

Research Paper

β_2 Adrenoceptors are underexpressed in peripheral blood mononuclear cells and associated with a better metabolic profile in central obesity

 Fernanda Leite^{1,2,3}, Margarida Lima^{2,3}, Franca Marino⁴, Marco Cosentino⁴, Laura Ribeiro^{1,5,6}✉

1. Department of Biomedicine, Faculty of Medicine, University of Porto, Portugal
2. Department of Clinical Haematology, Centro Hospitalar of Porto, Portugal
3. UMIB/ICBAS - Unit for Multidisciplinary Investigation in Biomedicine- Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal
4. Center of Research in Medical Pharmacology, University of Insubria, Varese, Italy
5. Department of Public Health Sciences, Forensic and Medical Education, Faculty of Medicine, University of Porto, Portugal
6. I3S-Instituto de Investigação e Inovação em Saúde, University of Porto, Portugal

✉ Corresponding author: Laura Ribeiro, Department of Biomedicine, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319, Porto, Portugal. Phone/ Fax: +351 22 5513624. E-mail address: lribeiro@med.up.pt

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Abstract

Background: Central obesity (CO) is an inflammatory disease. Because immune cells and adipocytes are catecholamines (CA)-producing cells, we studied the expression of adrenoceptors (AR) in peripheral blood mononuclear cells (PBMCs) hypothesizing a distinct adrenergic pattern in inflammatory obesity.

Methods: AR expression was assessed in blood donors categorized by waist circumference (WC) (CO: WC \geq 0.80 m in women and \geq 0.94 m in men). Following a pilot study for all AR subtypes, we measured β_2 AR expression in fifty-seven individuals and correlated this result with anthropometric, metabolic and inflammatory parameters. A ratio (R) between AR mRNA of CO and non-CO $<$ 0.5 was considered under and $>$ 2.0 over expression.

Results: The pilot study revealed no differences between groups, except for β_2 AR mRNA. CO individuals showed underexpression of β_2 AR relatively to those without CO (R=0.08; $p=0.009$). β_2 AR expression inversely correlated with triacylglycerol ($r=-0.271$; $p=0.041$), very low-density lipoprotein-cholesterol ($r=-0.313$; $p=0.018$) and leptin ($r=-0.392$; $p=0.012$) and positively with high-density lipoprotein-cholesterol ($r=0.310$; $p=0.045$) plasma levels. Multiple logistic regression analysis showed a protective effect of β_2 AR expression ($\geq 2 \times 10^{-6}$) [odds ratio (OR) 0.177 with respective confidence interval of 95% (95% CI) (0.040- 0.796)] for the occurrence of CO. A higher association was found for women as compared to men (Ξ :1) [OR 8.972 (95% CI) (1.679–47.949)].

Conclusion: PBMCs β_2 AR, underexpressed in centrally obese, are associated with a better metabolic profile and showed a protective role for the development of CO. The discovery of β_2 AR as a new molecular marker of obesity subphenotypes in PBMCs might contribute to clarify the adrenergic immunomodulation of inflammatory obesity.

Key words: beta2-adrenoceptor, immune cells, central obesity, inflammation, catecholamines.

Introduction

Obesity, notably visceral or central, is a major risk factor for cardiovascular disease (CVD) increasing the incidence of hypertension, type 2 diabetes and dyslipidemia [1] which are linked to reduced life expectancy and premature death.

Central adiposity, as measured by waist circumference (WC), is highly correlated with visceral fat, as measured by computed tomography [2]. Visceral obesity and its comorbidities are characterized by increased concentrations of a large panel of

cytokines, chemokines and acute-phase proteins in circulation, which are in turn closely associated with low-grade chronic inflammation, although the pathophysiological mechanisms underlying this association are not completely understood [3].

Remarkably, immune cells, neurons and adipocytes share common signalling pathways. These pathways are mediated by the catecholamines (CA), adrenaline (AD) and noradrenaline (NA), through the activation of adrenoceptors (AR) [4, 5]. There are three major types of AR ($\alpha 1$, $\alpha 2$, β), each of which is further divided into three subtypes. These receptors are involved in essential metabolic and central nervous system functions. There is ample evidence that AR, and more specifically adrenoceptor β_2 (β_2 AR), have a role in immunomodulation. Endogenous CA produced by immune cells regulate, through autocrine/paracrine mechanisms, several immune cell functions [6], modulating inflammatory responses in monocytes and lymphocytes, among other immune cells, during health and disease [5, 6]. The global outcome of β_2 AR triggering in inflammation seems to be beneficial [6].

In obesity, visceral adipose tissue (AT) becomes infiltrated by a large number of immune cells, namely, macrophages [7] and lymphocytes [8]. Most of these originate from circulating peripheral blood mononuclear cells (PBMCs) [9]. These cells seem to possess the full cellular machinery for *de novo* synthesis, release, and inactivation of CA [6] and are referred as potential sources of biomarkers of early homeostatic imbalance that would be useful for the study and prevention of metabolic disorders as obesity [10]. Recent studies have reported that adipocytes are likewise capable of CA *de novo* synthesis suggesting a role of adipocyte CA in metabolic processes [11]. Our group demonstrated that CA release is differently affected by dietary unsaturated fatty acids [12].

Adrenergic modulation of immunity remains a non-appreciated issue in obesity. We recently described for the first time that tyrosine hydroxylase, the rate limiting step of CA synthesis, and dopamine receptors in PBMCs are underexpressed in central obesity (CO) [13]. We hypothesize that the adrenergic signature is distinct under these conditions, because AD and NA, important metabolic and immune regulators, may mediate inflammatory obesity.

In the present study, we looked for the expression of AR in circulatory immune cells and its correlation with anthropometric, endocrine/metabolic and inflammatory parameters in a well-defined group of blood donors (BD) to establish: i) whether central obesity, a surrogate marker of abdominal fat mass [14], is associated with variable AR expression in PBMCs and ii) the extent to which this association is

explained by anthropometric/metabolic/endocrine/inflammatory factors.

This study may give rise to new therapeutic interventions to manage inflammatory central obesity and its co-morbidities.

Methods

Participants and experimental design

This study was conducted in 57 blood donors from the Blood Bank of Clinical Haematology Department of Centro Hospitalar of Porto (CHP), Portugal; it meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 [JAMA 1997;277:925-926], and was approved by the Ethical Committee and Research Office, and authorized by the administration board of CHP, being registered with the identifier 072/09 (047-DEFI/065-CES). All participants signed a written informed consent, after being aware about the objectives of the study and the confidentiality of the data. The individuals met the selection criteria for blood donation and were not under any medicines during the previous month. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice and the mean values were calculated. The total sample was studied for anthropometric, metabolic/endocrine and inflammatory parameters and PBMCs AR expression. The characteristics of the enrolled subjects are shown in Table 1.

Anthropometrics

Body mass index (BMI) was calculated by dividing weight by squared height, expressed in kg.m^{-2} . BMI categories were defined according to the guidelines of the World Health Organization [15]. WC was measured at the level midway between the lowest rib and the iliac crest. Height (in m) was confirmed by medical record. The participants, all Caucasians, were divided in two groups, according to the International Diabetes Federation criteria of CO defined as WC ≥ 0.80 m in women and ≥ 0.94 m in men [16].

Biochemical analysis

Blood samples were taken from all subjects under standardized conditions. Fasting plasma glucose, triacylglycerol (TAG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and very low-density lipoprotein-cholesterol (VLDL-C), were measured with turbidometry and spectrophotometry methodology using the Cobas® 8000 autoanalyzer (Roche, Rotkreuz, Switzerland). Glycosylated hemoglobin (HbA1c) measurements were done by

High Performance Liquid Chromatography (HPLC), using the Hi-Auto A1c HA-8140 HPLC (Menarini Diagnostics, Florence, Italy). Plasma cortisol was performed with an electrochemiluminescence immunoassay (Elecsys Systems analyser Roche, Roche Diagnostics International Ltd Rotkreuz, Switzerland) and leptin was measured in serum by solid phase two-site enzyme immunoassay (Merceodia Leptin ELISA, Mercoodia AB, Sylveniusgatan 8A, Uppsala, Sweden). High-sensitivity C-reactive protein (hsCRP) (mg/L) determined by nephelometry (CardioPhase® hsCRP-BnProSpec SiemensHealthcare Diagnostics Inc. New York, United States) was categorized by the following cardiovascular event risk groups: <1- low, ≥1 to <3 -intermediate and above 3 - high risk, as described before [17].

Table 1. Characteristics of the study participants (n=57)

Parameter	Unit	Reference values (range)	mean± SEM/*	Min-Max
Age	years		40±2	20-63
Weight	Kg		77±2	51-120
Height	m		1.67±0.01	1.50-1.87
BMI	Kgm ⁻²		27.6±0.6	19.6-40.1
WC	m		0.97±0.02	0.75-1.24
SBP	mmHg	<130	134±2	103-177
DBP	mmHg	<85	80±1	61-107
Glycemia	mg/dL	70 - 105	85±1	67-115
HbA1c	%	3.8 - 5.6	5.2±0.1	4.5-5.8
TC	mg/dL	0 - 200	194±5	117-283
LDL-C	mg/dL	0 - 130	123±4	60-209
HDL-C	mg/dL	35 - 55	50±2	25-91
VLDL-C	mg/dL	3 - 56	21±2	6-58
TAG	mg/dL	40 - 160	109±10	32-475
hsCRP*	mg/L	< 1.0 low risk 1.0 <3.0 intermediate risk >3.0 high risk	1.38 (0.66- 3.01)	0.0-17.8
Noradrenaline*	pmoL/L	709 - 4019	684 (395-1552)	40-3760
Adrenaline*	pmoL/L	<328	151 (81-225)	55-473
Cortisol*	µg/dL	6.2 - 19.4	15.0 (12.6-17.3)	6.4-28.4
Leptin*	ng/mL	2.0 - 5.6	0.615 (0.245-1.075)	0.003-5.300
Leucocytes	cells/µL	4500 - 13000	6432±216	3500-11600
Monocytes	cells/µL	400 - 500	440±22	82-964
Lymphocytes*	cells/µL	1000-4800	1898 (1538-2299)	1007-5069

BMI, Body Mass Index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; TAG, triacylglycerol; hsCRP, high-sensitivity C reactive protein; Min, minimum; Max, maximum. Data are presented as mean ± standard error of the mean (SEM), unless otherwise indicated by * corresponding to data presented as median, 25th and 75th percentiles.

Assay of monocytes by Flow Cytometry

Monocytes from fresh EDTA-K3 anti-coagulated whole blood samples were determined by means of flow cytometry, as previously described [18].

Immunophenotypic studies were performed using a whole blood stain-lyse-and-then-wash method and a direct immunofluorescence technique with the following four-color panel of monoclonal antibodies: mouse anti-human CD36 conjugated with FITC (clone FA6.152, IgG1), mouse anti-human CD16 conjugated with PE (clone 3G8, IgG1), mouse anti-human CD14 conjugated with PE-Cy5 (IgG2a, clone RMO52) all obtained from Beckman Coulter (catalogue numbers IM0766U, IM1238U and IM2640U, respectively), and mouse anti-human CD11b conjugated with APC (IgG2a, clone D12) obtained from Becton Dickinson (BD) (catalogue number 333143).

Data acquisition was carried out on a FACS-Calibur flow cytometer (BD), using the Cell Quest software program (BD). A minimum of 2x10⁵ events were used for each staining and stored as list mode data. The Paint-a-Gate Pro software program (BD) was used for data analysis. Monocytes were quantified based on CD14 expression. CD16 was used to differentiate classical (CD16-) and non-classical ("pro-inflammatory") (CD16+) monocyte populations. The median fluorescence intensity (MFI) of CD14, CD36 and CD11b was assessed in each subset and expressed as fluorescence arbitrary units (AU). The forward light scatter (FSC) and sideward light scatter (SSC) of cell subsets were also determined. To overcome inter-individual variations and maturation process, in each subject, the ratio for each parameter between CD14+CD16+ non-classical monocytes and CD14+CD16- classical monocytes was calculated.

Expression of AR in PBMCs by Real time PCR

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll method), as previously described [19]. Total RNA was extracted by PerfectPure™ RNA Cell & Tissue kit (5Prime), and the amount of extracted RNA was estimated by spectrophotometry at 260 nm. Total RNA was reverse transcribed using the High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. Real-time PCR was performed with an ABI prism 7000 apparatus (Applied Biosystems) using the Assay on demand kits for the genes of interest (Applied Biosystems), according to the manufacturer's instructions. Gene sequence data were obtained from the Reference Sequence collection (RefSeq; www.ncbi.nlm.nih.gov/projects/RefSeq). For each gene, the thermal profile was as follows: stage 1, 2 min at 50°C; stage 2, 10 min at 95°C; stage 3, 40 cycles including 15s at 95°C and 1 min at 60°C. Table 2 contains the details about real-time PCR conditions.

Table 2. Real-time PCR gene expression

Gene Symbol	UniGene ID	Interrogated Sequence RefSeq/GenBank mRNA	Translated protein	Exon boundary RefSeq/GenBank mRNA	Assay location RefSeq/GenBank mRNA	Amplicon length	Annealing temperature (°C)	Efficiency (%)
α_{1A} -AR	Hs. 709175	NM_033302.2	NP_150645.2	1-2	1324	112	60	99.98
α_{1B} -AR	Hs. 368632	NM_000679.3	NP_000670.1	1-2	1126	61	60	100.04
α_{1D} -AR	Hs. 557	NM_000678.3	NP_000669.1	1-2	1166	68	60	100.08
α_{2A} -AR	Hs. 249159	NM_000681.3	NP_000672.3	1-1	1960	116	60	101.00
α_{2B} -AR	Hs. 247686	NM_000682.5	NP_000673.2	1-1	823	117	60	100.05
α_{2C} -AR	Hs. 123022	NM_000683.3	NP_000674.2	1-1	646	93	60	99.08
β_1 -AR	Hs. 99913	NM_000684.2	NP_000675.1	1-1	863	79	60	99.00
β_2 -AR	Hs. 2551	NM_000024.5	NP_000015.1	1-1	778	65	60	100.02
β_3 -AR	Hs. 2549	NM_000025.2	NP_000016.1	1-2	1401	65	60	99.87
18S rRNA	X03205.1	N.A.	N.A.	N.A.	N.A.	187	60	98.80

Linearity of real-time PCR assays were tested by constructing standard curves by use of serial 2-fold dilutions of a standard calibrator cDNA and regression coefficients (r^2) were always >0.900 (data not shown). Relative expression was determined by normalization to 18S rRNA (housekeeping gene) by means of AB Prism 7000 SDS softwareTM. Gene expression levels in a given sample were represented as $2^{-\Delta Ct}$ where $\Delta Ct = [Ct(\text{gene}) - Ct(18S\ rRNA)]$.

Table 3. Pilot study on peripheral blood mononuclear cells adrenoceptor mRNA expression

	Central obesity		Ratio Yes/No
	No	Yes	
n	5	10	
F/M	2/3	8/2	
Age	31 \pm 3	40 \pm 3	
BMI	21.1 \pm 0.7	30.1 \pm 2.4	
AR subtype			
α_{1A}	6.95 $\times 10^{-9} \pm 1.45 \times 10^{-9}$	6.87 $\times 10^{-9} \pm 4.63 \times 10^{-10}$	0.99
α_{1B}	1.11 $\times 10^{-7} \pm 2.02 \times 10^{-8}$	1.56 $\times 10^{-7} \pm 3.66 \times 10^{-8}$	1.41
α_{1D}	7.76 $\times 10^{-6} \pm 2.09 \times 10^{-6}$	1.1 $\times 10^{-5} \pm 2.21 \times 10^{-6}$	1.42
α_{2A}	3.72 $\times 10^{-9} \pm 2.89 \times 10^{-9}$	5.74 $\times 10^{-9} \pm 1.47 \times 10^{-9}$	1.54
α_{2B}	not detected	not detected	
α_{2C}	4.39 $\times 10^{-9} \pm 6.76 \times 10^{-10}$	4.57 $\times 10^{-9} \pm 3.69 \times 10^{-10}$	1.04
β_1	not detected	not detected	
β_2	7.54 $\times 10^{-5} \pm 5.98 \times 10^{-5}$	3.14 $\times 10^{-5} \pm 1.84 \times 10^{-5}$	0.42
β_3	2.56 $\times 10^{-8} \pm 5.51 \times 10^{-9}$	2.13 $\times 10^{-8} \pm 5.51 \times 10^{-9}$	0.83

Analysis of AR mRNA expression in peripheral blood mononuclear cells in a subgroup of subjects without and with central obesity selected as those having highest or lowest BMI values. Data are presented as mean \pm standard error of the mean (SEM). F/M, female male ratio; BMI, body mass index; AR, adrenoceptor. For details see Methods.

We performed a pilot study of all AR subtypes expression in 15 individuals divided in two groups: with and without CO, representing the lowest and the highest BMI of the total sample in order to know which AR subtype(s) showed the highest differences between the opposite fat groups (in Table 3). The ratio (R) was calculated between AR mRNA expression between individuals with and without CO. R <0.5 was considered under and >2.0 over expression. Our technical variance in that set of experiments is 0.03 cycles² for all adrenoceptor gene expression (so the

standard deviation, SD is 0.17 cycles); also consider that we measure samples in triplicates, so standard error (SE) for the mean ct is $SE = \sqrt{0.03/3} = \sqrt{0.01}$. By error propagation, the SE of a mean difference (Δct) is $\sqrt{2 \times 0.01}$ and the SE for a difference of such differences ($\Delta \Delta ct$) is $\sqrt{2 \times 2 \times 0.01} = \sqrt{0.04} = 0.2$. This SE is determined on $2 \times (3-1) = 4$ degrees of freedom, so the 95% confidence interval has a half width of $t[0.025;4] \times 0.2 = 2.77 \times 0.2 = 0.554$ or roughly half cycle. The total width of the interval is thus 1 cycle. This means that a 2-fold difference ($\Delta ct=1$) is considerably larger than the 95% CI obtained without any biological variance when 3 replicates are measured. For this reason, only differences exceeding 1 cycle were thereafter considered.

Statistical analysis

The modified Kolmogorov-Smirnov test with the correction of Lilliefors was used to evaluate the fit of the data to a normal distribution. Unless otherwise indicated, variables were presented using relative and absolute frequencies, means \pm standard error of the mean (SEM). Non-normal distributed data was presented as median, 25th and 75th percentiles. To compare the quantitative independent variables, we used bivariate statistical analysis ANOVA or non-parametric tests Mann-Whitney (comparison between 2 groups) or Kruskal-Wallis (comparison of more than 2 groups) tests for normal and non-normal distributed data, respectively. The Pearson Chi-Square test was used to compare qualitative independent variables. Correlations were assessed by Pearson test to determine the relationship between normal distributed quantitative variables and by non-parametric Spearman rank analysis for non-normal distributed quantitative data. The strength of association between variables was estimated by odds ratio (OR) and their respective confidence interval of 95% (95% CI) using multiple logistic regression. Variables that in the univariate analysis showed statistical significance below 10% ($p < 0.10$) were included in the logistic regression model.

Data analysis was performed using the SPSS version 22.0 (SPSS, Chicago, IL, USA). P-value lower than 0.05 was considered statistically significant.

Table 4. Comparison of anthropometric, metabolic/endocrine parameters between groups with and without central obesity (n=57)

Factor	F	df	Mean \pm S.E.M.		p
			CO	No CO	
F/M (n)	8.596	1, 55	23/19*	2/13*	0.006
Age (years)	8.640	1, 55	42 \pm 2	37 \pm 3	0.194
Weight (Kg)	3.077	1, 55	79 \pm 2	72 \pm 3	0.085
Height (m)	5.699	1, 55	1.65 \pm 0.01	1.71 \pm 0.01	0.020
BMI (Kg/m ²)	14.126	1, 55	28.7 \pm 0.6	24.4 \pm 0.7	<0.001
WC (m)	26.869	1, 55	1.00 \pm 0.016	0.86 \pm 0.016	<0.001
SBP (mmHg)	0.000	1, 55	134 \pm 2	134 \pm 4	0.983
DBP (mmHg)	2.606	1, 55	82 \pm 2	77 \pm 2	0.112
Glycemia (mg/dL)	0.114	1, 55	85 \pm 1	86 \pm 1	0.737
HgA1c (%)	1.386	1, 55	5.1 \pm 0.1	5.2 \pm 0	0.244
TC (mg/dL)	4.131	1, 55	200 \pm 5	178 \pm 9	0.047
LDL-C (mg/dL)	2.420	1, 55	127 \pm 5	112 \pm 8	0.126
HDL-C (mg/dL)	0.065	1, 55	50 \pm 2	51 \pm 4	0.800
VLDL-C (mg/dL)	4.596	1, 55	23 \pm 2	16 \pm 2	0.036
TAG (mg/dL)	3.726	1, 55	120 \pm 12	79 \pm 8	0.059
NA (pmol/L)	2.136	1, 55	1054 \pm 129	706 \pm 168	0.150
AD (pmol/L)	0.198	1, 55	167 \pm 15	181 \pm 30	0.658
Cortisol (μ g/dL)	0.024	1, 55	15 \pm 0.9	15 \pm 1.0	0.877
Leptin (ng/mL)	8.116	1, 38	1.30 \pm 0.25	0.15 \pm 0.05	0.007

CO, central obesity; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; TAG, triacylglycerol; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; NA, noradrenaline; AD, adrenaline. F/M, female/male ratio; F, Snedcor's distribution; df, degrees of freedom; p, level of significance.

*Pearson Chi-Square test was applied for the comparison of two categorical variables. Data are presented as mean \pm standard error of the mean (SEM).

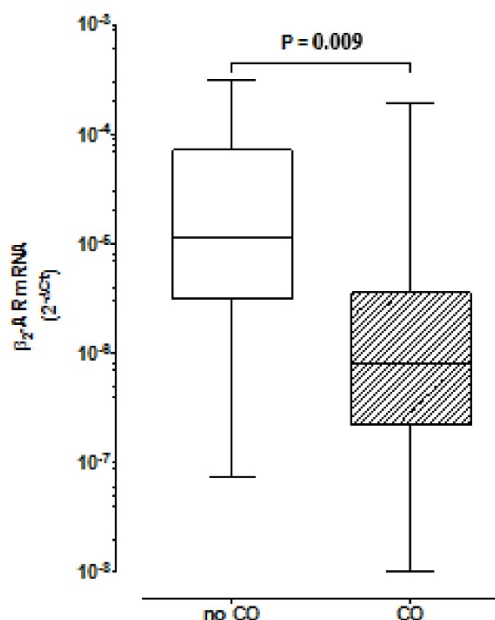


Figure 1. Comparison of β_2 Adrenoceptors expression in peripheral blood mononuclear cells between groups with and without central obesity. Boxes indicate medians with 25th–75th percentiles and whiskers indicate minimum and maximum values. Mann-Whitney test was used for comparison between the two groups. CO, Central Obesity; non-CO, without CO; P, level of significance.

Results

Characteristics of the study participants

The BD, with mean age of 40 years (minimum 20 and maximum 63), showed a prevalence of CO of 71.9%. Twenty-five individuals were female (44%) with a higher percentage of CO compared with men, respectively 92 % vs 59% ($p=0.006$). The total group presented a median hsCRP level, reflecting an intermediate cardiovascular risk, low median plasma leptin levels and mean SBP values of systolic hypertension. The other metabolic/endocrine parameters were within the normal range (Table 1).

Anthropometric and metabolic/endocrine parameters

The CO group showed higher leptin, TC and VLDL-C values in comparison with the group without CO (Table 4). WC was correlated with leptin ($r=0.524$; $p=0.001$), VLDL-C ($r=0.391$, $p=0.003$) and TAG ($r=0.319$, $p=0.016$) plasma levels. In CO, as well in all population, WC was correlated with SBP [$(r=0.495$, $p=0.001)$; ($r=0.385$, $p=0.003$), respectively] and with DBP [$(r=0.477$, $p=0.001)$; ($r=0.493$, $p<0.001$), respectively]. In both total and CO groups, AD was associated with VLDL-C [$(r=0.336$, $p=0.011)$; ($r=0.455$, $p=0.002$), respectively] and with TAG plasma levels [$(r=0.323$, $p=0.014)$; ($r=0.428$, $p=0.005$), respectively], and in CO was found to be inversely correlated with HDL-C ($r=-0.346$; $p=0.025$). In both total and CO groups, we also found significant correlations between NA and TC [$(r=0.277$, $p=0.037)$; ($r=0.344$, $p=0.026$), respectively] and with LDL-C [$(r=0.301$, $p=0.023)$; ($r=0.354$, $p=0.022$), respectively].

Monocytes subsets and hsCRP

The total sample showed a mean of 440 ± 22 monocytes/ μ L ($7.1 \pm 0.4\%$ of total leucocytes), of which 390 ± 19 cells/ μ L ($89 \pm 0.9\%$ of all monocytes) were CD16- and 50 ± 7 cells/ μ L were CD16+ monocytes ($11 \pm 0.9\%$ of all monocytes). Neither the number (Table 5) nor the percentage of CD16+ ($11.5 \pm 0.9\%$ vs $9.9 \pm 1.9\%$; $p=0.410$) and of CD16- monocytes ($89 \pm 0.9\%$ vs $90 \pm 1.9\%$; $p=0.408$) were different between groups with and without CO, respectively. However, the ratio between non-classical CD16+ and classical CD16- monocytes, calculated to overcome inter-individual variations, showed differences between these two groups. In particular, centrally obese showed lower CD14 and SSC ratios comparatively to non-CO subjects, reflecting a more inflammatory phenotype pattern of non-classical monocytes (Table 5). Despite hsCRP plasma levels were similar between CO and non-CO groups, in centrally obese hsCRP was correlated with leptin values ($r=0.397$, $p=0.011$). In addition, plasma

levels of leptin were significantly different when comparing the hsCRP cardiovascular risk groups ($p=0.011$): high risk group [3.100 (0.800-5.170) mg/L] showed higher levels of leptin relatively to the intermediate [0.615 (0.480- 1.140) mg/L] and to the low risk groups [0.320 (0.100- 0.850) mg/L]. The number of CD16+ monocytes was correlated with hsCRP ($r=0.372$; $p=0.005$) and with NA plasmatic level ($r=0.341$; $p=0.01$).

AR expression in PBMCs

The pilot study considered PBMCs expression of all the 9 AR in a subgroup of subjects with and without CO selected as those having the highest or the lowest BMI values (Table 3). Results from the pilot study revealed that there were no differences between groups except for β_2 AR mRNA levels. Indeed, the expression of β_2 AR in subjects with CO was less than half in comparison to those without CO. This finding led us to study β_2 AR expression in a large number of subjects.

Table 5. Comparison of inflammatory markers hsCRP and monocyte subsets (counting and phenotype) between groups with and without central obesity (n=57)

				No CO	CO	
		F	df	Mean \pm SEM/ *	Mean \pm SEM/ *	p
hsCRP*	(mg/L)	-	-	0.757 (0.469-2.090)	1.530 (0.764-3.560)	0.160
Monocytes subsets						
cells/ μ L	CD16+	0.055	1, 54	53 \pm 18	49 \pm 6	0.816
	CD16-	1.747	1, 54	431 \pm 38	375 \pm 21	0.192
	Ratio	0.404	1, 54	0.12 \pm 0.03	0.13 \pm 0.01	0.528
FSC	CD16+	0.227	1, 54	547 \pm 17	556 \pm 10	0.636
	CD16-	0.080	1, 54	549 \pm 17	554 \pm 10	0.778
	Ratio	0.833	1, 54	1 \pm 0.01	1 \pm 0.01	0.365
SSC	CD16+	3.998	1, 54	431 \pm 16	400 \pm 8	0.051
	CD16-	0.371	1, 54	485 \pm 13	477 \pm 7	0.545
	Ratio	9.684	1, 54	0.89 \pm 0.01	0.84 \pm 0.01	0.003
CD14	CD16+	0.285	1, 50	968 \pm 167	867 \pm 93	0.596
	CD16-	1.292	1, 53	1865 \pm 326	2423 \pm 264	0.261
	Ratio	8.974	1, 54	0.50 \pm 0.05	0.36 \pm 0.02	0.004
CD36	CD16+	0.108	1, 54	327 \pm 52	313 \pm 18	0.743
	CD16-	2.330	1, 54	670 \pm 68	765 \pm 28	0.133
	Ratio	3.826	1, 54	0.50 \pm 0.04	0.42 \pm 0.02	0.056
CD11b	CD16+	0.000	1, 24	80 \pm 30	81 \pm 18	0.996
	CD16-	0.621	1, 24	125 \pm 48	241 \pm 78	0.438
	Ratio	2.311	1, 24	0.69 \pm 0.07	0.51 \pm 0.06	0.141

CO, central obesity; FSC, forward scatter; SSC, side scatter; Values of CD14, CD36 and CD11b expressed as fluorescence arbitrary units (AU); Ratio SSC, ratio between side scatter (SSC) of CD16+monocytes and SSC of CD16- monocytes in each individual; Ratio CD14, ratio between the expression of CD14 on CD16+monocytes and the expression of CD14 on CD16- monocytes in each individual; Ratio CD11b, ratio between the expression of CD11b on CD16+monocytes and the expression of CD11b on CD16- monocytes in each individual; Data are presented as mean \pm standard error of the mean (SEM); F Snedcor's distribution; df degrees of freedom; p level of significance. * Data presented as median (25th-75th percentiles) and Mann-Whitney test used for comparison between the two groups.

We have found lower expression of β_2 AR in the CO group in comparison to the group without CO ($R=0.08$; $p=0.009$) (Fig. 1). In the CO model, the logistic regression analysis demonstrated a lower association for the development of CO for β_2 AR mRNA expression $\geq 2 \times 10^{-6}$ [OR 0.177 with respective confidence interval of 95% (95% CI) (0.040- 0.796)] and a higher association for women, relatively to men [$\geq 9:1$ [OR 8.972 (95% CI) (1.679-47.949)]].

To evaluate the clinical relevance of decreased expression of β_2 AR, we correlated β_2 AR mRNA levels with metabolic/endocrine parameters. When considering all the individuals, the expression of β_2 AR in PBMCs inversely correlated with VLDL-C ($r=-0.313$; $p=0.018$), TAG ($r=-0.271$; $p=0.041$) and leptin ($r=-0.392$; $p=0.012$), whereas in CO it was correlated with plasmatic HDL-C ($r=0.310$; $p=0.045$). After adjusting for gender, β_2 AR mRNA correlated with HDL-C ($r=0.298$; $p=0.026$) and inversely with VLDL-C ($r=-0.361$; $p=0.006$) and TAG plasmatic levels ($r=-0.311$; $p=0.020$).

Discussion

Our study addressed for the first time the expression of adrenoceptors in PBMCs in inflammatory obesity. The main findings are fourfold. Firstly, PBMCs from CO individuals showed underexpression of β_2 AR in comparison to non-CO subjects. Secondly, CO individuals showed higher TC, VLDL-C and leptin plasma levels and a higher inflammatory pattern of monocytes relatively to non-CO subjects. Thirdly, β_2 AR expression was inversely correlated with a dyslipidaemic lipid profile and with leptin plasma levels. And the fourth is that the multiple logistic regression analysis showed a lower and higher association, respectively for β_2 AR expression ($\geq 2 \times 10^{-6}$) and female for the occurrence of CO.

β_2 ARs are the most expressed AR on immune cells and considered the main mediators of CA immune effects; their activation usually results in anti-inflammatory effects [6, 20, 21]. Indeed, stimulation of β_2 AR modulates cytokine production by activated innate immune cells, primarily inhibiting proinflammatory cytokines, such as TNF- α , IL-12 and IL-6, and by increasing IL-10 and IL-33 release by these cells [22- 24].

The PBMCs underexpression of β_2 AR in CO may have resulted, through mechanisms as desensitization and down-regulation, from the action of circulating or endogenously produced CA, leptin and other cytokines and lipids. Indeed, apart from circulating CA, with similar values in subjects with and without CO, several of these molecules are elevated in this condition [25]. Our findings are in line with a

decreased number of β_2 AR in PBMCs in inflammatory immune mediated diseases such as systemic lupus erythematosus [26], multiple sclerosis [27], rheumatoid arthritis (RA) [26], juvenile RA [28], Crohn's disease [29] and myasthenia gravis [30]. Critical heart disease, a chronic low-intensity inflammation condition, was also associated with reduced β -AR on lymphocytes due to a non-regulated increased release of proinflammatory cytokines [31].

An important point for discussion is whether the altered pattern of β_2 AR and monocytes observed in an early phase of CO is a cause or consequence of inflammatory obesity. Further studies are needed to elucidate this association. Nonetheless, some findings support a role of circulating leptin and lipids in the association between visceral fat and PBMC β_2 AR expression: a) centrally obese present higher plasma levels of leptin, TC and VLDL-C and a more inflammatory pattern of monocytes comparing to those without CO and b) β_2 AR expression inversely correlated with plasma TAG, VLDL-C and leptin and positively with HDL-C plasma levels.

Cellular lipid homeostasis can indeed influence the level and function of immune cells [32]. Remarkably, Devèvre et al. (2015) [33] recently described that HDL-C negatively correlated with molecules involved in chemotaxis, proposing that decreased HDL could therefore be directly linked to changes in monocyte phenotype and function. This is consistent with our findings, since in centrally obese HDL-C levels were positively related with β_2 AR mRNA and inversely with AD. On the other hand, NA, an independent factor for the development of metabolic syndrome [34], correlated not only with TC and LDL-C, but also with the number of "pro-inflammatory monocytes" and hsCRP, findings that corroborate a putative role in inflammation [35].

β_2 ARs have been pointed out as a promising target in the pharmacotherapy of Multiple Sclerosis (MS) [36]. In MS patients, β_2 AR expression in lymphocytes increases after treatment with beta-interferon [37]. It would be interesting to see if during weight loss there is an increase of β_2 AR expression in immune cells.

Down regulation of AR-mRNA may also occur as a result of the effects of inflammatory cytokines produced by immune cells in response to fatty acids binding to toll-like receptor 4 (TLR 4) [38].

Leptin, besides its main role in metabolism, is also an immune mediator, promoting the activation, chemotaxis and survival of both innate and adaptive immune cells [39]. As stated before, β_2 AR mRNA inversely correlated with leptin plasma levels, and leptin receptors, found in monocytes and lymphocytes [40] mediate the production of

proinflammatory cytokines by these cells [39]. Interestingly, a deficiency of leptin receptor has been described as leading to a decreased expression of proinflammatory cytokines as tumour necrosis factor α (TNF- α), interleukin 6 (IL-6) and C-C motif chemokine ligand 2 (CCL2) and decreased infiltration of macrophages [41]. In centrally obese, leptin plasma values were also correlated with the hsCRP, also synthesized by adipocytes [42] and likewise described as able to affect β_2 AR function [43].

As previously highlighted [44], because intra-abdominal fat is not readily available for clinical assessment, and AT-infiltrating immune cells are originated from bone marrow, circulatory immune cells could serve as markers of intra-abdominal fat inflammation and ultimately of obesity associated cardio-metabolic risk. The present work identifies one more molecular marker of obesity subphenotypes and contributes to the continued search necessary for their better definition.

β_2 AR underexpression could be considered as a molecular signature of PBMC in obese patients. Indeed, modified mRNA expression of several genes involved in cytokines production, chemotaxis, fatty acid storage and glucose metabolism and pathogen recognition, was already related to monocyte function in obesity [33].

Our hypothesis is that proinflammatory monocytes (here also characterized by low cellular complexity (low SSC) and CD14 ratios [13] and underexpressing β_2 AR) of centrally obese would sense metabolic/inflammatory circulating factors. In response, these cells secrete more inflammatory cytokines and are probable more prone to migrate into AT where they could differentiate into macrophages. Both processes perpetuate a vicious cycle of inflammatory cell recruitment and secretion of deleterious adipokines and free fatty acids by AT that predispose to metabolic dysfunction.

The current study has some limitations that merit comment. β_2 AR expression was evaluated in PBMCs as a population. In future studies, it would be valuable to investigate possible differential expression on distinct mononuclear cell subsets (e.g. lymphocytes vs monocytes, T helper (TH) 1 vs Th2, regulatory T cells vs Th17). Furthermore, we only studied mRNA levels of β_2 AR and it would be relevant to also measure its protein level. Even though, Guereshi et al. (2013) [45] found that β_2 AR mRNA levels were higher in naive T cells than in Treg cells and protein expression confirmed the results for β_2 AR transcripts. Our group also showed in human lymphocytes that dopaminergic receptors responsiveness is better predicted by mRNA rather than membrane receptor expression [46].

Conclusion

Association of CO with a higher activation of innate immune response and a lower β 2-adrenoceptor expression suggests that circulating peripheral mononuclear immune cells sense inflammatory obesity, with β 2AR expression being less associated with the occurrence of CO.

An important challenge now is to understand how this receptor functions on PBMCs in inflammation related to obesity before we can truly apply this knowledge in a rational manner in clinical conditions. In this sense, evaluation of β 2-AR agonists as potential anti-inflammatory drugs is strongly warranted. Functional studies should be also planned in the near future to determine the migratory and inflammatory functions of these circulatory cells.

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Ethics approval and consent to participate

This work was approved by the Ethical Committee of Centro Hospitalar do Porto (Porto, Portugal). All participants signed their written informed consent as described in the study protocol approved by the Ethics committee. The clinical study is registered at local research department with the identifier 072/09 (047-DEFI/065-CES).

Competing interest

The authors declare that they have no competing interests.

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