus, the statin induced normalization of the serum lipid levels in the severe T2D patients may have coacted with the hyperglycemia to force the monocytes into a clear pro-inflammatory state.

This report also describes that the two non-diabetic control groups of hospital staff and caretakers collected in Rotterdam and Quito differed regarding body weight. It has been reported that overweight/obesity is very common in the Ecuadorian general population, increases with age, and occurs in the population over 30 years old, predominantly in women [20]. While in Western European countries, the urban lower socioeconomic classes show a higher prevalence of overweight/obesity, in Southern America the urban middle and higher classes show the highest prevalence of overweight/obesity, while urban lower socioeconomic classes and indigenous people are not overweight/obese [20].

The increased adipose tissue observed in overweight and obese people is involved in chronic inflammation and alters glucose homeostasis through the secretion of a diversity of cytokines and adipokines [23]. In obese tissue, adipocytes change in structure and function, exhibiting an increase in number and size. This is accompanied by an insufficient vascularization resulting in hypoxia, which triggers apoptosis and elevated secretion of cytokines, adipokines, and chemokines that induce infiltration of immune cells that contribute to lipolysis, increased inflammation, and consequently insulin resistance [23]–[25]. The low-grade inflammatory microenvironment induces the polarization of tissue regulatory macrophages towards an M1 phenotype [26]. As a result, the balance changes from a controlled anti-inflammatory immunological state to an extremely inflammatory state, with excessive production of many pro-inflammatory cytokines, including IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), and IL-1β [24], [27]–[29].

It is thus not surprising that the Ecuadorian non-diabetic controls had raised serum levels of some of the pro-inflammatory cytokines, such as IL-6 and IL-8, as compared to the Dutch non-diabetic control group which had a much lower prevalence of overweight/obesity (table 2). In addition, their risk of developing T2D was indicated by reduced expression of resistin.

Interestingly and despite the systemic pro-inflammatory condition, the Ecuadorian non-diabetic overweight controls showed anti-inflammatory monocytes in their circulation. It is tempting to speculate that high serum lipid levels may have forced the circulating monocytes to adopt an anti-inflammatory phenotype to counteract the inflammation process. However, we are unfortunately not informed of these parameters in the Ecuadorian non-diabetic controls.

## 5. CONCLUSIONS

In conclusion, this study shows a pro-inflammatory monocyte gene expression profile in a group of severe T2D as compared to a group of milder T2D with milder hyperglycemia and of shorter duration. The pro-inflammatory state of the more severe T2D cases, as evidenced by an increased expression of transcripts for many pro-inflammatory cytokines, was supported by an increase in pro-inflammatory cytokines in serum.

## 6. LIMITATION

The fact that the patients have different genetic backgrounds coming from different ethnic groups is a limitation. Also, relatively few patients and controls were studied and therefore confirmation in larger groups of patients is required, as well as functional studies to establish a defective CAC like character of the monocytes of the severe cases in relation to the reduced HGF gene expression.

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**Institutional Review Board Statement:** All protocols used to create the Diabetes Pearl and all generic String of Pearl biobanking, and the ethics committee of the VU University Medical Center reviewed data procedures as a multicenter study. Ethical Committees of all seven other academic centers provided positive advice for participation [14]. The Medical Ethical Review Committee of the Ecuadorian Corporation of Biotechnology in Quito, Ecuador, and the Ethic Committee of the Biomedicine Center at Central University approved the Ecuadorian study. The Ecuadorian Ministry of Health (MSP) validated the ethics approval: MSP-DIS-0082-2012-O. Approval Date: 04-12-2012 and issued the necessary permit to export and process the samples at the Erasmus MC, Rotterdam, The Netherlands.

**Informed Consent Statement:** Writing Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available because that was not asked during the informed consent that the data was going to be available online.

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**Key message:** Adipose tissue in obesity induces a state of chronic low-grade inflammation leading to a metabolic disorders, including diabetes. Monocytes are believed to play an important role in regulation and vascular repair, however, the inflammatory process of obesity, shows also an effect on monocytes, hampering their regulatory activity. Consequently, understanding monocyte activity could be used to manage the complications of diabetes.

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