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Insulin resistance and obesity affect monocyte-derived dendritic cell phenotype and function

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ABSTRACT

Aim: Cardiovascular disease (CVD) is prevalent in women after menopause, which may be associated with obesity, insulin resistance and metaflammation. Despite the recognized role of immunological mechanisms in vascular remodeling, the role of dendritic cells (DCs) is still unclear. The aim was to characterize monocyte-derived DCs (Mo-DC) in post-menopausal patients with type 2 diabetes (T2DM) and obese woman, without clinical manifestations of atherosclerosis.

Methods: Obese post-menopausal women with or without T2DM were enrolled and were compared to age-matched healthy women. DCs obtained from patients were phenotypically and functionally characterized by flow cytometry and mixed lymphocyte reaction. MRNA integrins expression was assessed by real time RT-PCR; circulating fetuin-A and adiponectin levels were measured by ELISA.

Results: Phenotypic dysregulation of Mo-DC reported was related to a defective allogenic lymphocyte stimulation and to an increased mRNA of CD11c, CD18 and DC-SIGN/CD209 which regulate their adhesion to vascular wall cells. Fetuin-A and adiponectin levels were significantly altered and negatively correlated. Hyperglycaemia significantly impaired $CD14^+$ transdifferentiation into Mo-DC.

Conclusions: These data show a dysfunction of Mo-DCs obtained from precursors isolated from T2DM obese post-menopausal woman without any documented clinical CV event. Association of obesity to diabetes seems to worsen DC's phenotype and function and increase vascular inflammation.

1. Introduction

Obesity and Type 2 diabetes mellitus (T2DM) are considered two chronic low-grade inflammatory illnesses [1]. According to World Health Organization, about 13% and 8% of world's adult population suffers of obesity or T2DM, respectively, with 87.5% of T2DM patients that are obese or overweight. Both conditions represent independent risk factors for

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Abbreviations

BMI	body mass index
CAD	coronary artery disease
CV	cardiovascular
CVD	cardiovascular disease
DCs	dendritic cells
FCS	Fetal Calf Serum
MetS	metabolic syndrome
Mo-DCs	monocyte-derived dendritic cells
PBMCs	peripheral blood mononuclear cells
ROS	reactive oxygen species
T2DM	type 2 diabetes mellitus

cardiovascular diseases (CVD), whose prevalence in women, during the reproductive period, is lower compared to men. After the loss of estrogen protection [2], gender differences in CVD risk fade. However mortality for cardiovascular events is lower in females of any age class [3].

Clinical and preclinical data highlight the role of adipose tissue and insulin resistance in the development of a systemic inflammatory state. Among several pathways involved, impaired adipokine and hepatokine levels are reported [4]. Adiponectin and fetuin-A seem to have an important role. Adiponectin plays a major role in glucose and lipid metabolism, it has important metabolic and anti-inflammatory actions with a protective role in T2DM development [5]. Fetuin-A is a physiological inhibitor of insulin receptor activation and thus associated with insulin resistance, metabolic syndrome (MetS) and an increased risk for T2DM [6,7]. In this context, the role of dendritic cells (DCs) is intriguing. DCs are antigen-presenting cells that have a significant role in the pathogenesis of atherosclerosis and autoimmune diseases [8]. Together with other leukocytes DCs are involved in low grade inflammation and influence the atherogenic process by modulating cellular and humoral immune response. DCs accumulate in the arterial intima where they contribute to local inflammation and lesion destabilization [9]. However, their role in early stage of vascular remodeling is still under investigation. It is reported that diabetes and obesity modulate DC function. Adipokines released by visceral adipose tissue (VAT), such as chemerin [10], are reported to recruit circulating DCs and other immune cells contributing to metaflammation [11–13]. In our previous work some characteristics of circulating and monocyte-derived dendritic cells (Mo-DCs) collected from obese and diabetic postmenopausal women were explored, in order to find markers of altered immune response before the onset of any possible cardiovascular event [14]. We reported that Mo-DCs obtained from circulating precursors display a defective phenotype compared to controls and demonstrated a higher ability to adhere to coronary vascular smooth muscle cells, suggesting that insulin-resistance and obesity modulate DC function. In this paper, DCs from obese and T2DM postmenopausal patients were further characterized. Fetuin-A and adiponectin were also measured, in order to assess any difference between T2DM and T2DM-obese patients. Moreover, the effect of hyperglycaemia on in vitro immature DCs (iDCs) yield from circulating precursors and their maturation was assessed.

2. Material and methods

2.1. *Patients*

Fiftytwo post-menopausal women, (to avoid potentially confounding factors related to menstrual cycle-related fluctuation in plasma estrogen concentration), were recruited from the Diabetology, Careggi University Hospital, (AOU-Careggi) Table 1. Eleven patients were obese with body mass index (BMI) > 30, seventeen were T2DM obese while ten were only T2DM. All T2DM subjects were treated with insulin sensitizer oral drugs, such as metformin or pioglitazone. All patients have been evaluated for their lipidic and glycaemic profile (fasting plasma glucose). They had an age of 66.34 ± 8.55 years. Exclusion criteria included: presence of hepatic, cardiac or renal insufficiency, cancer, insulin therapy, any previous cardiovascular event. The control group consisted of 14 age matched healthy post-menopausal women. For each participant 3 samples of about 5 ml of fresh peripheral blood in test-tubes with EDTA were collected. Written consent was obtained from each participant. The protocol was approved by the local Ethical Committee (prot. N. 0011762).

2.2. *Blood sampling and ex-vivo generation of Mo-DCs.*

$CD14^+$ cells were separated performing a positive selection with CD14 micromagnetic beads by magnetic cell sorting (MACS) according to the manufacturer's instructions. DCs were generated by culture of $CD14^+$ in RPMI 1640 as previously reported [15].

To assess glucose effect on Mo-DC generation, two protocols were used. (1) iDCs and Mo-DCs were obtained by growing $CD14^+$ in the presence of increasing D-glucose concentrations. (2) high glucose effect was only evaluated on iDC maturation, by growing them in the presence of 33 mM D-glucose during the 24 h-stimulation with 100 nM LPS. Mannitol was added to the medium to bring total osmolality to a value equivalent to 33 mM glucose.

2.3. *Flow cytometry*

The following fluorescent monoclonal antibodies were used for flow cytometry analysis of iDCs and Mo-DCs: CD80-isothiocyanate (FITC), CD83-phycoerythtrin (PE), CD86-allophycocyanin (APC), (BD Pharmingen; San Diego, CA). Isotype-matched antibodies were used as negative control.

Table 1 – Clinical characteristics of enrolled patients. Mean ± SD or median ± range. *P < 0.05, , ***P < 0.001 vs obese. ^P < 0.001 vs T2DM. °P < 0.01, °°P < 0.001 vs T2DM and obese.

Variables	Healthy	Obese (n = 11)	T2DM (n = 10)	T2DM Obese (n = 17)
Age (years)	65,40 ± 8,23	64,36 ± 8,23	69,20 ± 7,70	65,94 ± 9,25
BMI (Kg/m2)	24,20 ± 4,50	37,54 ± 6,81	26,33 ± 3,49*** °	33,52 ± 4,02
Total cholesterol (mg/dL)	160,00±20,00	219,22 ± 35,72	186,95 ± 32,00	176,18 ± 26,37*
HbA1c (%)	4,60 ± 0,80	5,63 ± 0,19^°°	7,56 ± 0,66	7,24 ± 0,56
HDL-C (mg/dL)	50,00± 15,01	61,22 ± 14,94	58,60 ± 10,61	54,53 ± 12,66
LDL-c (mg/dL)	95,20 ± 15,00	135,97 ± 35,98	107,84 ± 33,07	91,95 ± 25,51***
Glycemia (mg/dL)	90,10 ± 5,00	103 [62–128]	186 [104–240]*	123 [89–242]*
Triglycerides (mg/dL)	84,00 ± 10,00	108 [77–181]	81 [69–171]	141 [75–223]

7-amino-actinomycin D (7-AAD; Sigma-Aldrich, St Louis, Mo, Usa, San Diego, CA) was used to exclude dead cells from analysis. For lymphocyte proliferation assay the following monoclonal antibodies were used: anti-CD3- PerCP-Cy 5.5, CD4-PE and -CD8-APC. After selection of $CD3^+CD4^+$ or $CD3^+CD8^+$ subsets, the shift of CFSE-FITC stained proliferating cells versus the unstimulated lymphocytes was compared

2.4. *Isolation of human peripheral blood mononuclear cells and mixed lymphocyte reaction (CFSE)*

Allogeneic peripheral blood mononuclear cells (PBMCs) were seeded in RPMI 1640 and 10% FBS and monocytes were let to adhere 45 min. Suspended leukocytes were collected, centrifuged at 1200 rpm for 10 min and used for mixed leucocyte proliferation with Mo-DC. Briefly, 10^7 lymphocytes were stained with 5 μM of CFSE (Molecular Probes, Eugene, Oregon, USA) according to manufacturer instructions. Stained lymphocytes seeded into black 96-well plates at $2x10^5$ cells/well and 4×10^4 Mo-DCs/well were added to each well and incubated for 5 days at 37 °C in a humidified chamber in air and 5% CO_2. Lymphocytes treated with phytohemagglutinin 5μg/ml (PHA) or untreated were used as positive and negative control of proliferation, respectively. At the end of 5 days of mixed reaction, cells were harvested and stained for flow cytometry analysis of lymphocyte proliferation [15].

2.5. *Real time PCR*

RNA was isolated from Mo-DC obtained from patients with TRI Reagent (Sigma–Aldrich, Milan, Italy) and quantified spectroscopically with NanoDrop (Thermo-fisher scientific, Waltham, Massachusetts, USA). One μg of RNA was retrotranscribed using Prime Script RT reagent Kit with gDNA eraser (Takara, Otsu, Japan) and 50 ng of cDNA were amplified with specific primers described below. For CD11c mRNA fw: CCAGTGTGGCTACAGCACTGGTGCC and rev: TGGGTGAGCTGGGTGGGGCCC; CD18 fw: CTGTCTGAGGACTCCAGCAATGTGG and rev: CACCTGGAAGGTGATCGGGACATTGAT; DC-SIGN (CD209) fw: CCAGCAGATACATGGCCACAAGAGC and rev: CTGCAGCTTTAAGCTGGGTCAGGTTCT; PCR amplification was carried out as using SYBR Premix Ex Taq (Takara, Otsu, Japan) according to manufacturer instructions on Rotorgene RG-3000A cycle system (Qiagen, Germany) platform.. PCR amplification of 18 s rRNA was used as the normalizer. Real-time PCR assays were performed using the Rotorgene RG-3000A cycle system (Qiagen, Germany). PCR amplification of 18 s rRNA was used as the normalizer fw: ATTAAGGGTGTGGGCCGAAG and rev: GGTGATCACACGTTCCACCT. The cycle was set at 95 °C for 5 s followed by 30 s at 60 °C and repeated 40 times.

2.6. *ELISA*

One ml of fresh peripheral blood was collected in test-tubes with EDTA and serum harvested for fetuin-A and adiponectin measurement by means commercially available enzyme-linked immunosorbent assay kits (R&D System).

2.7. *Statistical evaluation*

Parametric data were reported as means ± SEM and differences between groups were tested with ANOVA test (followed by Bonferroni's and Dunnett's Multiple Comparison Test) as appropriate. Non-parametric measures were reported as median ± range and differences among groups were tested with Kruskal Wallis test. Association among non-parametric variables were tested with Spearman correlation. Alpha value was set at 0.05.

3. Results

3.1. *Functional characterization of Mo-DCs of obese and T2DM patients*

We previously demonstrated that DC precursors isolated form T2DM obese patients displayed less capability to trans-differentiate into mature monocyte-derived DCs (Mo-DCs). To confirm their phenotype, their ability to stimulate allogenic lymphocytes was assessed. Mo-DCs obtained from T2DM obese patients were significantly less able to induce cytotoxic lymphocytes $CD8^+$ proliferation, compared to control- and obese-derived Mo-DCs, in accord to phenotypic analysis [14]. A defective function, although not statistically significant, was observed for Mo-DC obtained from T2DM-not obese patients. Moreover, no differences were observed for $CD4^+$ proliferation (Fig. 1).

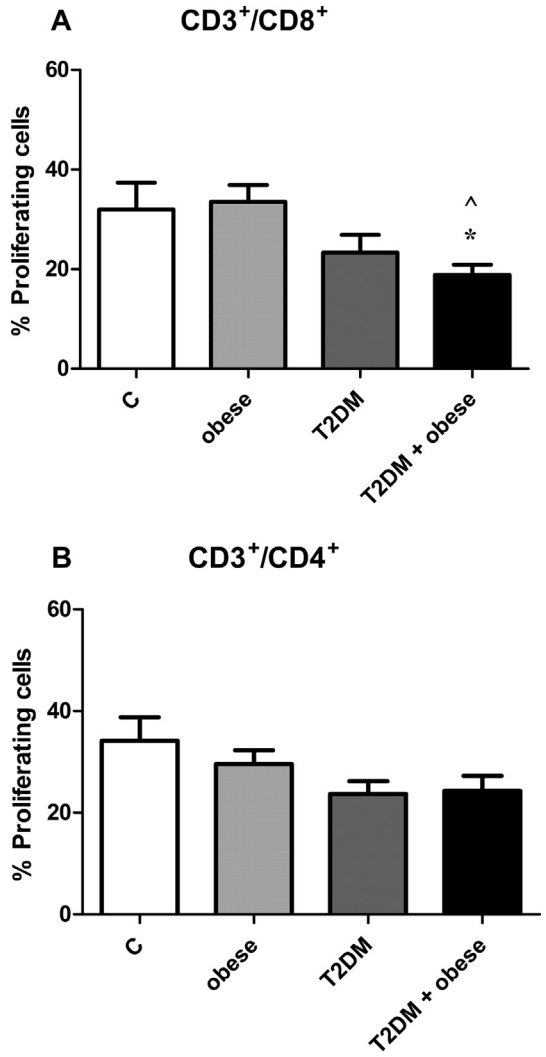

Fig. 1 – Mixed lymphocyte reaction (CFSE). Allogeneic lymphocytes were co-cultured with Mo-DCs obtained from obese, T2DM and T2DM + obese patients and control subjects. Their proliferation was assessed by flow cytometry. Means ± SEM, n = 9; ^P < 0.01 vs obese; *P < 0.05 vs control healthy subjects (C).

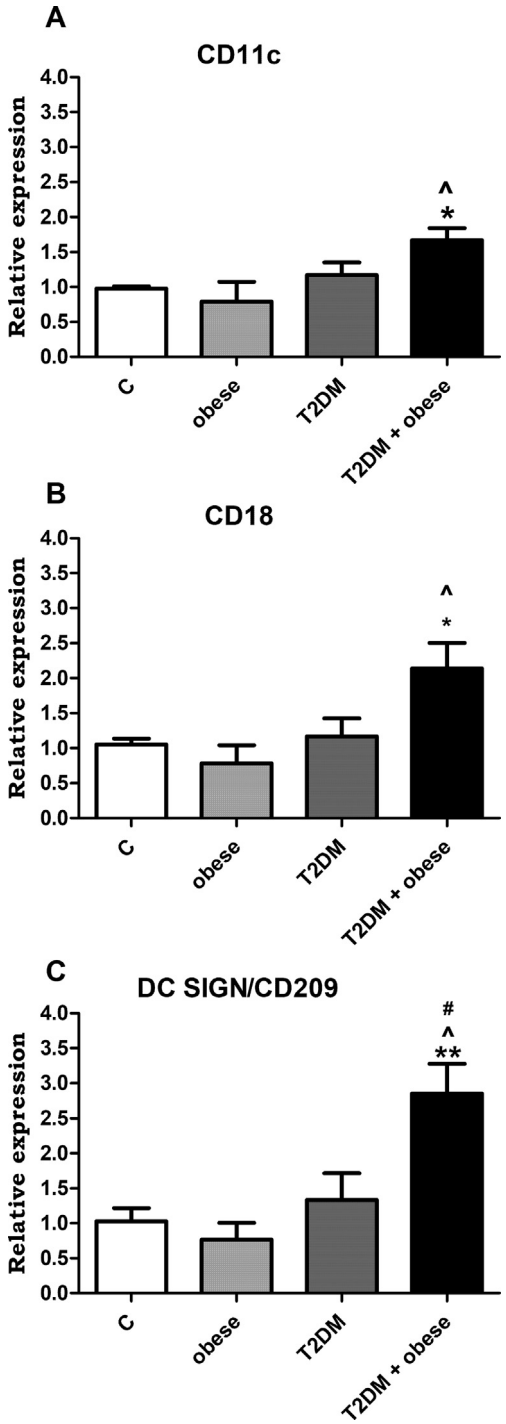

Fig. 2 – Real-time PCR for CD11c, CD18 and DC-SIGN/CD209 mRNA expression. Total RNA was isolated from Mo-DCs of obese, T2DM, and T2DM + obese patients and control subjects. Data are expressed as relative expression vs control healthy subjects (C). Means ± SEM, n = 8; *P < 0.05, **P < 0.01 vs control healthy subjects (C). ^P < 0.05 vs obese; #P < 0.05 vs T2DM.

3.2. *Integrin expression in obese and T2DM patient-derived Mo-DCs*

Obese and T2DM patients are characterized by low-grade inflammation with increased adhesion molecule at vascular levels which mediate leucocyte recruitment. It was demonstrated that Mo-DCs isolated from T2DM obese patients displayed higher ability to adhere to human coronary vascular smooth muscle cells (CASMC) [14]. Consistently, Mo-DCs isolated from healthy subjects significantly increased their adhesion to CASMC treated with IFNγ and TNFα, compared to control-untreated CASMC [15]. We then investigated mRNA expression of counter-receptors of vascular adhesion molecules, in Mo-DCs obtained from enrolled patients. As reported in Fig. 2, CD11c, CD18 and DC-SIGN/CD209 mRNA expression was significantly higher in T2DM obese patients than obese alone, T2DM alone or control DCs, supporting the hypothesis that low grade inflammation affects DC phenotype at vascular level.

3.3. Effect of high D-glucose on CD14+ trans-differentiation and iDC maturation

Since Mo-DCs obtained from T2DM obese patients display defects on phenotype and function compared to obese and control ones, we investigated the effect of high D-glucose concentrations on CD14+ trans-differentiation into iDC and then their maturation (Mo-DCs) in response to LPS. For this purpose, CD14+ isolated from healthy subjects were grown in the presence of increasing concentration of D-glucose and let to obtain Mo-DCs. We used 33 mM mannitol as a high-osmolarity control treatment. Flow cytometry analysis showed that 33 mM D-glucose significantly impaired Mo-DCs yield, although did not affect CD14+ trans-differentiation into iDCs, demonstrated by a significant decrease of cells expressing CD83 (Fig. 3A). This impairment was not due to high osmolarity, since 33 mM mannitol was devoid of any effect. However, hyperglycaemia did not impair iDC maturation into Mo-DCs, when they were obtained from CD14+ in normoglycaemic conditions and then were let to maturate into Mo-DCs in the presence of 33 mM D-glucose (Fig. 3B).

3.4. Fetuin-A and adiponectin expression

Fetuin-A is a multifunctional plasma protein associated with insulin resistance. Its plasma levels are higher in T2DM or obese patients, but its role in the pathogenesis of diabetes and obesity is still discussed [16,17]. We then characterized fetuin-A and adiponectin levels in enrolled patients. As reported in Fig. 4A, adiponectin levels were significantly lower in T2DM obese patients compared to controls (p value = 0.0083). Consistently, T2DM obese patients displayed significant higher concentration of fetuin-A, compared to controls (p value = 0.0457, Fig. 4B). No differences were found in fetuin-A when patients were divided into 2 subgroups, according to HbA1c: T2DM obese with HbA1c < 7.0 and T2DM obese with HbA1c > 7.0 (data not shown). A negative correlation between fetuin-A and adiponectin was found in all population (r = -0.492p value = 0.00108, Fig. 4C), and in population stratified according to the presence/absence of T2DM and obesity (Fig. 4D). A negative (r = -0.416p value = 0.0199) and positive significant correlation (r = 0.549p value = 0.00139) with BMI was obtained for adiponectin and fetuin-A, respectively (Fig. 4E and F).

4. Discussion

T2DM is as a chronic, low-grade inflammatory disease caused by long-term immune system imbalance, and is often associated with obesity or MetS. Increasing evidences support the role of autoimmunity and adaptive immune system in the pathogenesis of T2DM and its vascular complications [18]. The impact of DCs on the initiation and progression of

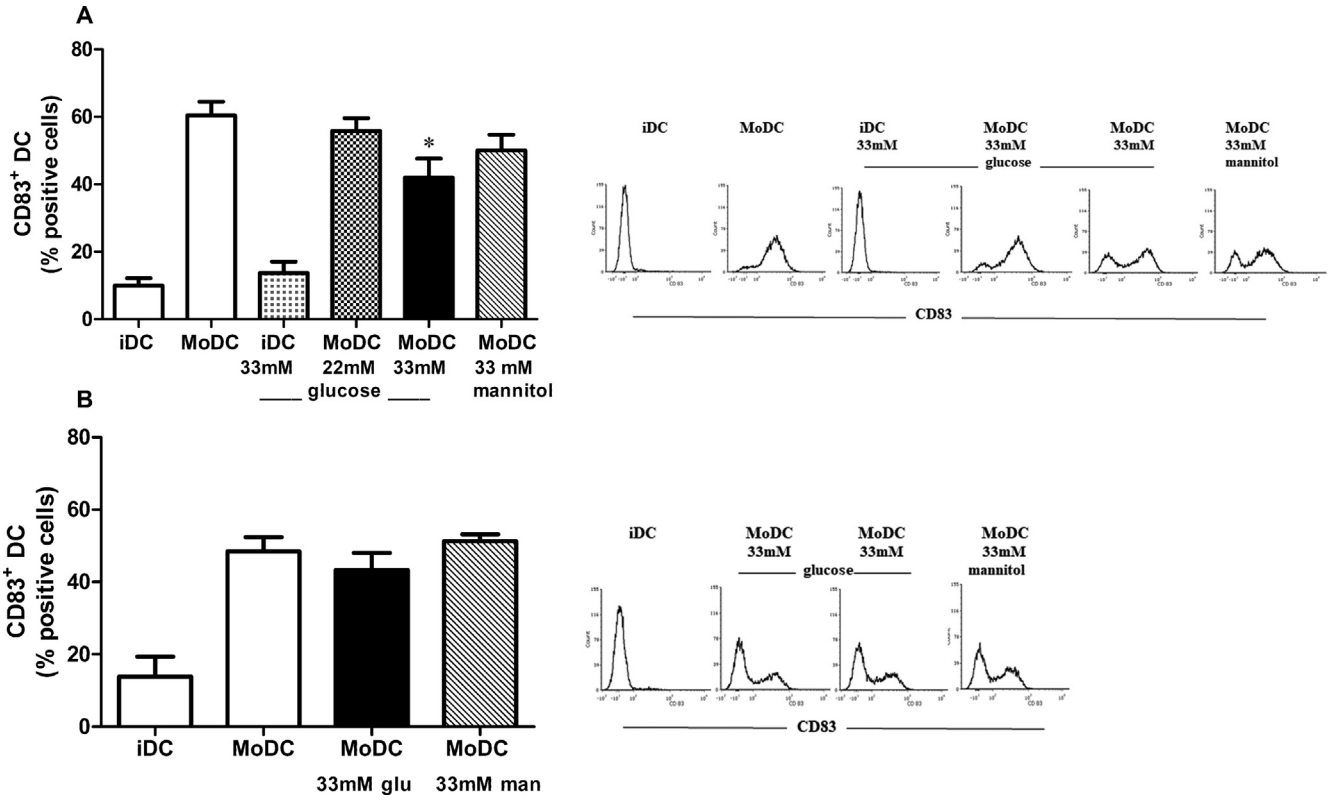

Fig. 3 – Effect of high D-glucose on CD14+ trans-differentiation (A) and iDC maturation (B) into Mo-DCs. (A) Mo-DCs were obtained from CD14+ grown for 7 days in the presence of increasing D-glucose concentrations. (B) iDC obtained from CD14+ grown in standard medium were then let to maturate into Mo-DCs in the presence of 33 mM D-glucose for 24 h. Means ± SEM, n = 7; Close to each bar graph: representative histograms. *P < 0.05 vs Mo-DC. iDC: immature DC; MoDC: mature DCs; glu: D-glucose; Man: D-mannitol.

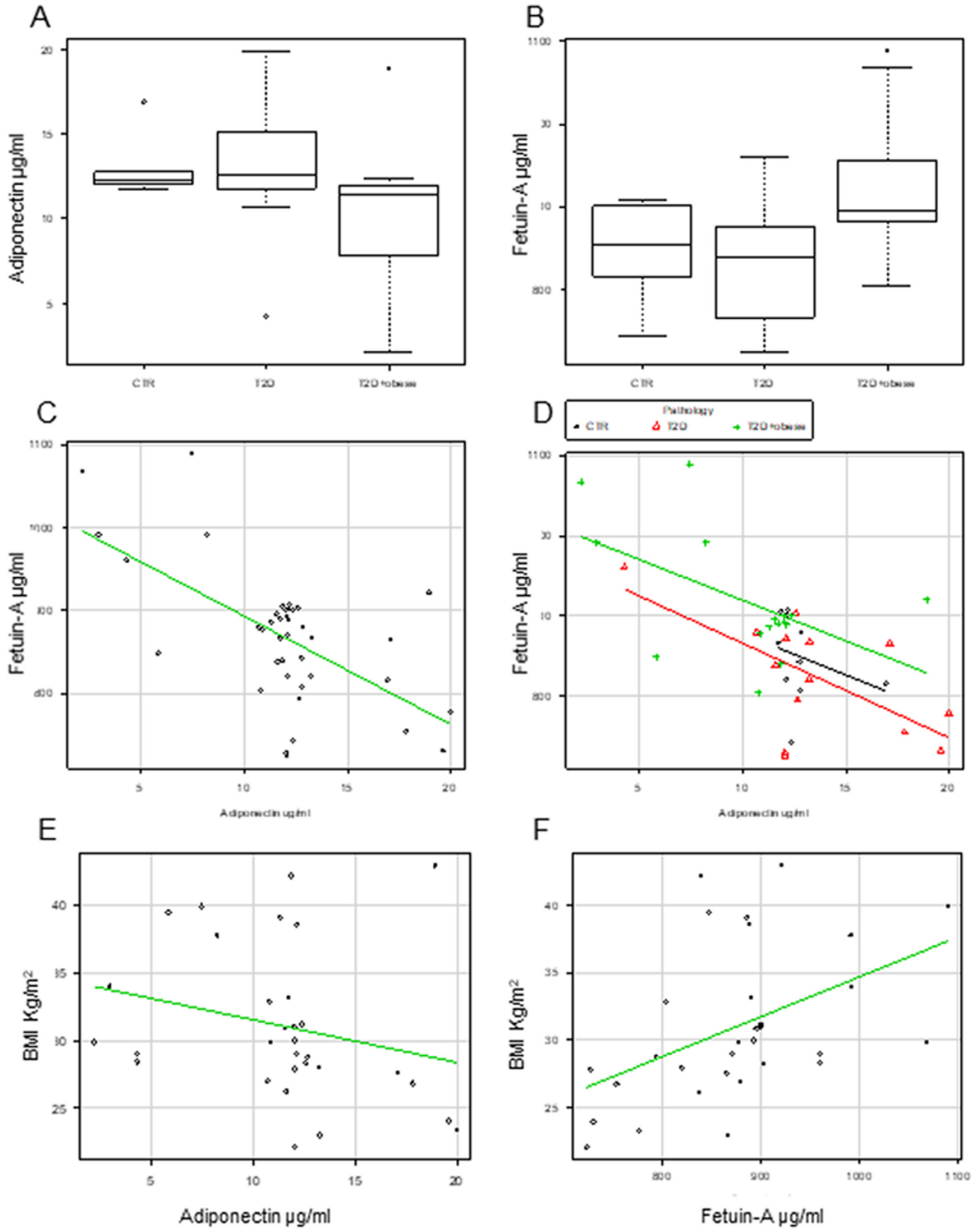

Fig. 4 – Adiponectin (A) and Fetuin-A (B) levels in controls and enrolled patients. (C) Spearman rank correlation between fetuin-A and adiponectin in all population (r = −0.492p value = 0.00108, (C) and in population stratified according to the presence/absence of T2DM ± obesity (D). Adiponectin (E) and Fetuin-A (F) correlation with BMI in T2DM ± obese patients. Spearman's rank correlation coefficient r = −0.416p value = 0.0199 for adiponectin and r = 0.549 with p value = 0.00139 for fetuin-A.

atherosclerosis has been under investigation. DCs at vascular levels are reported to contribute to vascular remodeling, and among them conventional DCs (cDCs) seem to have a pivotal role, although plasmacytoid DCs (pDCs) can be found in atherosclerotic lesions in apolipoprotein E–deficient ($Apoe^{-/-}$) and low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) mice [19,20]. In human tissue, cDCs, corresponding to the two subsets of myeloid DC as previously described [21], and $CD123^+$ pDC numbers were increased in unstable compared with stable lesions, mainly localizing to the shoulder region of plaques [22]. In patients with peripheral artery disease Kretzschmar and co-authors (2015) [23] have reported a decrease in circulating DC precursors, suggesting an increase of their accumulation into vessel lesions. Despite these data, it is unclear the pathogenetic role of DCs in the initiation of vascular remodeling associated with high risk CVD. For this purpose, we selected post-menopausal obese woman with or without diabetes or patients with diabetes alone without any previous major CV event, in order to characterize DC function. We previously reported that Mo-DCs obtained from T2DM obese post-menopausal woman display a defective phenotype compared to obese and control ones. Present paper demonstrates the latter DC population being less able to stimulate $CD8^+$ cytotoxic T lymphocytes, compared to Mo-DCs obtained from obese or T2DM and control ones. These data are a new observation and reflect the immune state of these patients, in which DC dysfunction may contribute to an increased susceptibility to infections and to higher risk of CVD [24,25]. It is known that obesity and T2DM diseases share T cell alterations that may contribute to their additional metabolic disturbances. In T2DM and obesity there is a shift of $CD4^+$T cell subsets toward a proinflammatory phenotype, such as $CD4^+$ Th1 and Th17 and a regulatory T cells (Treg) [26]. Proinflammatory Th1 cells producing IFN-γ are correlated with adiposity [27] and patients with diabetes display an increase of $CD4^+CD28$null T lymphocytes, a subpopulation exerting direct cytolytic and proapoptotic effects on endothelial cells and vascular smooth muscle cells [28,29].

According to published data, our present paper strengthens metanflammation in enrolled population, since Mo-DCs obtained from T2DM obese patients express higher mRNA levels of integrins, such as CD18 and CD11c, compared to obese or T2DM ones, which may modulate their adhesion to vascular wall cells, as previously published [14]. Among integrins binding vascular adhesion molecules, beta2 integrin CR4 (CD11c/CD18) seems to be important for a strong Mo-DC adhesion to fibrinogen in order to mediate their transendothelial migration [30]. Retention of leukocytes to vascular wall is involved in the worsening of inflammatory condition, leading to the progression and complication of vascular remodeling. Consistently, hyperglycaemia up-regulates adhesion molecule expression [31] modulating immune cells recruitment, as reported in subcutaneous abdominal adipose tissue of obese women without metabolic complications [32]. Despite this, the effect of hyperglycaemia on DC phenotype and function is still controversial. Present data show defective Mo-DCs yield when hyperglycaemia was applied to their circulating precursors, $CD14^+$, during all their culture conditions (7 days). Nevertheless, when iDC were obtained in normoglycemic condition and then were let to maturate in the presence of high D-glucose for 24 h, Mo-DCs yield was not impaired. This result is apparently in contrast to that of Lu et al (2013) [33], who reported an increase of DC yield in response to high D-glucose concentration. However, their experimental design was different, since they applied high D-glucose to $CD14^+$ after a-5 days of normoglycemic condition, without adding LPS for their maturation. More recently, it was reported that hyperglycemic sera from T2DM patients impaired DC differentiation through ROS production [34], highlighting DC dysfunction in these patients which may be responsible to their defective immune response.

Circulating fetuin-A and adiponectin were also measured in enrolled patients. Fetuin-A is an endogenous inhibitor of insulin receptor and epidemiological and clinical studies reported that high plasma fetuin-A levels are associated with insulin resistance (IR), MetS and risk of T2DM [16,35], the latter seemed more evident in women than in men [36]. In contrast adiponectin protects against insulin resistance/diabetes and atherosclerosis and its plasma levels decrease in T2DM or obese patients [37]. Our results show an inverse correlation between adiponectin and fetuin-A in enrolled patients, being fetuin-A significantly higher and adiponectin significantly lower in T2DM obese patients compared to healthy subjects. These results extend previous cross-sectional study in which fetuin-A and adiponectin were measured in a new diagnosed T2DM patients (either male and female), younger than patients in our study and with a BMI > 25 [38]. Interestingly, our present data showed that fetuin-A and adiponectin levels were not significantly different in T2DM patients compared to controls. This result, apparently in contrast with previous published, could be explained by pharmacological therapy. All T2DM enrolled woman were indeed treated with insulin sensitizers drugs, such as metformin or pioglitazone, which were reported to modulate adiponectin and/or fetuin in obese and/or T2DM patients [39–42], whose mechanisms, still under investigation, are not likely dependent on improved glycemic control alone.

5. Conclusions

All together these data confirm a dysfunction of Mo-DCs obtained from circulating precursors of T2DM obese post-menopausal woman without any clinical CV event documented. The association of obesity with diabetes seems to worsen DC phenotype and function with potentially greater ability to mediate inflammation. Our data underline the importance of the multiple CV risk factors coexistence in a patient. Pharmacological treatment is almost certainly able to revert fetuin-A and adiponectin dysregulation in post-menopausal T2DM woman, while is not in T2DM obese ones.

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Author contributions

AP, LP, BC and CMR designed the study. CMR, LP and BC enrolled patients; SP, AS, AG and MC conducted the research. AP and SP analyzed the data and wrote the manuscript. The final manuscript was read and approved by all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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