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Expression of catecholaminergic receptors in peripheral immune cells in human subclinical inflammatory obesity

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“If we knew what it was we were doing, it would not be called research, would it?”
“The important thing is to not stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day”. *In* "Old Man's Advice to Youth: Never Lose a Holy Curiosity” LIFE Magazine (2 May 1955) pg. 64.

Albert Einstein

I never stopped studying since I was 5 and one day my sister asked me: why not doing a PhD? I like questioning, collecting data, writing abstracts at light velocity and presenting the results. “Is not enough?” I said OK. I felt crazy and foolish because I knew my big deal: not able to focus!
A couple of days after having applied for Metabolism Doctoral Program at the Faculty of Medicine of the University of Porto (where I graduated) I received a telephone call at home from Professor Isabel Azevedo. She asked why I wanted to study metabolism if my background was related to hematology, immunology. At that time, I could not exactly answer but now I believe that this question is answered in this thesis.
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“Stay hungry...Stay foolish”

“Because the people who are crazy enough to think they can change the world are the ones who do.”

-Steve Jobs

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I declare that I have contributed for the investigation herein described to this thesis by collection of part of the data and by handling the data collected except for the real-time PCR experiments performed by the Center of Research in Medical Pharmacology, University of Insubria, and the flow cytometry analysis executed by Cytometry Laboratory of Clinical Haematology Department of Centro Hospitalar of Porto. I also participated in the conceptualization of the research questions, performed statistical analysis and wrote the drafts of all manuscripts included.

No part of this work has been submitted as application for other degree or qualification of this or any other university or institution of learning.

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Abstract

Obesity, mainly central obesity (CO), is an inflammatory condition and represents a major threat to human health and lifespan. Assessment of health risk associated with excess adiposity requires, for one hand, an accurate measurement of obesity and for another hand identification of predictors of subclinical inflammatory obesity, which can be important tools for early therapeutic interventions in obesity-related comorbidities.

The immunological-metabolic crosstalk regulates metabolism and immunity. It is not clear at present which neuroendocrine regulators or metabolic factors affect specifically the immune system in obesity. Sympathetic nervous system overactivity and a state of chronic immune activation are characteristics of obesity-related comorbidities. Catecholamines (CA) regulate both metabolism and immune response through activation of adrenergic (AR) and dopaminergic receptors (DR). Whether peripheral catecholaminergic system is involved in the low-grade inflammation linked to obesity is unknown. The migration of peripheral blood mononuclear cells (PBMC) towards sites of inflammation such as the adipose tissue is crucial to the development of sustained tissue inflammation. Circulating PBMC are exposed to systemic factors, such as dyslipidemia and inflammatory molecules and are directly involved in the low-grade chronic inflammation related to obesity. The expression of both AR and DR in immune cells under an obesogenic state is unknown.

Herein, we aimed to identify subclinical inflammatory obesity predictors hypothesizing that body mass index (BMI) is less sensitive for early metabolic markers and inflammatory changes than waist circumference (WC) obesity-defined. We studied the catecholaminergic system in peripheral immune cells in inflammatory obesity, hypothesizing different expression pattern of catecholaminergic receptors with different metabolic outcomes

associations. For those purposes, a low-cardiovascular risk population was studied concerning anthropometric, metabolic, endocrine and inflammatory parameters. With a flow cytometry approach, we studied the signature of monocytes subsets. The expression of catecholaminergic receptors and tyrosine hydroxylase (TH), the rate-limiting step in CA synthesis, was assessed by real-time PCR in PBMC. Our study has the novelty of identification and prediction of subclinical inflammatory obesity contributing to: i) predict CO in more than one third with a model that includes the determination of plasma levels of leptin and very low-density lipoprotein-cholesterol (VLDL-C) and CD14 expression in monocytes subsets; ii) suggest that in subclinical inflammatory obesity, dyslipidemia and leptin induce monocytes maturation process rather than an independent arise of CD16+ monocytes; iii) suggest CA immunomodulation in obesity; iv) explain the stronger association of CO measures with cardiometabolic risk in comparison with general obesity assessed by BMI and v) recommend, in a public health setting, the implementation of WC as a priority tool for inflammatory obesity screening in low cardiovascular risk population. Focused on the expression of AR in immune cells, we performed a pilot study considering PBMC expression of all the 9 AR (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3) in a subgroup of subjects with and without CO. The preliminary results revealed that there were no differences between groups except for β_2AR mRNA levels. This finding led us to study β_2AR expression in whole population showing that β_2AR in PBMC were under expressed in CO individuals in comparison to no-CO subjects. The multiple logistic regression analysis showed a lower and higher association, respectively for β_2AR expression and female gender for the occurrence of CO. PBMC from subjects with CO showed a reduction of the expression of DR D2, DR D4 and DR D5 as well as lower levels of TH mRNA in comparison to cells from the non-obese group. Logistic regression analysis revealed that DR

D_2 expression may have a protective role for the existence of CO. β_2AR expression inversely correlated with plasma triacylglycerol (TAG), VLDL-C and leptin and positively with HDL-C plasma levels. CO individuals showed higher TC, VLDL–C and leptin plasma levels and a higher inflammatory pattern of monocytes relatively to no-CO subjects. AD and NA plasma levels were associated with dyslipidemia. In addition, our work suggests an inflammatory role for NA supported by the association of its plasma values with "pro-inflammatory monocytes" number and with hsCRP levels. DR D2 and DR D5 expression in PBMC strongly correlates with lower weight, BMI and WC, lower plasma levels of leptin and with a lower inflammatory pattern, while DR D4 mRNA correlates with less HbA1c and TH mRNA correlates with lower WC and leptin levels.

In conclusion, we identified predictors of subclinical inflammatory obesity, the expression of catecholaminergic receptors in PBMC has a different pattern in inflammatory obesity and associate with a better metabolic profile and with a lower inflammatory state.

The findings of a predictor model and the protective role of β2AR and DR D2 expression in peripheral immune cells for inflammatory obesity are expected to impact, first, on early diagnosis with implementation of WC measurement at primary care settings to define obesity and secondly, on therapeutic interventions in inflammatory obesity.

Resumo

A obesidade, sobretudo a obesidade central (OC), é uma condição inflamatória e representa uma ameaça para a saúde e longevidade humanas. A avaliação do risco para a saúde associado ao excesso de adiposidade exige, por um lado, uma medição precisa e, por outro lado, a identificação de preditores da obesidade inflamatória subclínica. Estes podem representar ferramentas importantes para as intervenções terapêuticas precoces nas co-morbilidades da obesidade.

A interligação entre metabolismo e imunidade regula a resposta metabólica e a resposta imunológica. Contudo, ainda são desconhecidos os reguladores neuroendócrinos ou os fatores metabólicos que afetam especificamente o sistema imunológico na obesidade. A obesidade e as suas co-morbilidades caracterizam-se por apresentarem aumento da atividade do Sistema Nervoso Simpático e ativação crónica do sistema imunitário. As catecolaminas (CA) regulam o metabolismo e a resposta imunológica através da sua ligação aos recetores adrenérgicos (RA) e dopaminérgicos (RD). Desconhece-se se o sistema catecolaminérgico periférico está envolvido na inflamação crónica de baixo grau associada à obesidade. A migração das células mononucleares do sangue periférico (PBMC) para os locais de inflamação, como o tecido adiposo, é crucial para o aparecimento e cronicidade da inflamação associada à obesidade. As PBMC, expostas a fatores sistémicos como a dislipidemia e moléculas inflamatórias, estão diretamente envolvidas na inflamação da obesidade. Porém, a expressão dos RA e RD nas células imunológicas no estado obesogénico é desconhecida.

Assim, o objetivo inicial foi a identificação de preditores da obesidade inflamatória subclínica, colocando a hipótese de que o perímetro abdominal (PA) é mais sensível que o

índice de massa corporal (IMC) para marcadores metabólicos e alterações inflamatórias das PBMC na obesidade. Estudámos o sistema catecolaminérgico nas células imunitárias periféricas na obesidade inflamatória, tendo como hipótese a existência de um padrão diferente de expressão dos recetores das CA com associação a diferentes consequências metabólicas.

Com esta finalidade, uma população de baixo risco cardiovascular foi estudada relativamente a parâmetros antropométricos, metabólicos, endócrinos e inflamatórios. Por citometria de fluxo analisámos a assinatura imunofenotípica das subpopulações de monócitos. A expressão dos recetores catecolaminérgicos e da hidroxilase de tirosina (TH), a enzima limitante da taxa de síntese das CA, foi avaliada por PCR em tempo real nas PBMC. O nosso estudo tem a novidade de identificação e predição de obesidade inflamatória subclínica contribuindo para: i) prever OC em mais de um terço com um modelo que inclui a determinação dos níveis plasmáticos de leptina e de *very low-density lipoprotein- colesterol* (VLDL-C) e a expressão de CD14 nas subpopulações de monócitos; ii) sugerir que na obesidade inflamatória subclínica, a dislipidemia e a leptina induzem um processo de maturação nos monócitos em vez da expansão do número de monócitos CD16 +; iii) sugerir imunomodulação pelas CA na obesidade; iv) explicar a maior associação da OC com risco cardiometabólico em comparação com a obesidade geral avaliada pelo IMC e v) recomendar, em saúde pública, a implementação do PA como um instrumento prioritário para o rastreio da obesidade inflamatória nas populações de baixo risco cardiovascular. Tendo em conta a expressão de RA nas células do sistema imunológico, realizamos um estudo piloto nas PBMC relativamente à expressão de todos os 9 RA (α_1A, α_1B, α_1D, α_2A, α_2B, α_2C, β_1, β_2 e β_3) num subgrupo de indivíduos com e sem OC. Os resultados preliminares não revelaram diferenças entre os grupos exceto para os níveis de ARNm dos

RA β_2. Estes dados levaram-nos a estudar a expressão dos RA β_2 em todos os indivíduos mostrando que os RA β_2 estavam sub-expressos nas PBMC nos indivíduos com OC comparativamente aos indivíduos sem OC. A análise de regressão logística múltipla mostrou uma associação menor e maior entre a existência de OC e, respetivamente, a expressão dos RA β_2 e o sexo feminino. As PBMC de indivíduos com OC mostraram uma redução da expressão dos RD D2, RD D4 e RD D5, bem como níveis mais baixos dos transcritos da TH em comparação com as células do grupo sem OC. A análise de regressão logística revelou que a expressão do RD D2 pode ter um papel protetor para a existência da obesidade central. A expressão dos RA β_2 correlacionou-se inversamente com os níveis plasmáticos de triacilglicerol, VLDL-C e leptina e positivamente com os níveis plasmáticos de *high-density lipoprotein- colesterol* (HDL-C). Os indivíduos com OC apresentaram valores plasmáticos mais elevados de colesterol total, VLDL-C e de leptina e monócitos com um padrão mais inflamatório relativamente aos indivíduos sem OC. Os níveis plasmáticos de adrenalina (AD) e de noradrenalina (NA) mostraram associação com a dislipidemia. Além disso, o nosso trabalho sugere um papel inflamatório para a NA suportado pela associação dos seus valores no plasma com o número de "monócitos pró-inflamatórios" e com os níveis de proteína C reativa de alta sensibilidade. A expressão dos RD D2 e RD D5 nas PBMC correlacionam-se fortemente com menor peso, IMC e PA, menores níveis plasmáticos de leptina e com um padrão inflamatório mais baixo. A expressão do RD D4 correlaciona-se com valores mais baixos da hemoglobina glicosilada e o ARNm da TH correlaciona-se com níveis mais baixos do PA e da leptina.

Em conclusão, foram identificados preditores de obesidade inflamatória subclínica, a expressão dos recetores catecolaminérgicos nas PBMC tem um padrão diferente na

obesidade inflamatória e associa-se a um melhor perfil metabólico e a um estado inflamatório mais baixo.

Os resultados de um modelo preditor e o papel protetor da expressão do RA β_2 e do RD D2 nas células imunológicas periféricas para a obesidade inflamatória apresentam potencialmente impacto, em primeiro lugar, no diagnóstico precoce com a implementação da medida do PA para definir obesidade nos cuidados de saúde primários e, em segundo lugar, nas intervenções terapêuticas precoces na obesidade inflamatória.

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List of abbreviations

AD	Adrenaline
ANOVA	Analysis of variance
APC	Antigen presenting cells
AR	Adrenoceptor
AT	Adipose tissue
ATM	Adipose tissue macrophages
AU	Fluorescence arbitrary units
BMI	Body mass index
CA	Catecholamines
cAMP	cyclic adenosine monophosphate
CCL-2	Chemokine (C-C motif) ligand 2 or MCP-1
CD	Cluster of differentiation
CMR	Cardiometabolic risk
CNS	Central Nervous System
CO	Central obesity
CV	Cardiovascular
DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine-β-hydroxylase
DR	Dopamine receptors
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

FFA	Free fatty acids
FSC	Forward light scatter
GPCR	G protein coupled receptor
HDL-C	High-density lipoprotein- cholesterol
HFD	High fat diet
HPLC-EC	High pressure liquid cromatography with electrochemical detection
hsCRP	High-sensitivity C reactive protein
IFN	Interferon
IL	Interleukin
IRS-1-PI3K	Insulin-receptor substrate 1-phosphoinositide 3 kinase
JAK-STAT	Janus kinase-signal transducer and activation of transcription
JNK	c-Jun N-terminal protein kinase
LDL-C	Low-density lipoprotein- cholesterol
LPS	Lipolysaccaharide
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MFI	Median fluorescence intensity
MHO	Metabolically healthy obesity
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NEFA	Non-esterified fatty acids

NET	Noradrenaline transporter
NF-kB	Nuclear factor kB
NLRP	Nucleotide binding domain leucine rich repeat containing inflammasomes
oxLDL	Oxidized LDL
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SNS	Sympathetic Nervous System
SSC	Sideward light scatter
TAG	Triacylglycerol
TC	Total cholesterol
T2D	Type 2 Diabetes Mellitus
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cells
VAT	Visceral adipose tissue
VLDL-C	Very low-density lipoprotein- cholesterol
VMAT	Vesicular monoamine transporters
WC	Waist circumference
WHO	World Health Organization

Chapter 1: Introduction

1.1 Humanity on the fat track

Humanity is getting fat and this trend appears to be continuing (1). We are facing a worldwide obesity epidemic, independently of gender, age, economic status, race or ethnicity that shows little sign of slowing (1). Obesity is associated with an increased risk of insulin resistance, type 2 diabetes (T2D), fatty liver disease, dyslipidemia, neurodegenerative disorders, including dementia, airway disease, certain types of cancers (2) and with premature death (3) (Figure 1). Because the extent of the increase in the obesity prevalence over the last 25 years was not predictable from the obesity prevalence data prior to 1980 (4), the term "obesity epidemic" is used with the meaning of a "rapid spread" instead of as a communicable disease.

Worldwide, the proportion of overweight/obese adults (body mass index, BMI $\geq$ 25 $kg.m^{-2}$) increased between 1980 and 2013 from 28.8% to 36·9% in men, and from 29.8% to 38.0% in women. In developing countries with emerging economies, the rate of increase of childhood overweight and obesity has been more than 30% higher than that of developed countries (5).

In 2014, more than 1.9 billion adults were overweight and of these over 600 million were obese. Overall, about 13% of the world's adult population (11% of men and 15% of women) was obese.

Overweight and obesity are linked to more deaths worldwide than underweight (5). Consequently, with the shift in the population age structure to older demographics, the burden of obesity and its associated comorbidities are expected to shrink the healthy lifespan (6, 7).

The rapidity with which the incidence of obesity has increased worldwide is unlikely explained by genetic changes. Human obesity is due to a mismatch between adaptive biological characteristics of our species and the modern environment, which has changed dramatically from the one that we evolved. In evolution, selective pressures do not favor the development of countermeasures against excess nutrients and energy, but rather select for phenotypes that ensure survival in the face of deficiencies. In a physiological perspective, food intake is regulated by internal mechanisms defending homeostasis. Multiple factors contribute to obesity, including but not limited to genetic, dietary, economic, psychosocial, reproductive, and pharmacological factors. The main negative features of contemporary lifestyle include stress (long-term and continuous, psychological), positive energy balance (excessive energy intake and low physical activity, called "the big two"), low-quality food (both high fat and energy dense, and at the same time poor in micronutrients), and disruption of chronobiology (9). While the cause of this epidemic remains unclear, obesity can be conceived as a failure of total body energy homeostasis. Most energy stores are in the form of lipids stored in adipose tissue, so the homeostatic model define obesity as failure of lipostatic mechanisms, and thus considered as pathology.

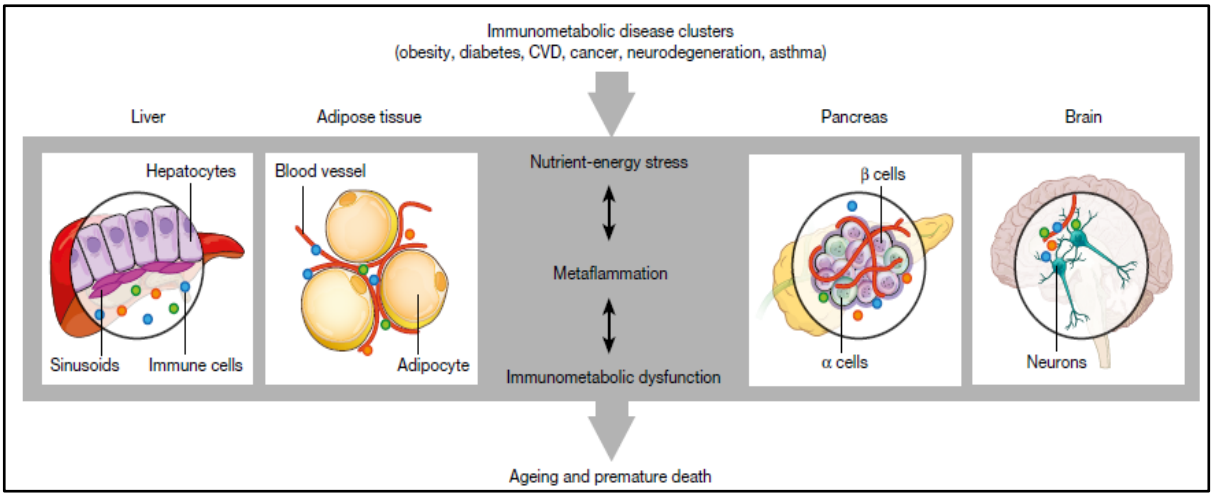


Figure 1 Immunometabolic impact on health. Chronic metabolic inflammation, metaflammation, in multiple organs is implicated in metabolic disease. In metabolic organs including the liver, brain, pancreas and adipose tissue, inflammatory cell action and interactions within the stromal components is an important determinant of maintenance of tissue homeostasis as well as metabolic disease pathogenesis. Owing to their proximity,

immune cells may sense and react to changes in nutrient availability, and in turn influence the intrinsic metabolic action within neighboring cells critical for metabolic homeostasis, for example by altering lipid metabolism or inhibiting glucose uptake. These tissues and mediators produced from them also cause systemic inflammatory responses and disrupt metabolic homeostasis. As a result, immunometabolic diseases often emerge as clusters and promote ageing, disability and premature death. [Copyright with License Number 4073871408191 from Nature Publishing Group of the manuscript Gökhan S. Hotamisligil. *Nature* (2017) 542:177–185].

1.2 Yin and yang of measuring obesity: Disease and obesity paradox

In 2013, the American Medical Association classified obesity as a disease (10). Assessment of health risk associated with excess adiposity requires, for one hand, an accurate measurement of obesity and for another hand the identification of early markers of adult obesity in order to prevent its comorbidities. Finding sensitive tools towards the identification of obesity inflammatory conditions is a challenging assignment.

Traditionally, obesity is defined based on the BMI as equal to or greater than 30 $kg.m^{-2}$ according to the World Health Organization (WHO) (11). Waist circumference (WC) greater than 0.94 m for men and 0.80 m for women (11) in Caucasians, defining central obesity (CO) is an alternative anthropometric index of obesity. Despite the well-documented association between visceral adipose tissue (VAT) and cardiometabolic risk (CMR), there is discordance between BMI and WC driven by the substantial variability in VAT for a given BMI. Waist circumference was associated with VAT, liver fat, and CMR factors (12). Indeed, BMI neither indicates fat distribution nor does distinguish fat mass from fat-free mass, which have opposite associations with health risk (13). In addition, the validity of BMI as a marker of fatness in elderly persons is limited because fat-free mass decreases with aging, even without a change in overall weight (14). In contrast, WC is highly correlated with visceral fat measured by computed tomography (15), and better estimates obesity-associated cardiovascular risk in comparison to BMI (12).

More sensitive indicators than BMI, like body adiposity and its distribution, should be projected on mortality to yield more accurate associations with an increased health risk.

The observation that overweight and even obese patients might be protected against adverse cardiovascular events has generated the term ‘metabolically healthy obesity (MHO)’, to describe the absence of components of the metabolic syndrome (hypertension, dyslipidemia, high fasting glucose levels and T2D) in the presence of an increased BMI (13). However, there are no MHO criteria definition and many authors have questioned the existence and benign nature of such an entity, because MHO definition criteria are not consistent across the literature (14). Most of the studies have incomplete information on key confounding variables, including CO. Obese patients often transit from a healthy to an unhealthy phenotype (15) and studies with follow-up periods longer than 10 years demonstrate no benefit – or even an increased risk for cardiovascular events – compared with metabolically healthy normal weight individuals (16, 17). This paradox underlines the importance of an accurate measurement of obesity and the identification of its early pathological markers in order to promote early therapeutic interventions to prevent comorbidities obesity-associated including premature death.

1.3 Obesity is an inflammatory systemic disease

It is widely accepted that chronic systemic low-grade inflammation is associated with the development of obesity and metabolic dysfunction. Compared with lean tissue, obese adipose tissue (AT) secretes inflammatory cytokines inhibiting insulin signaling (18). This observation contributed to the concept of the link between inflammatory and metabolic responses, and the research field of immune cells- adipocytes crosstalk gave rise to the term “immunometabolism” (19). These studies first demonstrated the common pathways of immune and metabolic systems and the detrimental effects that this crosstalk can have on cellular and systemic metabolism.

The hallmarks of obesity-induced inflammation have been described as different from the Celsius paradigm. In obese state, inflammation is moderate-low-grade and local expression of inflammatory mediators induced by stress sensors triggered by metabolic signal, a nutrient-induced inflammatory response is orchestrated by adipocytes. These metabolic cells create a modified milieu with altered composition of immune cells favoring a proinflammatory tissue environment. This chronic maintenance of the inflammatory state without apparent resolution is, unlike the classic paradigm, associated with a reduced metabolic rate (20). In contrast Asterholm *et al.* (2014) (21) suggest that AT inflammation is an adaptive response that enables safe storage of excess nutrients and contributes to a visceral depot barrier that effectively filters gut-derived endotoxin. Upon high fat diet (HFD) exposure, the expansion of VAT is associated with decreased intestinal barrier function, increased hepatic steatosis and metabolic dysfunction. An impaired local pro-inflammatory response in the adipocyte leads to increased ectopic lipid accumulation, glucose intolerance and systemic inflammation.

The precise temporal sequence of inflammatory cell infiltration into AT is still unclear. Similarly, it is unknown the initiating events that link overnutrition to inflammation.

The systemic effects of obesity are linked to an imbalance in homeostatic and proinflammatory immune responses. Obesity triggers inflammatory pathways in the brain and in AT dysregulating physiological responses that maintain insulin and leptin sensitivity. Independent of obesity, hypothalamic inflammation can impair insulin release from β cells, impair peripheral insulin action and potentiate hypertension. The sympathetic nervous system (SNS) originates most of these effects being able, as well, to induce inflammatory changes in AT in response to neuronal injury (22). Over time, ectopic lipid accumulation in muscle, liver, and blood vessels activates tissue leukocytes, contributes to organ-specific

disease, and exacerbates systemic insulin resistance. Cellular- and cytokine-mediated inflammation in pancreatic islets accelerates the progression toward diabetes. With HFD, metabolites such as diacylglycerols and ceramides accumulate in the hypothalamus and induce leptin and insulin resistance in the central nervous system (CNS) (23, 24). Saturated fatty acids, which activate neuronal c-Jun N-terminal protein kinases (JNK) and nuclear factor-κB (NF-κB) signaling pathways with direct effects on leptin and insulin signaling mediate part of this effect (25).

Visceral adiposity is predictive of T2D, cardiovascular disease and hepatic steatosis, whereas subcutaneous fat increase does not show, clearly, association to a higher risk of these conditions (21). Visceral and subcutaneous AT differ in immune cell composition, particularly macrophages. Increased macrophage accumulation has been demonstrated in AT of humans with obesity and/or with T2D, with significantly more macrophages in visceral fat depot compared to subcutaneous depot (21). Increased macrophage accumulation in VAT may be a key pathologic feature of obesity and its comorbidities mentioned before. Dysfunctional AT is central to obesity and associated disorders. In addition to a matrix of extracellular proteins, adipocytes are surrounded by a variety of cells including immune cells, endothelium, fibroblasts, preadipocytes, and stem cells. Mature lipid-laden adipocytes represent 20%–40% of the cellular content of a fat pad; every gram of AT contains 1–2 million adipocytes but 4–6 million stromal-vascular cells, of which more than half are leukocytes (26). Adipose tissue is an important source of tumor necrosis factor α (TNF-α) and other cytokines, being their production magnified by overnutrition (18, 27). Many of these cytokines are produced by macrophages within the fat pad rather than the adipocytes themselves (28, 29). These macrophages can be observed histologically as ‘‘crown-like structures’’ surrounding adipocytes, particularly in obese visceral fat; their distribution has

been attributed to clustering around dead or dying adipocytes (30). Phenotypically, macrophages exist along a spectrum, the poles of which have been designated M1 (‘‘classically activated’’) and M2 (‘‘alternatively activated’’). M1 macrophages have a proinflammatory phenotype expressing cytokines like TNF-α, interleukin 6 (IL-6), and IL-1β in response to lipopolysaccharide (LPS) and interferon δ (IFN-δ). M2 macrophages, on the other hand, play a role in tissue remodeling and wound healing and secret anti-inflammatory cytokines like IL-10 and IL-1 receptor antagonist. As obesity progresses, more M1 macrophages infiltrate the fat pad, causing insulin resistance (31). Adipose M2 macrophage numbers do not diminish in obesity and may even increase; but there is a major shift in the M1/M2 ratio favoring a proinflammatory state (32).

Virtually all known classes of immune cell have been implicated in setting the inflammatory environment in AT. During obesity, the expansion of effector T cells population and recruitment of AT macrophages is paralleled by an increase in the number of B-2 cells with no change in the frequency of B-1a cells (33, 34). Inflammatory T cells (T helper type 1 cells and cytotoxic CD8+ T cells) contribute to metabolic dysfunction by producing potent M1 macrophage–inducing cytokines, including IFN-δ, TNF-α and IL-6. Lean AT shows considerable enrichment for regulatory T cells (Treg cells) that express the transcription factor Foxp3, and these cells have been suggested to dampen proinflammatory responses and the recruitment of M1 macrophages through the release of IL-10 and transforming growth factor-β (26).

Adipocyte ‘‘stress’’ due to overnutrition has been linked to oxidative stress, endoplasmic reticulum stress, and toll-like receptors (TLRs) activation due to fatty acids and/or bacterial LPS (metabolic endotoxemia) which may be elevated in the serum of obese subjects (35-37). These insults cause upregulation of various chemokines in adipocytes, which recruit immune

cells to the fat pad. These chemokines include monocyte chemotactic protein 1 (MCP-1) among others. A recent study suggests that adipocytes may act as the antigen-presenting cells (APCs) that activate resident T cells shortly after the initiation of HFD (38), though others suggest that AT macrophages (ATM) are the relevant APCs (39).

An intriguing example of adipose-macrophage crosstalk is the newly discovered role of M2 macrophages to promote browning of white AT. Cold exposure was found to polarize macrophages toward the alternatively activated form in an IL-4-dependent manner, leading to the formation and secretion of catecholamines (CA) (40).

As stated before, cells of the immune system chiefly contribute to the persistent state of inflammation responsible for metabolic dysfunction during obesity but the identity of the specific immune receptors that sense metabolic-stress signals and trigger inflammation remain largely unknown. Recruitment of macrophages into AT is an early event in obesity-induced adipose inflammation (31). Tissue macrophages are derived from circulating monocytes, and the infiltration of monocytes into tissues is a complex phenomenon with increased expression of adhesion molecules, transmigration of monocytes across the endothelium, migration along a chemotactic gradient into underlying tissues, and, finally, differentiation of monocytes into tissue macrophages (31).

1.4 Monocyte subsets reveal another link between systemic inflammation and obesity

Monocytes originate in bone marrow and are released into peripheral blood, where they circulate for several days. They are part of the mononuclear-phagocyte system, including macrophages, dendritic cells, and their bone-marrow precursors. Monocytes represent 5–10% of peripheral leucocytes serving as a systemic reservoir of myeloid precursors that are

needed for the renewal of tissue macrophages and dendritic cells. However, they also have other well-documented functions in immune response against infection (41) and in pathogenesis of several inflammatory disorders, including obesity and atherosclerosis (42). Although initially perceived as a homogeneous population, it has become increasingly apparent that monocytes display considerable heterogeneity with respect to their phenotype and function (43, 44).

Two major subsets have been described for monocytes, according to their immunophenotype: classical CD14+CD16- (CD16-) and non-classical, CD14+CD16+ (CD16+) subsets (41). CD16- and CD16+ monocytes are also differentiated by respectively higher and lower expression of both CD36 and CD11b (44). CD16-positive monocytes were first described as a single subpopulation (43) that, compared with CD16-negative monocytes, were smaller, had somewhat diminished expression of CD14, contained fewer cytosolic granules, and exhibited a lower rate of phagocytosis (43). CD16+ monocytes are considered "proinflammatory" cells since they exhibit a macrophage-like phenotype with enhanced antigen-presenting capacities, higher endothelial affinity and production of proinflammatory cytokines (44). Accordingly, CD16+ monocytes expansion has been described in inflammatory states, such as sepsis, rheumatoid arthritis, infections (41) and in patients with a high cardiovascular mortality risk (45, 46).

In humans, over the years, monocytes have been divided into three subtypes based on relative surface expression of LPS co-receptor CD14 and FCγIII receptor CD16 (44) that binds IgG complexed with modified low-density lipoprotein (LDL) cholesterol (47). The most predominant of the three, termed "classical monocytes", do express high levels of CD14 on their surface, are devoid of surface CD16, and account for approximately 80% of the total monocyte population. The remaining 20% express CD16 and they have been further

classified into two subtypes. The more abundant “nonclassical monocytes”, are characterized by very low expression of surface CD14 and high levels of CD16, whereas the third monocyte subtype, called “intermediate monocytes”, express intermediate levels of both the receptors (44). As stated before, CD16+ monocytes are phenotypically heterogeneous, expressing a different set of chemokine receptors and adhesion molecules, and therefore have different homing characteristics and functional properties than CD16-negative monocytes (48). The greater chemokine receptor expression and enhanced in vitro migratory capacity strongly infer that the increase in macrophages in the AT of humans with obesity results, partially, from recruitment of new cells into the tissue. Krinninger *et al*. (2014) (49) demonstrated that monocytes, the circulating precursors of macrophages, from obese women show increased chemokine receptor expression comparatively to lean women and that these differences could promote accumulation of proinflammatory macrophages in AT.

Based on this original classification scheme, CD16-positive monocytes were associated with cardiovascular disease. Patients with coronary artery disease exhibited higher circulating numbers of CD16-positive monocytes than healthy controls (50), and those in the highest quartile for circulating CD16-positive monocytes also had significantly higher serum TNF-α concentrations.

Over the years, several studies, often contradictory, aimed at functional characterization of the monocyte subsets have been undertaken by different research groups (51, 52). Two main reasons have been pointed out: lack of a standardized gating strategy for immunophenotyping by flow cytometry and functional studies have been performed with purified monocytes, contributing to highly variable results depending on method of isolation employed (53). ‘Classical’ monocytes are phagocytic with no inflammatory characteristics,

'non-classical' subtype display 'inflammatory' characteristics on activation and exhibit properties for antigen presentation while 'intermediate' monocytes constitute a very small percentage in circulation and appear to be a minor transitional subset that displays both phagocytic and inflammatory function (53) (Figure 2). Mukherjee *et al*. (2015) (53) demonstrated unequivocally that nonclassical (but not intermediate) monocytes are the primary inflammatory monocytes and further validate by studying patients with acute and chronic inflammatory diseases. However, the temporal relationship of the change in monocyte phenotype with respect to development of obesity remains to be investigated. The pathophysiological significance of monocytes subsets fluctuations both in number and phenotypic pattern in inflammatory diseases is unknown. Moreover, in inflammatory obesity, it is uncertain whether there is an expansion of CD16+ cells or a maturation process from classical to non-classical monocytes and if CA, important immunomodulators (developed in item 1.6), are associated with monocytes characteristics observed in that condition.

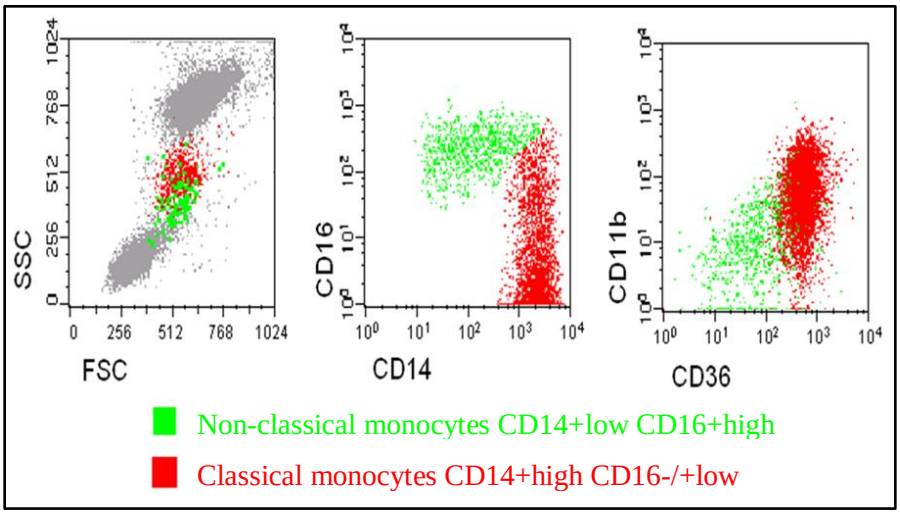


Figure 2 Representative example of monocyte subsets: whole blood protocol; monocyte scatter properties were gated and monocyte subpopulations were defined according to their surface expression pattern of the LPS receptor CD14 and the Fcδ receptor CD16. CD16- and CD16+ monocytes are also differentiated by respectively higher and lower expression of both CD36 and CD11b. Monocytes subsets are a continuum cell population.

1.5 Pathways of adipocytes-immune cells crosstalk

1.5.1 Leptin immunomodulation

The discovery of leptin brought the concept of adipocytes as active endocrine cells, identifying a specific biological cause of obesity (at least in mice), and subsequent target for a rational drug therapy. Though small amounts may be produced by other tissues in specific contexts (54), the main source of leptin comes from adipocytes, and serum levels are associated with fat mass (55). Many factors regulate leptin expression and secretion, including nutrients, steroid and thyroid hormones, and cytokines (54).

The adipokine leptin is the product of the obese gene (*ob*; also, known as *Lep*), regulating feeding behavior through the CNS. Functionally, leptin affects the hypothalamus region of the brain, where it triggers satiety signals, regulates food intake by counter-acting ghrelin, the hunger hormone, but also promotes energy expenditure in peripheral tissues (56). Chronically high leptin levels can lead to leptin resistance and changes in the dynamics of fat storage, glucose metabolism and insulin signaling. However, the leptin receptor is expressed not only in the CNS, but also in peripheral tissues, such as hematopoietic and immune systems (57). Leptin receptor is expressed in human PBMC, namely in monocytes (58), mediating the leptin effect on proliferation and activation. Leptin shares structural similarity with IL-6 and acts on immune cells via the leptin receptor, which belongs to the cytokine receptor family. Stimulation of the leptin receptor activates Janus kinase- Signal Transducer and activation of transcription (JAK–STAT), insulin receptor substrate 1-phosphoinositide 3 kinase (IRS-1-PI3K) and mitogen-activated protein kinases (MAPK) signaling pathways, and it has been suggested that it modulates RNA metabolism (59). Because it shares a similar signal transduction mechanism as cytokines, leptin signaling can promote obesity-associated induction of pro-inflammatory mediators (60). Therefore, the

physiological role of leptin should not be limited to the regulation of food intake and energy expenditure. In addition, leptin stimulates the production of proinflammatory cytokines from cultured monocytes and enhances the production of Th1 type cytokines from stimulated lymphocytes. Moreover, leptin has a trophic effect on monocytes, preventing apoptosis induced by serum deprivation. These signal transduction pathways provide possible mechanisms whereby leptin may modulate activation of PBMC. Moreover, Cannon *et al*. (2014) (61) showed evidence that leptin promotes CD16 expression on human monocytes being a critical link between adiposity and inflammation.

Deficiency of leptin receptor led to decreased AT infiltration of inflammatory macrophages and to decreased expression of pro-inflammatory cytokines in AT. Therefore, leptin can be regarded as a proinflammatory cytokine with major role in the link between the nutritional status and the immune response with importance in the pathophysiology of obesity (62).

Contrary to its pro-inflammatory effects, leptin can also reduce AT inflammation by enabling a leptin-catecholamine signaling axis (63). Over-nutrition triggers leptin-mediated increases in sympathetic nerve activity that stimulate the mobilization of triglycerides from AT. Administration of exogenous leptin increased expression of noradrenaline in AT, which increased cyclic adenosine monophosphate (cAMP) production, ultimately leading to dephosphorization and nuclear translocation of a histone deacetylase that can inhibit NF-κB-mediated inflammation, dephosphorization, nuclear translocation, and association with genes that transcribe proinflammatory cytokines, namely TNFα and IL-12. Increased expression of pro-inflammatory cytokines in macrophages, as well as increased crown-like structure formation in AT contribute to metabolic dysfunction (64).

1.5.2 The nutritional mechanism in the link between immune and metabolic systems

Obesity is a chronic low-grade inflammatory condition promoted by hyperlipidemia involving cells of the innate and adaptive immune system. Moreover, the immune system affects lipids and lipoprotein levels and cellular lipid homeostasis can influence the level and function of immune cells (65).

Lipoproteins provide hydrophilic properties to lipids, allowing them to be transported within aqueous environments inside and outside of cells. Some well-studied lipoproteins include Apolipoprotein (Apo) A and E that bind lipids reversibly to form high density lipoprotein (HDL) and Apo B that binds lipids irreversibly to form LDL. One of the main functions of HDL is to promote cholesterol efflux from cells. As such, decreased HDL levels are indicative of increased atherosclerosis and cardiovascular events (64).

The fat molecule transporter HDL has anti-inflammatory properties including decreasing expression of adhesion molecules, TNF and chemokine (C-C motif) ligand (CCL2) in endothelial cells. LDL is also a fat molecule transporter; it differs from HDL in that it contains higher proportions of fat molecules. In conditions of oxidative stress, LDL is susceptible to oxidation, and can form aggregates. These oxidized LDL (oxLDL) aggregates form fat droplets that are recognized by scavenger receptors on monocytes/macrophages, such CD36, and lead to macrophage development into foam cells precipitating atherosclerotic events. Moreover, oxLDL, as well as cholesterol, cause dysfunction in macrophage lysosomal activity that contributes to processing of lipids (66).

Considering nutritional mechanisms, adipocytes safely store excess calories during periods of nutritional affluence and release them when there is nutritional deprivation. These calories come in the form of free fatty acids (FFA), which are liberated by lipolysis during fasting and are released into the circulation, where they are utilized by skeletal muscle and

other tissues. By enabling these tissues to switch to a lipid-oxidizing economy during fasting, glucose is spared for the CNS and red blood cells. This is usually achieved through the coordinated action of hormones, CA and insulin being the chief regulators of this balance (65).

Free fatty acids or, also named, non-esterified fatty acids (NEFAs) are chronically elevated in the circulation of obese subjects (67), activating intracellular inflammatory signaling pathways. An increase in concentration of circulating NEFA reflects the inability of the AT to buffer the excess nutrient intake and is related to the dyslipidemic state that characterizes obesity. When overload becomes present, the liver increases the production of apo-B containing particles that carry triacylglycerol to the AT resulting in low-density lipoprotein (LDL) formation (68). This occurs in VAT very efficiently and this depot is also more capable of releasing lipids in times of requirement. When the capacity of storing fat in AT is overwhelmed, the conversion of very low-density lipoprotein (VLDL) is delayed originating hypertriglyceridemia (69). Individuals with insulin resistance have increased secretion rates of VLDL-apoB and triglycerides. In obesity, the main regulators of abnormal apoB and triglyceride kinetics are visceral fat, hepatic fat, insulin resistance and plasma adiponectin. An increased amount of peritoneal fat results in an alteration of portal FFA flux, an altered pattern of adipocytokines and a disturbed ratio of proinflammatory to anti-inflammatory processes. Free fatty acids, adipocytokines and inflammation affect each other, being intimately linked. Although each factor may increase VLDL secretion by itself, it is the altered flow of FFA that probably has the greatest impact on driving hepatic VLDL overproduction (68). Furthermore, not fat store tissues such as liver, muscle and pancreas accumulate lipids (69), and the resulting lipotoxicity may induce insulin resistance.

Though most of the lipid stored in adipocytes comes from the diet, the fat cell is fully capable of synthesizing new lipids from carbohydrates using *de novo* lipogenesis. Lipolysis is the process that is required for fatty acids to be liberated from triglyceride so that they can be oxidized locally or by other organs. Classically, lipolysis is accepted as being driven by β-adrenergic signaling in the adipocyte, but other inducers (such as TNF-α) exist and may have physiological relevance (70).

Adipocytes are involved in innate immunity, expressing toll-like receptors (TLRs) in these cells. The two most widely studied TLRs are TLR2 and TLR4, which are activated by bacterial lipoproteins and by LPS, respectively. The engagement of either receptor leads to translocation of nuclear factor κB (NFκB) to the nucleus. In addition to their early recognized role in immunity, a participation in the regulation of metabolism is also being attributed to TLRs (70). Saturated fatty acids activate both TLR2 and TLR4 and, instead, unsaturated fatty acids inhibit TLR-mediated signaling and gene expression. Activation of TLRs results in synthesis of proinflammatory factors such as TNF-α, IL-6, and chemokines (74).

Lipid species appear to regulate inflammatory signaling in macrophages through non-TLR pathways as well. Overexpression of diacylglycerol transferase-1 (DGAT-1, which catalyzes the final stage in triglyceride synthesis) in macrophages protects against adipose macrophage infiltration, inflammation and insulin resistance (71). Diacylglycerols have been implicated in insulin resistance in liver and muscle (71). In addition, ceramides have recently been shown to activate the nucleotide-binding domain, leucine-rich-containing family, and pyrin-domain-containing (NLRP) inflammasome (72). NLRP activation ultimately leads to IL-1β and IL-18 secretion, a response that is generally stimulated by "danger signals" of non-microbial origin. Omega-3 fatty acids have anti-inflammatory and

antidiabetic effects in humans and mice. Activation of the omega-3 fatty acid receptor, G protein coupled receptor 120 (GPCR120), on macrophages and adipocytes reverses adipose inflammation and insulin resistance in obese mice (73).

1.5.3 Catecholaminergic regulation of metabolism and Obesity

The CA, adrenaline (AD) and noradrenaline (NA) are considered the major regulators of lipolysis (74) affecting differentiation and proliferation of adipocytes. Impairment of CA-induced lipolysis in adipocytes is thought to contribute to the development of obesity characterized by adrenergic over activity (74, 75).

In terms of metabolism, the SNS is fundamental in controlling daily energy expenditure via the regulation of resting metabolic rate and initiation of thermogenesis in response to physiological stimuli, such as, changing energy states, food intake, carbohydrate consumption, hyperinsulinemia, and exposure to cold. Sympathetic tone is an important mediator of cardiovascular function predominantly through its direct effects on β-adrenergic receptors in the heart to modulate cardiac output and on α-adrenergic receptors (α-AR) in blood vessels to modulate arteriolar resistance and venous capacitance. Activation of sympathetic nerves innervating the liver, pancreas, skeletal muscle, and AT can also elicit acute catabolic responses (i.e., glycogenolysis and lipolysis).

Individuals with CO demonstrate augmented sympathetic outflow when compared to non-central adiposity phenotype (76). Compared to healthy lean individuals, obese adults have elevated rates of whole-body NA spillover in plasma (77). Interestingly, the degree of sympathetic overactivity in obese individuals appears to be dependent on body fat distribution with CO shown to be associated with greater sympathetic nerve activation than subcutaneous obesity (77, 78). The underlying cause of the observed SNS overdrive in

obesity is not fully understood, but is likely multifactorial and starts as part of a homeostatic mechanism aimed at expending the excess energy as heat rather than additional storage of fat. SNS activation is seen in humans following overfeeding and is decreased with fasting (79). The chronic increase in basal sympathetic activity is presumably an adaptive physiological response aimed to stimulate β–adrenergic thermogenesis in order to prevent further fat storage (77), but can also stimulate lipolysis to increase NEFAs, contributing to insulin resistance. The catecholamine-producing monocytes/macrophages that infiltrate white AT induce the development of thermogenic beige AT (40, 80). This previously unrecognized function of macrophages in instructing beige AT development and subsequent energy expenditure has significant implications for the role of these cells in metabolic disorders.

The AT through the release of various adipokines may directly or indirectly increase sympathetic activity. A chronically elevated SNS outflow could in turn impair β–adrenergic signaling, reduce stimulation of metabolism, and evolve into a cycle that contributes to obesity, insulin resistance and morphologic changes in target tissues.

Compared to the liver, pancreas, and skeletal muscle, which are also under parasympathetic control, AT, as stated before, is mostly innervated by sympathetic nerves (81) making it an important regulator of lipid mobilization. Central sympathetic outflow directly stimulates adipocyte lipolysis by binding to β-adrenergic receptors in white AT to activate cAMP-dependent pathways to translocate inactive lipase. Equally, when α-AR are activated, lipolysis is inhibited.

Central obesity is characterized by hyperinsulinemia, increased leptin and NEFAs release from excess visceral fat and reduced baroreceptor sensitivity and activation of the hypothalamic-pituitary-adrenal axis.

Downregulation of β-adrenoceptors leading to a blunted thermogenic response to food can potentiate insulin resistance and perpetuate the negative feedback cycle between insulin governing sympathetic outflows. Evidence in support of weight gain being directly related to decreased β-adrenergic responsiveness comes from studies that show that both short-term (82) and chronic (83, 84) pharmacological blockade of beta-receptors leads to an increase in body weight.

Besides the central action of DA on the nervous system as a canonical neurotransmitter regulating cognition, motor control, mood, and reward systems, peripheral DA originated from other sources namely and importantly from the immune system, suggests a key role on neural-immune interactions (see item 1.6). Because DA does not cross the blood-brain barrier dopaminergic signaling in brain is functionally distinct from peripheral pathways. In the basal ganglia, DA is involved in the signaling of the rewarding effects secondary to food intake. In peripheral tissues, DA regulates pancreatic endocrine function including insulin release and also modulates the effects of insulin action on adipocytes. Indeed, peripheral DA can originate from at least three sources, i.e. neuronal fibers, adrenal medulla, and neuroendocrine cells found in kidney, pancreas and leukocytes with a role in regulation of blood pressure, sodium balance, adrenal and renal functions, as well as glucose homeostasis and body weight (85).

Changes in plasma DA levels are predominantly determined by the activity of sympathetic nerves, which may release the precursor DA along with NA into the parenchyma of target organs. Some DA may be released directly into the circulation from catecholamine synthesizing cells of the adrenal medulla, chromaffin cells, similarly to NA and AD.

Based on the distribution of TH immunoreactivity in gastric mucosa, many epithelial cells in the human stomach (including parietal cells), are capable of producing DA. Lower down in

the digestive tract, TH-positive epithelial cells were not detected; however, numerous lamina propria cells were positive for TH. This suggests that immune cells throughout the human gastrointestinal tract might also be capable of producing DA (86).

1.6 Immune cells-a catecholaminergic organ–Opening the Pandora Box of Inflammatory Obesity?

1.6.1 Pharmacology of adrenergic and dopaminergic pathways

Dopamine, NA and AD are CA, i.e. chemical compounds with a 3,4-dihydroxyphenyl group (catechol) and an amine function. They represent the main CA in humans and are all produced from the non-essential amino acid tyrosine which is both obtained from dietary proteins or in turn synthesized from the essential amino acid phenylalanine by the enzyme phenylalanine hydroxylase. The enzyme dopamine β- hydroxylase synthesizes NA from DA, and phenylethanolamine N-methyltransferase converts NA to AD (87) (Figure 3). Dopamine, NA and, to a lesser extent, AD act as neurotransmitters in the CNS. Noradrenaline is also the main neurotransmitter in postganglionic neurons on the sympathetic nervous systems, while AD (and, to a lesser extent, NA) is the neurohormone secreted by chromaffin cells in medulla of adrenal glands (87).

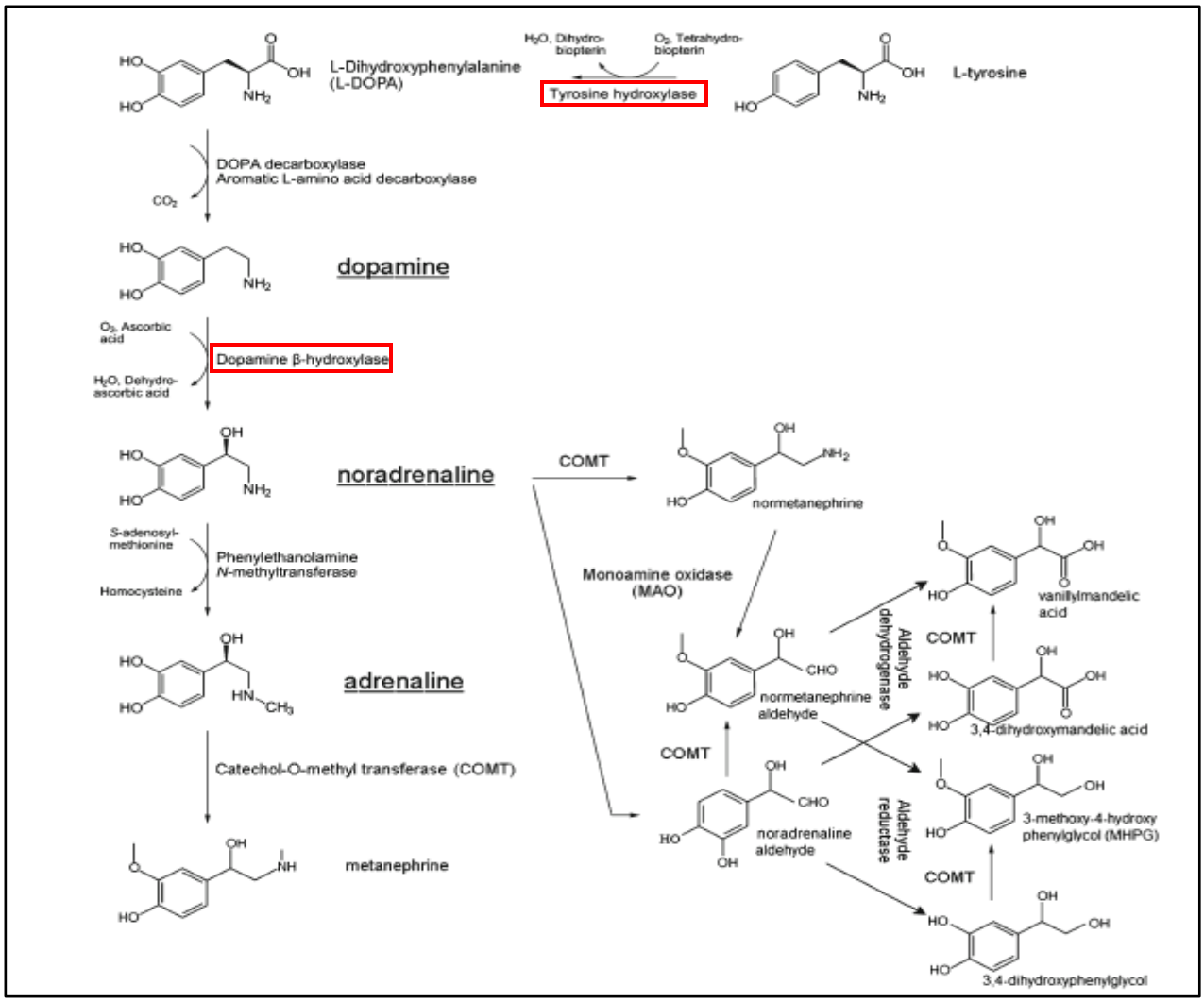


Figure 3 Pathways for biosynthesis and degradation of dopamine, noradrenaline and adrenaline. Synthesizing enzymes and enzyme cofactors are shown close to each arrow. Tyrosine hydroxylase (TH) is the rate-limiting step for catecholamines synthesis and dopamine-β-hydroxylase (DBH) which converts dopamine to noradrenaline is, also, a key enzyme for the synthesis of catecholamines. [Copyright with License Number 3970830471544 from Springer of the manuscript Cosentino M, Marino F. *J Neuroimmune Pharmacol* (2013) 8:163–179) (ref. 87)].

The immunological-metabolic crosstalk regulates metabolism and immune responses (88). Indeed, immune cells are considered biosensors for metabolic/endocrine factors (89) and adipocytes exhibit immune functions (90). It is not clear at present which neuroendocrine regulators or metabolic factors affect the immune system in obesity.

Scientific evidence has accumulated regarding the ability of immune cells themselves to produce and utilize DA, NA and AD (91).

Coexisting in the nervous as well as in the immune system, CA become a universal language of a neuro-endocrine-immune modulating network which enables the nervous, endocrine, and immune system to regulate and fine-tune their functional responses. Through direct communication via sympathetic nerve fibers that innervate lymphoid organs (92), CA can modulate lymphocyte proliferation, differentiation, and cytokine production of human PBMC expressing AR (93). Human lymphocytes and PBMC were described to contain intracellular levels of CA (94- 97). Endogenous CA regulate, through autocrine/paracrine mechanisms, several immune cell functions namely, proliferation, differentiation and apoptosis via an autocrine loop (94, 98).

Remarkably, immune cells, neurons and adipocytes share common signaling pathways mediated by CA, namely AD, NA and DA through activation of AR and dopaminergic receptors (DR) (93-101). Like neurons and endocrine cells, lymphocytes and phagocytes can produce, store, release and reuptake CA *de novo* (101). These cells regulate their CA-synthesis in response to various extracellular stimuli (93). Thus, immune cells seem to possess not only the full cellular machinery for *de novo* synthesis, but also are capable of intracellular CA-inactivation utilizing the same classical metabolic pathway described in nervous and endocrine systems.

1.6.2 The Adrenergic and Dopaminergic Receptors- Adrenergic and Dopaminergic modulation of immunity

The effects of DA, NA and AD are mediated by receptors belonging to the 7-transmembrane, G protein coupled receptors family. DA acts on 5 different DR grouped into two families according to their pharmacological profile and main second messenger coupling: the D1-like (D1 and D5) which activate adenylate cyclase and the D2-like (D2, D3

and D4) which inhibit adenylate cyclase (see Table 1) (97, 100). NA and AD act on 9 different AR, which include three major types - α1, α2 and β - each further divided into three subtypes (see Table 2) (100). DR and AR ligands are currently used in the treatment of several non-immune conditions. DR agonists are currently used in the treatment of Parkinson's disease, restless leg syndrome, and hyperprolactinemia, while antagonists are mainly used as antipsychotics and antiemetics. AR agonists and antagonists are used to treat a variety of diseases, including hypertension, angina pectoris, congestive heart failure, asthma, depression, benign prostatic hypertrophy, and glaucoma. Additional conditions where AR ligands proved useful include shock, premature labour, opioid withdrawal and as co-medications in general anesthesia. All the steps involved in DA, NA and AD synthesis, storage and release, uptake and metabolism represent the target of several drugs already in use for non-immune indications (e.g. cardiovascular, neurologic, neuropsychiatric).

The SNS is, together with the hypothalamic-pituitary-adrenal axis, the major pathway responsible for the crosstalk between the CNS and the immune system. The relevant role of adrenergic pathways in the modulation of the immune response has been an important target of extensive scientific investigation.

Table1 Physiopharmacology of dopaminergic receptors

Name	Transduction mechanisms (effectors)	Tissue distribution	Physiological functions	Examples of therapeutic drugs (main effect/indication)
D_1-like				
D_1	- G_s (adenylate cyclase stimulation, calcium channel)	brain, retina, other peripheral tissues[a]	*brain*: control of locomotor activity; reward and reinforcement mechanisms; learning and memory *periphery*: regulation of renin secretion; regulation of renal function; blood pressure regulation; vasodilation; and gastrointestinal motility	*agonists* - lisuride[b] (antiparkinson) - cabergoline[c] (antiparkinson)
D_5	- G_s (adenylate cyclase stimulation)	brain, other peripheral tissues[a]	*brain*: cognitive functions	
D_2-like				
D_2	- G_i/G_o (adenylate cyclase inhibition)	brain, retina, pituitary gland, adrenal gland, other peripheral tissues[a]	*brain*: control of locomotor activity; reward and reinforcement mechanisms; learning and memory *periphery*: regulation of prolactin secretion; regulation of aldosterone secretion; regulation of renal function; blood pressure regulation; vasodilation; and gastrointestinal motility	*agonists* - aripiprazole[d] (antipsychotic) - lisuride[b] (antiparkinson) - cabergoline[c] (antiparkinson) - bromocriptine[e] (antiparkinson) - pergolide[f] (antiparkinson) - quinpirole (antiparkinson) - pramipexole (antiparkinson)
D_3		brain, other peripheral tissues[a]	*brain*: control of locomotor activity; cognitive functions	*antagonists* - domperidone (antiemetic)
D_4		brain, retina, other peripheral tissues[a]	*brain*: cognitive functions *periphery*: regulation of renal function; blood pressure regulation; vasodilation; and gastrointestinal motility	- haloperidol (antipsychotic) - amisulpride (antipsychotic)

Legend: **a**: in periphery, all subtypes of dopamine receptors have been observed in varying proportions in the kidney, adrenal glands, sympathetic ganglia, gastrointestinal tract, blood vessels, and heart; **b**: agonist at D5 receptors, partial agonist at D1, D2, D3 and D4 receptors, also α2-AR antagonist; **c**: agonist at D1 and D5 receptors, partial agonist at D2, D3 and D4 receptors, also α2-AR antagonist; **d**: partial agonist; **e**: full agonist at D2, partial agonist at D3, antagonist at D4, also full/partial agonist at D1 and D5 and α2-AR antagonist; **f**: full agonist at D2, partial agonist at D3 and D4. **[**Copyright with License Number 3970830471544 from Springer of the manuscript Cosentino M, Marino F. *J Neuroimmune Pharmacol* (2013) 8:163–179) (ref. 87)].

Table 2 Physiopharmacology of adrenoceptors

Name	Transduction mechanisms (effectors)	Tissue distribution	Physiological functions	Examples of therapeutic drugs (main effect/indication)
α_{1A}	- G_q/G_{11} (phospholipase C stimulation, calcium channel) - phospholipase D stimulation - protein kinase C	human heart, liver, cerebellum, cerebral cortex, predominant subtype in human prostate and urethra	contraction of urethral smooth muscle, contraction of skeletal muscle resistance arteries	*agonists* - oxymetazoline[a] (vasoconstrictor) - phenylephrine (vasoconstrictor) - methoxamine (vasocostrictor)
α_{1B}	- mitogen activated protein kinases	human spleen and kidney, human somatic arteries and veins	contraction of arteries and veins	*antagonists* - tamsulosin (benign prostatic hyperplasia) - phentolamine[b] (vasodilator) - risperidone[c] (antipsychotic)
α_{1D}		human aorta, blood vessels of human prostate, human bladder	constriction of arteries, urethral contraction, contraction of corpus cavernosum	- ritanserin[d] (antipsychotic) *inverse agonists* - prazosin (vasodilator, smooth muscle relaxation)
α_{2A}	- G_i/G_o (adenylate cyclase inhibition, potassium channel, calcium channel, phospholipase A2 stimulation) - G_s (adenylate cyclase stimulation)	human brain>spleen> kidney>aorta=lung= skeletal muscle> heart=liver	presynaptic inhibition of noradrenaline release, hypotension, sedation, analgesia, hypothermia	*agonists* - dexmedetomidine (sedative) - clonidine (antihypertensive, anxiolytic, sedative) - brimonidine (ocular hypertension)
α_{2B}		human kidney >>liver> brain=lung=heart= skeletal muscle (also reported in aorta and spleen)	vasoconstriction	- guanfacine (antihypertensive, anxiolytic, sedative) - xylazine (veterinary sedation and analgesia) *antagonists* - lisuride[e] (antiparkinson) - yohimbine (erectile dysfunction)
α_{2C}		human brain ≥ kidney (also reported in spleen, aorta, heart, liver, lung, skeletal muscle)	presynaptic inhibition of noradrenaline release	- phentolamine[b] (vasodilator)
β_1	- G_s (adenylate cyclase stimulation) - G_i/G_o (guanylate cyclase stimulation)	pineal gland, skeletal muscle, liver, superior cervical ganglion, heart, lung, adrenal cortex, cardiac myocytes, brain	increase of cardiac output (heart rate, contractility, automaticity, conduction), renin release from juxtaglomerular cells, lipolysis in adipose tissue	*agonists* - xamoterol[f] (cardiac stimulant) - isoprenaline[g] (treatment of bradycardia and heart block) - prenalterol[f] (cardiac stimulant) - dobutamine[h] (cardiac stimulant) *antagonists* - carvedilol[i] (antihypertensive) - propranolol[i] (antihypertensive) - metoprolol (antihypertensive) - atenolol (antihypertensive)
β_2		lung, lymphocytes, skin, liver, heart	smooth muscle relaxation, striated muscle tremor and glycogenolysis, increase of cardiac output, increase of acqueous humor production in eye, glycogenolysis and gluconeogenesis in liver, insulin secretion	*agonists* - salmeterol (bronchodilator) - isoprenaline[g] (treatment of bradycardia and heart - block) - dobutamine[h] (cardiac stimulant) - salbutamol[f] (bronchodilator)

Table 2 Physiopharmacology of adrenoceptors (continued)

Name	Transduction mechanisms (effectors)	Tissue distribution	Physiological functions	Examples of therapeutic drugs (main effect/indication)
				- terbutaline[f] (bronchodilator) *antagonists* - carvedilol[i] (antihypertensive) - propranolol[i] (antihypertensive)
β_3		adipose, gall bladder> small intestine>stomach, prostate> left atrium> bladder	lipolysis, thermogenesis, relaxation of miometrium and colonic smooth muscle cells, vasodilatation of coronary arteries, negative cardiac inotropic effect	*agonists* - isoprenaline[g] (treatment of bradycardia and heart block) *antagonists* - carvedilol[i] (antihypertensive) - propranolol[i] (antihypertensive)

Legend: **a**: also an α2-AR partial agonist; **b**: inverse agonist at α1D-AR, also an α2-AR antagonist; **c:** also a DRD3 antagonist; **d**: also a 5-HT_2 antagonist; **e**: also DRD2-3 partial agonist; also dopamine receptor agonist/partial agonist; **f**: partial agonist; **g**: non-selective β-AR agonist; **h**: β1- and β2-AR partial agonist; **i:** non-selective β-AR antagonist. **[**Copyright with License Number 3970830471544 from Springer of the manuscript Cosentino M, Marino F. *J Neuroimmune Pharmacol* (2013) 8:163–179) (ref. 87)]

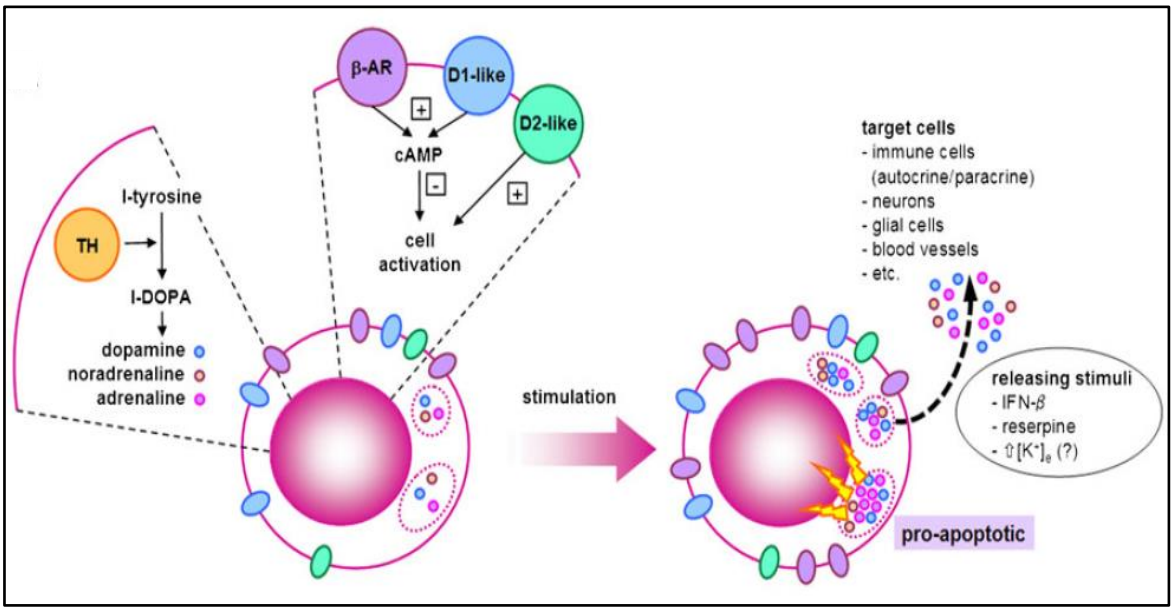


Figure 4 Schematic overview of β-AR and dopaminergic receptor pathways and of endogenous catecholamines in human lymphocytes in healthy subjects. Lymphocytes express both β-ARs (mainly of the β2 subtype) and D1-like and D2-like dopaminergic receptors, and can produce and store dopamine, noradrenaline and adrenaline. Cell stimulation results in β-AR (and, probably, also dopaminergic receptor) upregulation, as well as in increased intracellular storage of catecholamines which, upon release induced by appropriate stimuli, can act as autocrine/paracrine transmitters in the immune system in the nervous system as well as in different tissues. The process of cell activation is an integrated balance of – on one side – β-ARs and D1-like dopaminergic receptors, which exert an inhibitory influence, and – on the other side – by D2-like dopaminergic receptors, which exert a stimulatory influence. Intracellular catecholamines may also play a role in the regulation of activation-induced apoptosis. The scheme does not include information about α-ARs, as their role in the immunomodulation has received so far, few attention, and depicts the arrangement of pathways as it occurs in T effector lymphocytes. Different lymphocyte subsets may however exhibit distinct arrangements, such as in the case of CD4+CD25high T cells. **[**Copyright with License Number 3970830471544 from Springer of the manuscript Cosentino M, Marino F. *J Neuroimmune Pharmacol* (2013) 8:163–179) (ref. 87)].

Released by sympathoadrenergic terminals, NA acts on β_2-ARs expressed on immune cells inducing anti-inflammatory effects which result from both inhibition of Thelper (Th) 1 proinflammatory cytokines (e.g. IFN-γ, IL-12, TNF-α) and enhancement of Th2 cytokines (e.g. IL-10 and TGF-β) (102) (Figure 4). Activation of β-ARs on human lymphocytes may, however, result also in immune stimulatory effects, depending on the time of exposure and the degree of cell differentiation and activation. It has been shown that NA may promote IL-12-mediated differentiation of naïve CD4+ T cells into Th1 effector cells, and increase the amount of IFN-γ produced by Th1 cells (102). The occurrence of β_2-ARs, usually with inhibitory functions, has been reported also on monocytes/macrophages and neutrophils, as

well as in CNS resident immune cells. In contrast to the anti-inflammatory effect of β-AR signaling, treatment of human monocytes with the α1-adrenergic receptor agonist phenylephrine hydrochloride promoted LPS-induced IL-1β (103) (Figure 4).

In comparison to adrenergic pathways, dopaminergic pathways modulating immune responses (Figure 4) have been investigated only recently. It is now clear that DR are expressed in most if not all human immune cells, including T and B cells, dendritic cells, macrophages, microglia, neutrophils and NK cells, and that immune cells contact DA not only in brain but also in blood, lymphoid organs and in other peripheral tissues, such as the kidney and the hepatic vasculature (104). Dopamine induces activation of resting T cells in the absence of any other stimulating factor, however it inhibits stimulated T cells. In naïve human T cells, DA acts on DR expressed on these cells inducing β1 integrin-dependent adhesion to fibronectin (through DR D2/D3 receptors) (105), as well as increased production of TNF-α (through DR D3 and D1/D5 receptors) and IL-10 (through DR D2 and D1/D5 receptors) (106). The effect also depends on dopamine concentration as well as on DR subtype(s) and of the specific subpopulations of immune cells (107). For instance, activation of D1-like receptors expressed in CD4+CD25high T lymphocytes results in inhibition of the regulatory functions of this cell subset, which usually suppresses the activity of effector T cells (98). Evidence exists for an immunomodulatory role of DA regarding the functional responses of NK cells, splenic cells, macrophages, B cells and microglial cells (Figure 5) (104, 107, 108).

The existence of a classical pathway for the synthesis of DA, NA and AD in immune cells is suggested by the expression of the enzyme tyrosine hydroxylase (TH), the first and rate-limiting enzyme in the synthesis of CA which undergoes upregulation following cell

stimulation, as well as by the ability of the TH inhibitor α-methyl-p-tyrosine to prevent intracellular enhancement of CA levels (93, 95-114) (Figure 3).

In human lymphocytes CA are stored in reserpine-sensitive compartments, possibly through vesicular monoamine transporters (VMAT) similar to those expressed in neural and neuroendocrine cells (93, 108, 115), and CA release can be effectively induced by biological agents such as IFN-β (116) or by physiological stimuli such as elevation of extracellular K+ concentrations (117). Human lymphocytes may also express the DA transporter (DAT) (93, 118) and possibly the NA transporter (NET) (112). Several lines of evidence support the functional relevance of endogenous CA in rodent and human immune cells (101, 119). In human immune cells, intracellular levels of CA in circulating lymphocytes from healthy subjects correlated with both basal and isoprenaline-stimulated cAMP production. Studies in human PBMC showed that stimulation with PHA induces the synthesis of CA through induction of TH and its pharmacological inhibition with α-methyl-p-tyrosine results in decreased activation-induced apoptosis (114).

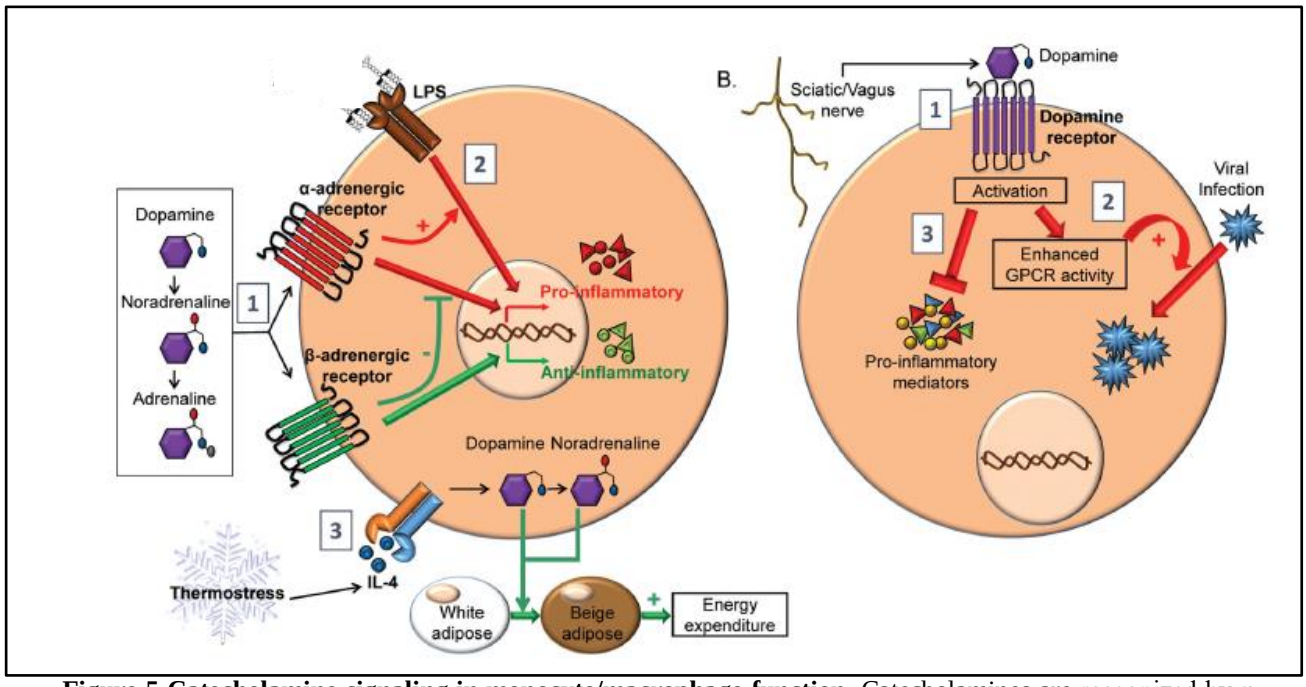


Figure 5 Catecholamine signaling in monocyte/macrophage function. Catecholamines are recognized by α and β-adrenergic receptors (1). Signaling through α-adrenergic receptors is pro-inflammatory and promotes LPS-induced gene expression whereas β-adrenergic receptor signaling inhibits this and induces expression of anti-inflammatory cytokines (2). Cold temperature induces macrophage synthesis of catecholamines which act to increase white to beige adipose tissue conversion and energy expenditure (3). B. Sciatic and vagus nerve stimulation promotes dopamine synthesis (1). Dopamine receptor signaling enhances GPCR activity leading to increased viral entry and replication (2). Dopamine signaling also inhibits pro-inflammatory cytokine production (3). *Abbreviations*: *LPS*, lipopolysaccharide; *GPCR*, G-protein-coupled receptors. [Source of figure: Barnes *et al*. Non-traditional cytokines: How catecholamines and adipokines influence macrophages in immunity, metabolism and the central nervous system *Cytokine* 2015; 72:210–219 (ref.64). Acknowledgments to *Cytokine* Editor-in-Chief, Dhan V. Kalvakolanu, M.S., PhD, for kindly allow copyright].

1.7 Rationale for Peripheral Blood Mononuclear Cells as model of obesity study

Circulating leucocytes, being an integral part of the immune system and the main source of immune cells infiltrating AT in response to obesity, are biomarker candidates for identifying forms of obesity (120).

Peripheral blood mononuclear cells (PBMC) have been proposed as representative of the inflammatory status in obesity (121), potential biomarkers of weight-loss strategies (122) and, as well, model to study the response of energy homeostasis-related genes to acute changes in feeding conditions (123). Leptin expression in human PBMC was suggested to

have a role in blood pressure regulation (124). However, using either total leucocyte counts or the main leucocyte classes has so far fallen short from being clinically useful in risk-stratification of the individual obese patient. The full repertoire of leucocyte subclasses has yet to be identified. Specific molecular markers are continuously being discovered and proposed to signify unique subclasses, which may prove to be more tightly associated with better defined obesity subphenotypes.

Studies attempting to link a specific circulating leucocyte population to AT inflammation are scarce. Since AT, particularly visceral fat, is usually not available for examination, identifying circulating blood cells that will signify the state of intra-abdominal fat inflammation would be very useful clinically.

1.8 Aims

Assessment of health risk associated with excess adiposity requires, for one hand, an accurate measurement of obesity and for another hand identification of predictors of subclinical inflammatory obesity that can be important tools for early therapeutic interventions in obesity-related co-morbidities.

It is not clear at present which neuroendocrine regulators or metabolic factors affect specifically the immune system in a complex multisystem disorder such as obesity. Whether peripheral catecholaminergic system is involved in the low-grade inflammation linked to obesity is unknown. The migration of PBMC towards sites of inflammation such as the AT is crucial to the development of sustained tissue inflammation. Circulating PBMC are exposed to systemic factors, such as dyslipidemia and inflammatory molecules and are directly involved in the low-grade chronic inflammation related to obesity. The expression of both AR and DR in immune cells under an obesogenic state is unknown. The major aims of this thesis are the following:

i. To identify subclinical inflammatory obesity predictors;
ii. To investigate whether WC defining obesity is more sensitive for early metabolic markers and inflammatory changes in monocytes subsets than BMI obesity defined;
iii. To investigate whether the expression of catecholaminergic receptors in PBMC has a different pattern in central obesity;
iv. To investigate whether the expression of catecholaminergic receptors in PBMC is associated with metabolic/inflammatory markers;

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Chapter 2: Paper 1- Predictors of subclinical inflammatory obesity: plasma levels of leptin, very low-density lipoprotein-cholesterol and CD14 expression of CD16 positive monocytes.

2.1 Preface to Paper 1

Our drive in this work was, in first place, to find early markers for inflammatory obesity in order to precociously intervene therapeutically and contribute for prevention of comorbidities obesity-associated that threaten human life span. The first manuscript identifies a prediction model of subclinical inflammatory obesity that is discussed.

2.2 Paper 1

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Predictors of subclinical inflammatory obesity: plasma levels of leptin, very low-density lipoprotein-cholesterol and CD14 expression of CD16 positive monocytes

Running title: Predictors of subclinical inflammatory obesity

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Key-words: Predictors; inflammatory obesity; CD16+ monocytes; leptin; catecholamines

Abstract

Background: Predictors of subclinical inflammatory obesity (SIO) can be important tools for early therapeutic interventions in obesity-related co-morbidities. Waist circumference (WC) and body mass index (BMI) have different SIO sensitivity. We aimed to (1) identify SIO predictors and (2) investigate whether CD16+ monocytes are associated with BMI-(general) or WC-(central) defined obesity. **Methods**: Anthropometric and metabolic/endocrine (namely catecholamines, adrenaline and noradrenaline) parameters were evaluated, and CD16+ monocytes were studied by flow cytometry in the peripheral blood from 63 blood donors, and compared and correlated to each other. Multiple linear regression analysis was performed to identify variables that best predict SIO. **Results**: CD16+ monocyte counts were similar in BMI and WC groups. CD16+ monocytes from centrally obese (CO) showed a more inflammatory pattern, as compared to non-CO subjects. WC was sensitive to lipidemia and, in CO subjects, lipidemia was associated with a more inflammatory phenotype of CD16+ monocytes. These differences were not noticed between BMI groups. Adrenaline was correlated with CD16+ monocyte expansion with a lower inflammatory pattern. Leptin, very low-density lipoprotein–cholesterol (VLDL-C), CD14 expression of CD16+ monocytes were found to be central obesity predictors. **Conclusions**: WC, but not BMI-defined obesity, was associated with a more inflammatory pattern of CD16+ monocytes, without monocyte expansion, suggesting that a monocyte maturation process rather than an independent arise of CD16+ monocytes occurs in CO. Thus, in a population with low cardiovascular low-risk, leptin, VLDL-C and CD14 expression of CD16+ monocytes predict CO, constituting a putative tool for screening of SIO.

Introduction

Obesity is a major threat to human health and lifespan. In the pathogenesis of obesity and its comorbidities, increasing evidence points to a pivotal role of the crosstalk between metabolic and immune systems, with adipocytes exhibiting immune functions and immune cells being influenced by endogenous metabolites, namely lipids [1, 2]. Obesity induces macrophage infiltration into white adipose tissue (AT) in both humans [3] and mice [4], orchestrating local and systemic inflammation, and consequently leading to insulin resistance, T2D and cardiovascular disease (CVD). The recruitment of circulating monocytes into the AT is an early event in obesity-induced AT inflammation [1]. Although monocyte counts have a strong and independent relationship with the overall cardiovascular (CV) risk [5], the role of the distinct circulating monocyte subsets in obesity is still undetermined.

Even though inflammation remains a key mechanism underlying this pathophysiology, finding sensitive tools towards the identification of subclinical inflammatory obesity (SIO) is challenging. In the context of our study, SIO is defined as the presence of inflammatory markers in a healthy population without obesity-associated metabolic or cardiovascular diseases [6]. Discovery of biomarkers to allow earlier detection of inflammatory obesity will help to assess therapeutic effectiveness for obesity-related comorbidities such as CVD and cancer, which are increasing in incidence worldwide [7].

Body mass index (BMI) is the most used and widely accepted method in epidemiological studies to measure obesity [8]. However, BMI neither indicates fat distribution nor does distinguish fat mass from fat-free mass, which have opposite associations with health risk [8]. In contrast, waist circumference (WC), a measure of central adiposity, has been found to be highly correlated with visceral fat measured by computed tomography [9], and better

estimates obesity-associated CV risk in comparison to BMI [9]. Obesity, mainly the central type, is associated with both a chronic inflammatory state and sympathetic nervous system overactivity [1, 10]. The catecholamines (CA), adrenaline (AD) and noradrenaline (NA), potent modulators of neuroendocrine/immune/inflammatory networks, regulate monocyte inflammatory response during health and disease [11]. The crosstalk between adipokines and CA suggests a role for these molecules linking obesity to inflammation [12]. Among immune cells, monocytes are key players in obesity-associated systemic inflammatory processes and in the development of CVD [1]. In obesity, visceral adipose tissue becomes infiltrated by a large number of monocytes mainly originated from the peripheral blood [4]. Two major peripheral blood (PB) monocyte subsets have been described, according to their immunophenotype: classical CD14+CD16- (CD16-) and non-classical CD14+CD16+ (CD16+) subsets [13]. CD16+ monocytes have also been shown to have lower expression of both CD11b (integrin alpha M, ITGAM, or, complement receptor type 3, CR3), and CD36 (fatty acid translocase, FAT, a scavenger receptor), as compared to CD16-monocytes, and they are considered "proinflammatory" cells since they exhibit a macrophage-like phenotype with enhanced antigen-presenting capacities, higher endothelial affinity and production of proinflammatory cytokines [13] [14]. Accordingly, increased percentages and/or absolute numbers of CD16+ monocytes have been described in the PB from patients with inflammatory pathologies, such as sepsis, rheumatoid arthritis, and infections [13], and in patients with a high cardiovascular mortality risk [15]. Rogacev *et al*. described a weak association between CD16+ monocyte counts and BMI in a healthy population [16]. However, the pathophysiological significance of these findings is still controversial. Moreover, in inflammatory obesity, it is uncertain whether there is an expansion of CD16+ cells or a maturation process from classical to non-classical monocytes and if CA (AD and

NA) are associated with monocytes characteristics observed in that condition. Previously, we have demonstrated that central obesity (CO) is associated with a higher immune inflammatory pattern and a downregulation of dopaminergic pathways in peripheral blood mononuclear cells [17].

The main aims of our study were (1) to identify predictors of SIO, (2) to investigate whether proinflammatory CD16+ monocytes are preferentially associated with BMI-defined or WC-defined obesity and (3) to evaluate which anthropometric, metabolic or endocrine parameters are associated with CD16+ monocyte characteristics.

Our work, for the first time, addresses the prediction of SIO, a major issue in public health given its comorbidities associated with a decrease in human life span.

Methods

Methods

Study population

This study was conducted in 65 blood donors from the Blood Bank of Clinical Hematology 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 ware 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. Two blood donors that were not fasting for, at least, 12 hours at the time of blood withdrawal were finally excluded. After 5 minutes of rest, blood pressure (BP) [systolic blood pressure (SBP) and diastolic blood pressure (DBP)] was measured twice and the mean values were used in the analysis.

Anthropometrics

Height (in meters) was based on an identification document and confirmed by medical record. Body weight was measured to the nearest 100 g on electronic weight scales. Body mass index was calculated by dividing weight by squared height and expressed in $kg.m^{-2}$. Participants, all Caucasian, were divided into three BMI categories that correspond to the definitions of normal weight (NW, BMI from 18.5 to 24.9 $kg.m^{-2}$), overweight (OW, BMI from 25.0 to 29.9 $kg.m^{-2}$), and obesity (OB, BMI≥30.0 $kg.m^{-2}$), according to the World

Health Organization guidelines (WHO) [8]. Waist circumference was measured with a flexible tape at the level midway between the lowest rib margin and the iliac crest. As stated by the International Diabetes Federation, CO was defined as WC ≥0.80 m in women and ≥0.94 m in men [18].

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 CA (NA and AD) were determined by HPLC with electrochemical detection (HPLC-ED) (Chromosystems Instruments & Chemicals GmbH, Munich, Germany). Determination of cortisol in plasma was performed with an electrochemiluminescence immunoassay (Elecsys Systems analyzer Roche, Roche Diagnostics International Ltd Rotkreuz, Switzerland). Leptin levels were measured in serum by solid phase two-site enzyme immunoassay (Mercodia Leptin ELISA, Mercodia AB, Sylveniusgatan 8A, Uppsala, Sweden). High-sensitivity C-reactive protein (hsCRP) (mg/L) was determined by nephelometry (CardioPhase™ hsCRP – BnProSpec Siemens Healthcare Diagnostics Inc. New York, United States), and was categorized by cardiovascular event risk groups: <1- low, ≥1 to <3 –intermediate and above 3 - high risk, as described before [19].

Flow cytometry assay of monocytes

Monocytes were analyzed in fresh ethylenediaminetetraacetic acid tripotassium (EDTA-K3) anti-coagulated blood samples by means of flow cytometry, using a previously described technique [20]. The enumeration and the immunophenotypic analysis of monocytes were performed. Immunophenotypic studies were done through a whole blood stain-lyse-and-then-wash method, a direct immunofluorescence technique using the following a four-color panel of monoclonal antibodies (mAbs): mouse anti-human CD36 conjugated with fluorescein isothiocyanate (FITC) (clone FA6.152, IgG1), mouse anti-human CD16 conjugated with phycoerythrin (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 allophycocyanin (APC) (IgG2a, clone D12) obtained from Becton Dickinson (BD) (catalogue number 333143). Data acquisition was carried out on a FACSCalibur flow cytometer (BD), using the Cell Quest software program (BD). Information on a minimum of $2x10^5$ events was acquired for each staining and stored as FCS data. The Paint-a-Gate Pro software program (BD) was used for data analysis. Monocytes were quantified based on the CD14 expression, while CD16 was used to differentiate classical (CD16-) and non-classical (CD16+) monocyte populations (fig. 1). 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), which is related to cell size, and sideward light scatter (SSC), which correlates with cell complexity/granularity, were also measured. The ratio for each parameter between CD14+CD16+ non-classical monocytes and CD14+CD16- classical monocytes in each individual was calculated, to overcome inter-individual variations.

Instrument setup, calibration and daily monitoring was performed with the protocols in use in our laboratory at the time of the study. Briefly, for instrument setup and calibration, unstained cells (auto-fluorescence controls) were used to adjust FSC and SSC detector settings so that cells of interest were displayed on scale. Next, also using unstained cells and gating on lymphocytes, FL1, FL2, FL3 and FL4 detector settings were adjusted so that auto-fluorescence background was roughly within the first decade of the log scale of the respective fluorescence histogram. Subsequently, electronic compensation was performed by running cell samples stained individually with FITC-, PE-, PE-Cy5- and APC-conjugated anti-CD8 mAbs antibodies (single stains). Finally, compensation was checked by running cell samples stained simultaneously with FITC-, -PE, -PE-Cy5 and -APC conjugated mAbs staining mutually exclusive cell populations. Daily control was performed using Calibrite beads and monitored by plotting MFI values in Levy Jennings charts.

Blood cell counts

Blood cell counts were obtained using a Coulter LH750 automated hematology analyzer (Beckman Coulter – BC, Miami, FL). Absolute counts of total, CD16- and CD16+ peripheral blood monocytes, expressed as numbers of cells / μl, were calculated using a dual platform method, in which total WBC counts derived from the hematology analyzer and the correspondent percentages of monocytes were obtained by flow cytometry.

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. Variables were summarized using relative and absolute frequencies, mean and standard deviation, median (interquartile range

(25th and 75th percentiles). To compare the quantitative independent variables, we used bivariate statistical analysis ANOVA with Bonferroni post hoc testing (comparisons between more than 2 groups), or non-parametric Mann-Whitney (comparison between 2 groups) and Kruskal-Wallis (comparison of more than 2 groups) tests for normal and non-normal distributed data, respectively. Correlations were assessed by non-parametric Spearman rank analysis. The Pearson Chi-Square test was used to compare the distribution of qualitative independent variables. Multiple linear regression analysis was performed to identify the variables that best predict central obesity after controlling for potential confounders. Linear regression models require only two subjects per variable for adequate estimation of regression coefficients, standard errors, and confidence intervals [21]. Variables that in the univariate analysis showed statistical significance below 5% ($p<0.05$) were included in the regression. Data analysis was performed using the SPSS version 22.0 (SPSS, Chicago, IL, USA). P-value lower than 0.05 was considered statistically significant.

Results

Baseline characteristics and anthropometrics, metabolic/endocrine parameters and hsCRP in BMI and WC groups

The baseline characteristics of the study sample are shown in table 1. The mean age (years) of the individuals divided either by BMI or CO groups was similar [(NW=38±11 vs OW=40±11 vs OB=44±11); F (2, 60) =1.199; p=0.309], [(with CO=42±10 vs without CO=36±11); F (1, 61) =3.855; p=0.054], respectively. Considering gender distribution, 55.6% were male. The prevalence of BMI-defined OW/OB and of WC-defined CO was 75% and 73%, respectively. Diastolic blood pressure (mm Hg) and leptin plasma levels (ng/mL) in OB were significantly higher than in both OW and NW [(89±9) vs (80±9) vs (73±8); F (2, 60) =12.748; p<0.001], [1.17 (0.85-3.47) vs 0.50 (0.30-1.05) vs 0.20 (0.04-0.63); p=0.002], respectively. Mean DBP in OW was also higher than in NW (p=0.025). OB individuals also presented higher SBP than NW [(144±16) vs (129±16); F (2, 60) =4.413; p=0.016]. Subjects with CO in comparison with those without CO had not only higher DBP (82±11 vs 76±7; F (1, 61) =4.244; p=0.044) and leptin levels [0.870 (0.510-1.300) vs 0.100 (0.040-0.210); p=0.006] but also showed differences in lipid profile. These include higher levels of TC (200±35 vs 178±35; F (1, 59) =4.978; p=0.029), TAG [100 (68-135) vs 78 (50-99); p=0.034] and VLDL-C [20 (14-27) vs 16 (10-20); p=0.033] (mg/dL). None of the other metabolic/endocrine parameters were significantly different between BMI and WC-groups. The plasma levels of hsCRP (mg/L) [data as median and interquartile range (25^{th} and 75^{th} percentiles)] were neither different between BMI-defined [1.410 (0.352-1.920) vs 1.190 (0.571-2.250) vs 3.020 (1.090-3.860); p=0.171, respectively for NW, OW and OB] nor between WC-defined groups [1.530 (0.764-3.560) vs 0.757 (0.469- 1.750); p=0.099,

respectively for groups with and without CO]. Plasma leptin values (ng/mL) were different between hsCRP-defined cardiovascular risk groups [0.320 (0.100-0.850) vs 0.615 (0.480-1.140) vs 3.100 (0.800-5.170); p=0.011, respectively for low, intermediate and high risk groups]. hsCRP-defined groups did not show sensitivity for other studied parameters. However, hsCRP plasma levels correlated with DBP, VLDL-C, TAG and leptin [(ρ=0.286; p=0.027); (ρ=0.276; p=0.033); (ρ=0.284; p=0.028); and (ρ=0.397; p=0.011), respectively].

Frequency and immunophenotype of monocyte subpopulations in BMI- and WC-defined groups

In the whole population, total monocytes were on average 439 cells/μL (table 1), and were not different in both BMI- (446±141 vs 445±151 vs 422±188; F (2, 60) =0.121; p=0.886, respectively for OB, OW and NW) and WC-defined groups (425±150 vs 479±174; F (1, 61) =1.488*;* p=0.227, respectively for CO and non-CO individuals). Classical CD16- monocytes accounted for 89% and inflammatory CD16+ monocytes 11% of total monocytes (table 2). As previously described [14], CD16+ monocytes showed lower cellular complexity/granularity (SSC) and lower expression of CD14, CD36 and CD11b, as compared to CD16- monocytes (table 2). The frequency and the immunophenotype of either CD16+ or CD16- monocytes in BMI-defined groups were similar. However, CD16+ monocytes from CO subjects showed a more inflammatory pattern, with lower SSC and lower expression of CD14, as compared with subjects without CO, as evaluated by the ratio of SSC and CD14 expression on CD16+ and CD16- monocytes in each individual (p = 0.003 and p=0.017, respectively) (table 3).

CD16+ monocytes and anthropometric and metabolic/endocrine parameters in BMI- and WC-defined groups

In the whole sample, plasma levels of adrenaline correlated positively with both CD16+ monocyte size (FSC) and CD14 expression (fig. 2). In BMI-defined OB subjects, CD14 expression was negatively correlated with plasma TC (ρ = -0.764; p = 0.006) and, similarly to whole population, adrenaline was correlated with the FSC of monocytes (ρ = 0.619*;* p = 0.032).

In CO subjects, AD correlated positively with the number and with the immunophenotype characteristics of CD16+ monocytes, namely, size (FSC), complexity (SSC), and CD14 and CD11b expression (table 4).

No other associations between anthropometric, metabolic/endocrine parameters, namely leptin, and CD16+ monocytes characteristics (counting and phenotype) were verified.

CD16+ monocytes and anthropometric and metabolic/endocrine parameters: impact of gender

The female/male ratio was different between CO groups and not verified among BMI groups (table 3); thus, we analyzed the differences between genders concerning the anthropometric, metabolic and endocrine variables in whole population (table 1). Relatively to the monocyte subsets, women, comparatively to men, showed lower numbers of CD16- monocytes (cells/μL) [343±99 vs 424±149; F (1, 60) =5.994; p=0.017] but similar numbers CD16+ monocytes [41±28 vs 61±63; F (1, 60) =2.529; p=0.117], respectively. The ratio of CD14 expression on CD16+ and CD16- monocytes was lower in female in comparison to male [F (1, 60) =4.953; p=0.030] (table 1), indicating a more inflammatory pattern of CD16+

monocytes in women. No other differences were noticed between genders in relation to monocytes characteristics.

The number of CD16+ monocytes did not correlate neither with the metabolic nor the endocrine parameters in both women and men. The plasma levels of LDL-C were found to correlate positively with a more inflammatory phenotype of the CD16+ monocytes in both female and male [SSC (ρ=-0.394; p=0.042); CD14 (ρ=-0.385; p=0.033) respectively]. Adrenaline, as in whole sample, also correlated positively with CD16+ monocyte size (FSC) in both females (ρ=0.472; p=0.013) and males (ρ=0.405; p=0.020). In females, AD was correlated with a less inflammatory pattern of CD16+ monocytes: [SSC (ρ=0.417; p=0.031); CD14 (ρ=0.411; p=0.041)]. On the contrary, in males, AD was inversely correlated with the expression of CD36 (ρ=-0.362; p=0.038). Noradrenaline was correlated with a higher inflammatory pattern of the CD16+ monocytes in females [CD11b (ρ=-0.618; p=0.019] but this correlation was not verified in males.

We performed a multiple linear regression analysis in order to find a predictive model for CO, controlling for gender. The independent variables weight, height, SBP, HDL-C, VLDL-C, TAG, adrenaline, leptin, total number of monocytes, classical monocytes (CD16-) counting and CD14 ratio were included in the analysis, as they were significantly different between genders. We found that 33.7% of the CO is explained by a model that includes leptin, CD14 ratio, VLDL-C (R^2 =0.337; Z=6.258; *df* 3; p=0.002).

Discussion

The main findings of the present study are threefold. First, we identified a prediction model of SIO identified by CO, but not by BMI; CO is predicted in more than one third with a model that includes the determination of plasma levels of leptin and VLDL-C, and CD14 expression in monocytes subsets. Second, in SIO, CD16+ monocytes showed a "more inflammatory" phenotype signature, although no increase in the frequency or absolute number of proinflammatory monocytes has been observed, suggesting that the maturation process from classical to proinflammatory monocytes already occurs in early stages of inflammatory obesity. Third, catecholamines (AD and NA) were associated with the characteristics of CD16+ monocytes with a putative immunomodulatory role in obesity.

A review of determinants for subclinical inflammation in obesity, namely in children, described a significant increase in C reactive protein (CRP), interleukin (IL)-6 and leptin with increasing adiposity, independent of age [22]. Total dietary fat and percentage of energy from fat were significant predictors of CRP concentration, independently of BMI [6]. Our work found that leptin and VLDL-C plasma levels, and CD14 expression on CD16+ monocytes are predictors of inflammatory obesity in a low cardiovascular risk population. For one hand, understanding that more than one third of inflammatory obesity is explained by these parameters, this data may contribute to clarify why the adiposity measurements (general and/or central) *per se* fail in prediction of CVD [22]. The interpretation of the results from a collaborative analysis of 58 prospective studies was that measures of general and central obesity did not improve CVD risk assessment when information is available on SBP, diabetes and lipids [23]. Moreover, it is widely accepted that the cluster metabolic syndrome is explained by underlying inflammation which trigger is not completely

understood. Our model predicts obesity-associated inflammation. Leptin has a main inflammatory / immunomodulatory role [24] and increased levels of VLDL-C is one of the first events of dyslipidemia associated to obesity [25]. In addition, the more inflammatory pattern observed on CD16+ monocytes from CO subjects may facilitate their transmigration to AT and subsequent local inflammation. Prospective studies would be necessary to evaluate our predictive model of inflammatory obesity in CVD risk assessment.

In the present work, a CV low-risk population was recruited to study SIO. Central obesity was associated with dyslipidemia and a distinct phenotypic more inflammatory pattern of CD16+ monocytes (with no expansion), while no significant differences were noticed between BMI groups. Using a flow-cytometric approach, we differentiated two monocyte subsets as described before [13]: classical CD16- monocytes and non-classical CD16+ monocytes (with lower SSC and lower expression of CD14, CD36 and CD11b comparatively to classical monocytes). Although an intermediate subset of CD16+ monocytes has been reported [26], sharing with the classical subset the gene expression profile [27] and a higher expression of CD14 [26], its biological role remains unclear. This intermediate subset, that in physiological states constitutes a very small percentage in circulation, seems to represent a transitional maturation stage from CD16- to CD16+ monocytes [26]. Moreover, it has been demonstrated that non-classical monocytes (and not the intermediate monocytes) are the primary inflammatory monocytes in acute and chronic inflammatory conditions [28]. Our findings, showing an association between CO and a subset of CD16+ cells with lower expression of CD14, would suggest that CD16+ monocytes more likely belong to the non-classical subset.

According to our results, several studies in human chronic pathologies associated with low grade inflammation did not find changes in CD16+ monocyte counts [29-31]. On contrary,

Rogacev *et al*. [16] have shown a weak correlation between BMI and CD16+ monocyte counts (total CD16+ and intermediate but not non-classical monocyte subsets). Curiously enough, Berg *et al.* [32] showed that elevated numbers of CD16- monocytes predicted cardiovascular events, independently of gender, age, current smoking, HDL-C and the presence of diabetes and hypertension.

The expression of adhesion molecules (CD11b) [33] and scavenger receptors (CD36) [34], in peripheral blood monocytes have been used to characterize the inflammatory response in metabolic disorders because of their involvement in the pathogenesis of diabetes and obesity [35, 36]. CD11b has been associated with high fat diet induced obesity [37] and carotid intima-media thickness [38], and CD11b expression was described as lowest for non-classical monocytes after LPS stimulation [39] and in a rodent model of T2D [40]. Results concerning CD36 atherogenicity are, also, contradictory, since an increase [35] or deficiency [41] of its expression have been associated with the pathogenesis of atherosclerosis and CVD.

These contradictory results relatively to monocytes subsets characteristics (counting and phenotype) may be explained by discrepancies in gating strategies for flow cytometric characterization and purification protocols contributing to significant changes in receptor expression. We have adopted whole blood and staining protocol to perform the flow cytometric study in a robust gating strategy.

Circulating monocytes undergo phenotypic modifications due to exposure to systemic environmental factors [2]. Plasma lipids and leptin are immune modulators [2, 24], and they could have affected the phenotype of CD16+ monocytes. Circulating lipids may also influence the function of the immune cells [2]. Despite the regulatory role of leptin on human monocytes [42], it seems unlikely that this adipokine could be the only factor

explaining the more inflammatory pattern observed on CD16+ monocytes from CO subjects, as its levels were also elevated in general obese. On the other hand, individuals with CO showed significantly higher levels of TAG, TC and VLDL-C comparing to those without CO. Furthermore, BMI, frequently associated with dyslipidemia [1], was not sensitive for differences in lipid plasmatic profile. Apart from BMI-defined obesity has missed the detection of subclinical inflammation in our study, it has also been reported that visceral fat measurements elucidate relationships between obesity and cancer otherwise no significant when evaluated by BMI [43].

CD16+ monocytes seem to reside in a marginal pool mobilized by a catecholamine-dependent pathway [44]. In CO, AD was correlated with the number, size and with a lower inflammatory pattern of CD16+ monocytes. This suggests that AD is associated with the expansion of this monocyte subset, as already described in stressful conditions such as coronary artery disease [45], but with a less inflammatory phenotype, in accordance with Takahashi *et al*. [46]. Our work also suggests that catecholamines, AD and NA, have an immunomodulatory role in obesity. The adrenergic regulation of human monocytes was recently reviewed [47]. On human monocytes, even if under certain conditions proinflammatory responses may arise, the predominant view is that β adrenoceptors (β-AR) activation is usually anti-inflammatory and immunosuppressive. In this context, the putative immune effects of β-AR agonists in CO requires further investigation.

Usually correlated with adiposity [48], hsCRP was not a useful marker for SIO in our population with low cardiovascular risk. Others have also described a non-linear relation between hsCRP concentration and adiposity [49]. Although we have found association between hsCRP and DBP, VLDL-C, TAG and leptin, the cardiovascular risk groups defined

by hsCRP only differentiated plasma levels of leptin. Indeed, leptin may mediate the relation between hsCRP and obesity [49].

Our study describes a stronger association of inflammation and obesity in females, which is in line with the results of Thorand *et al* [50]. Indeed, women showed higher levels of leptin usually associated to inflammatory immune responses [24]. Moreover, catecholamines behave differently by gender [51]. Adrenaline plasma levels were associated with a less pro-inflammatory CD16+monocyte phenotype in females and with a higher level of inflammation in males. In opposite, NA was associated with a higher inflammatory phenotype only in females. These data could be explained by higher percentage of adiposity in females and differences in endogenous hormones.

Our work suggests that in SIO, dyslipidemia and leptin could induce a monocyte maturation process rather than an independent arise of CD16+ monocytes. Moreover, our model explaining a significant part of CO can be an important tool for early therapeutic interventions to reduce health risks associated to obesity.

In a low CV risk population, the central, contrary to general, adiposity measure was sensitive to metabolic risk factors and to a more pro-inflammatory pattern of CD16+ monocytes (reflecting a higher activation of the innate immune system). Our findings seem very challenging to further investigate in an early phase of CO the phenotypic pattern of circulating monocytes, and its relationship with migratory and inflammatory functions.

Conclusions

Central, and not general, obesity identified subclinical inflammation. A model, that includes the determination of plasma leptin and VLDL-C levels and CD14 expression in monocytes subsets, predict and explain a significant part of CO. Our work suggests that, in SIO,

dyslipidemia and leptin induce a monocyte maturation process rather than an independent arise of CD16+ monocytes and, also suggests catecholamines (AD, NA) immunomodulation in obesity. The stronger association found between CO and cardiometabolic risk factors in comparison with general obesity assessed by BMI should consider WC a priority tool for the screening of inflammation in populations with low cardiovascular risk.

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Conflict of interests

The authors declare to disclose all financial and commercial conflicts of interest.

All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest.

All authors approved the final manuscript.

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Table 1 Baseline characteristics of study participants (n=63)

Variable	*Data*	*min-max*	*Normal range*	*F/M*
Age(years)	40±11	20-63	-	ns
Weight(kg)	77.1±13.3	51-120	-	**(70±10 /83±13)
Height (m)	1.67±0.09	1.50-1.87	-	**(1.60±0.07 /1.73±0.06)
BMI(kg.m^{-2})	27.6±4.2	19.6-40.1	-	ns
WC (m)	0.97±0.12	0.75-1.24	-	ns
SBP(mm Hg)	134±15	103-177	<130	**(127±13 /140±14)
DBP(mm Hg)	80±10	61-107	<85	ns
Glycemia(mg/dL)	86±9	67-115	70-105	ns
HbA1c (%)	5.2±0.3	4.5-5.8	3.8-5.6	ns
TC (mg/dL)	194±36	117-283	0-200	ns
HDL-C (mg/dL)	50±15	25-91	35-55	*(57±15 /45±12)
LDL-C (mg/dL)	123±33	60-209	0-130	ns
VLDL-C (mg/dL)[a]	19 (13-25)	6-58	3-56	*[15 (10-20) /23 (15-28)]
TAG (mg/dL)[a]	94 (67-124)	32-475	40-160	*[75 (51-99) /116 (76-140)]
NA (pmol/L)[a]	690 (395-1552)	40-3760	709-4019	ns
AD (pmol/L)[a]	154 (81-227)	55-965	<328	*[151 (81-185) /163 (103-279)])
Cortisol(μg/dL)[a]	15 (13-18)	6.4-28.4	6.2-19.4	ns
Leptin (ng/mL)[a, b]	0.63 (0.28-1.10)	0.003-5.3	2-5.6	**[1.05 (0.70-2.09) /0.31 (0.10-0.50)]
hsCRP (mg/L)[a, c]	1.460 (0.617-3.015)	0.150-17.800	< 1.0 low risk 1.0 <3.0 intermediate risk >3.0 high risk	ns
Leucocytes (cells/μL)	6494±1585	3500-11600	4500-13000	ns
Monocytes (cells/μL)	439±157	82-964	400-500	*(384±107 /483±177)
CD14 expression ratio (CD16+/CD16- monocytes)	0.40±0.17	0.14-0.87	-	*(0.35±0.14 /0.44±0.17)

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; 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; hsCRP, high-sensitivity C reactive protein. Data are shown as: mean ±standard deviation (SD) for normally distributed; (a) non-parametric data as median (interquartile range); or percentage as appropriate. (b) n=41. (c) n=60. ANOVA or non-parametric Mann-Whitney test was used to compare quantitative independent variables by gender for normal or non-normal distributed data, respectively. [F/M, female/male ratio = 28/35; ns -nonsignificant; * p (level of significance) <0.05 and ** = p<0.001].

Table 2 Immunophenotypic characterization of CD16+ and CD16- monocytes in the whole population of study participants

	CD16+	*CD16-*	*ratio CD16+/ CD16-*	*p*
Cells/μL	52±51	387±134	0.14±0.10	**<0.001**
% of total monocytes	11.4±6.8	88.6±6.8	0.14±0.10	**<0.001**
FSC	557±65	556±63	1.0±0.03	0.654
SSC	410±53	481±45	0.85±0.06	**<0.001**
CD14	892±558	2384±1522	0.40±0.17	**<0.001**
CD36	317±138	740±205	0.44±0.15	**<0.001**
CD11b	82±72	212±301	0.59±0.32	**0.006**

Abbreviations: FSC, forward light scattered; SSC, sideward light scattered; p, level of significance. The median fluorescence intensity of CD14, CD36 and CD11b was assessed in each subset and expressed as fluorescence arbitrary units. Data are presented as mean ±standard deviation (SD) or percentage, as appropriate. T test paired was used for the comparison of paired variables. Significant differences are in bold face.

Table 3 Immunophenotypic characteristics of CD16+ and CD16- monocytes in the study participants categorized by BMI and by WC

				BMI				Waist Circumference				
				NW	OW	OB	*p*			Without CO	With CO	*p*
	F/M (n)			9/7	12/21	7/7	0.377[a]			3/14	25/21	0.009[a]
		F	*df*		Mean±SD			F	*df*	Mean±SD		
	CD16+	0.371	2, 59	53±68	48±29	62±70		0.011	1, 60	51±66	53±45	
cells/μL	CD16-	0.227	2, 59	369±145	397±141	386±106		2.165	1, 60	428±137	372±131	
	Ratio CD16+/CD16-	0.313	2, 59	0.14±0.10	0.13±0.07	0.15±0.14	NS	1.076	1, 60	0.11±0.10	0.14±0.10	NS
	CD16+	0.160	2, 59	11.6±7.2	11.0±5.5	12.2±9.4		1.437	1, 60	9.7±6.9	12.0±6.7	
% cells	CD16-	0.160	2, 59	88.4±7.2	89.0±5.5	87.8±9.4		1.432	1, 60	90.3±6.9	88.0±6.7	
	Ratio CD16+/CD16-	0.306	2, 59	0.14±0.10	0.13±0.08	0.15±0.14	NS	1.045	1, 60	0.11±0.10	0.14±0.10	NS
	CD16+	0.615	2, 59	570±59	556±71	544±53		0.005	1, 60	558±71	557±63	
FSC	CD16-	0.837	2, 59	571±60	555±67	540±55		0.064	1, 60	559±16	555±62	
	Ratio CD16+/CD16-	0.257	2, 59	1.0±0.04	1.0±0.03	1.01±0.03	NS	0.755	1, 60	1.0±0.02	1.0±0.003	NS
	CD16+	0.784	2, 59	424±48	406±57	404±46		3.799	1, 60	431±58	402±49	
SSC	CD16-	0.938	2, 59	494±37	477±51	476±38		0.354	1, 60	487±46	479±45	
	Ratio CD16+/CD16-	0.213	2, 59	0.86±0.07	0.85±0.05	0.84±0.04	NS	9.356	1, 60	0.88±0.06	0.84±0.05	**0.003**
	CD16+	0.226	2, 55	976±691	863±529	861±473		1.191	1, 60	1027±626	845±532	
CD14	CD16-	1.354	2, 58	2855±2055	2194±1331	2292±1161		0.299	1, 60	2116±332	2362±240	
	Ratio CD16+/CD16-	1.051	2, 59	0.37±0.17	0.43±0.18	0.36±0.11	NS	6.003	1, 60	0.48±0.19	0.37±0.15	**0.017**
	CD16+	0.004	2, 59	315±194	316±120	320±110		0.060	1, 60	324±190	313±116	
CD36	CD16-	0.720	2, 59	755±196	713±221	790±175		1.926	1, 60	681±250	762±184	
	Ratio CD16+/CD16-	1.191	2, 59	0.41±0.16	0.47±0.16	0.40±0.08	NS	2.544	1, 60	0.49±0.04	0.42±0.02	NS
	CD16+	1.745	2, 27-	121±97	70±63	59±25		0.059	1, 28	87±63	80±76	
CD11b	CD16-	3.817	2, 27	442±492	127±147	132±49		0.091	1, 28	184±181	222±72	
	Ratio CD16+/CD16-	2.978	2, 27	0.44±0.22	0.71±0.36	0.45±0.11	NS	0.094	1, 28	0.62±0.22	0.58±0.08	NS

Abbreviations: BMI, body mass index; NW, normal weight; OW, overweight; OB, obese; WC, waist circumference; CO, central obesity; FSC, forward light scattered; SSC, sideward light scattered. The median fluorescence intensity of CD14, CD36 and CD11b was assessed in each subset and expressed as fluorescence arbitrary units. Data are presented as mean ±standard error of the mean (SEM) or percentage as appropriate. ANOVA was used for comparison between the 3 groups. (a)Pearson Chi-Square test was used to determine differences of F/M (female/male) n (number of individuals) between groups; F, Snedcor's distribution; *df*, degree of freedom; *p* level of significance.

Table 4 Correlation between plasma level of adrenaline and CD16+ monocytes number and phenotype in central obesity.

	CD16+monocytes									
	Number		FSC		SSC		CD14		CD11b	
	ρ	*p*	ρ	*p*	ρ	*p*	ρ	*p*	ρ	*p*
Adrenaline	0.309	**0.044**	0.488;	**0.001**	0.307	**0.045**	0.307	**0.050**	0.468	**0.033**

Abbreviations: FSC, forward light scattered; SSC, sideward light scattered; The median fluorescence intensity of CD14, CD36 and CD11b was assessed in each subset and expressed as fluorescence arbitrary units. ρ, Spearman's rho; p, level of significance. Significant correlations are in bold face.

Figure legends

Figure 1 Gating strategy of the monocyte subsets- Total monocytes (red dots) were selected in the SSC vs. CD14 dot plot, based on the expression of CD14 (panel A). Afterwards, classical CD14+CD16- monocytes (green dots) and proinflammatory CD14+CD16+ monocytes (blue dots) were identified in the CD16 vs. CD14 dot plot, based on the expression of CD14 and CD16 (panel B). Subsequently, these two monocyte populations were gated and separately analyzed for the levels of CD11b, CD14 and CD36 expression, using the median fluorescence intensity obtained for each marker; the forward light scatter (FSC) and sideward light scatter (SSC) were also measured. Finally, the ratio between the values obtained on CD14+CD16+ and CD14+CD16- monocytes was calculated, for each parameter and in each individual. CD14 and CD16 positivity were defined using fluorescence minus one controls. Other leukocytes are represented as gray dots. Dot plots showed in this figure were obtained using the Infinicyt software (Cytognos).

Figure 2 Correlation between plasma levels of adrenaline and CD14 expression (A) and forward light scattered (FSC) (B) of CD16+ monocytes. Abbreviations: ρ, Correlation coefficients, calculated by Spearman test; p, level of significance.

Figure 1

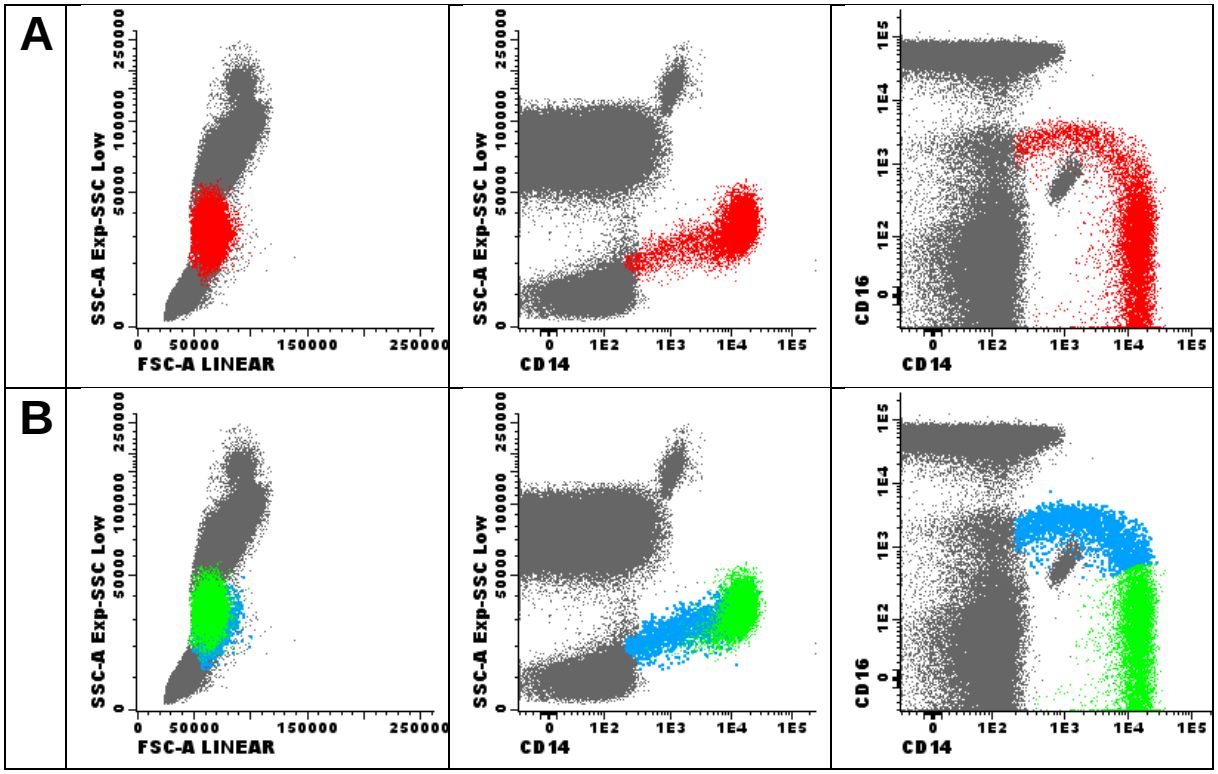
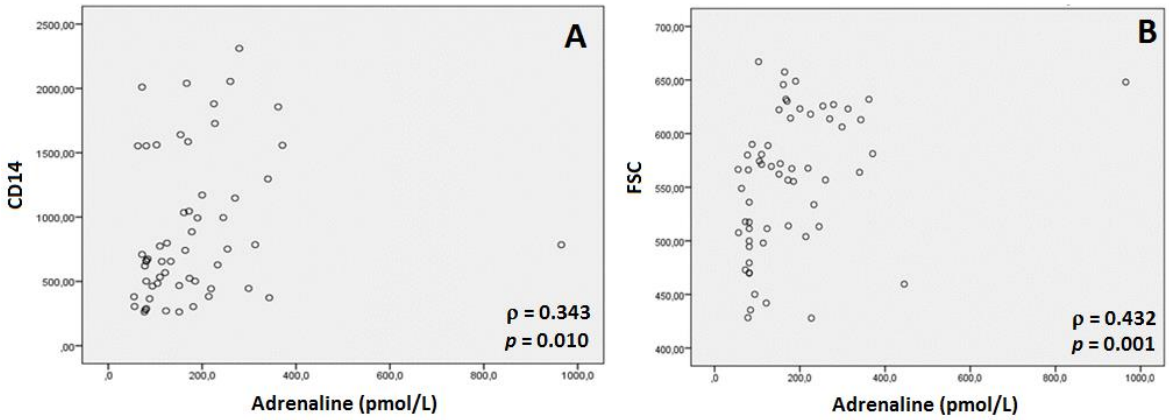

A
B
SSC-A Exp-SSC Low
FSC-A LINEAR
CD14
CD16
0
50000
150000
250000
1E2
1E3
1E4
1E5

Figure 2


A
CD14
2500,00
2000,00
1500,00
1000,00
500,00
,00
,0
200,0
400,0
600,0
800,0
1000,0
Adrenaline (pmol/L)
ρ = 0.343
p = 0.010
B
FSC
700,00
650,00
600,00
550,00
500,00
450,00
400,00
,0
200,0
400,0
600,0
800,0
1000,0
Adrenaline (pmol/L)
ρ = 0.432
p = 0.001

Chapter 3: Paper 2- β_2 Adrenoceptors are under expressed in Peripheral Blood Mononuclear Cells and associate with a better metabolic profile in Central Obesity.

3.1 Preface to Paper 2

In this paper, we present the study of the expression of adrenergic receptors (AR) on peripheral mononuclear cells in human obesity-related subclinical inflammatory state, based on the results of the manuscript 1 that suggested immunomodulation by adrenaline and noradrenaline in this condition. First, we performed a pilot experiment studying all adrenoceptors subtypes. β_2-adrenoceptors (β_2AR) were showed to be under expressed in subjects with central obesity. In view to explain the molecular results of the distinct adrenergic pattern in obesity, statistical analysis with multiple logistic regression was performed. Detailed results are described concerning the relationships between β_2AR expression in peripheral immune cells and anthropometric, metabolic, endocrine and inflammatory parameters.

3.2 Paper 2

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

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

Running head: Immune cells underexpression of β_2 adrenoceptors in obesity

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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 (PBMC) hypothesizing a distinct adrenergic pattern in inflammatory obesity. Methods: AR expression was assessed in blood donors categorized by waist circumference (WC) (CO: WC≥0.80 m in women and ≥0.94 m in men). Following a pilot study for all AR subtypes, we measured β_2AR 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_2AR mRNA. CO individuals showed underexpression of β_2AR relatively to those without CO (R=0.08; p=0.009). β_2AR 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_2AR expression (≥2x10-6) [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 (≅9:1) [OR 8.972 (95% CI) (1.679–47.949)]. Conclusion: PBMC β_2AR, 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_2AR as a new molecular marker of obesity subphenotypes in PBMC might contribute to clarify the adrenergic immunomodulation of inflammatory obesity.

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 ARs (α1, α2, 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_2AR), 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_2AR 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 (PBMC) [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 PBMC 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 PBMC 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 ware 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 PBMC 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 $\geq$0.80 m in women and $\geq$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 (Merecodia Leptin ELISA, Mercodia AB, Sylveniusgatan 8A, Uppsala, Sweden). High-sensitivity C-reactive protein (hsCRP) (mg/L) determined by nephelometry (CardioPhase® hsCRP–BnProSpec Siemens Healthcare 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].

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 $2x10^5$ 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 PBMC 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.

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 software™. Gene expression levels in a given sample were represented as $2^{-\Delta Ct}$ where ΔCt = [Ct(gene)–Ct (18S rRNA)].

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) = sqrta (0.01). By error propagation, the SE of a mean difference (delta-ct) is sqrt (2*0.01) and the SE for a difference of such differences (delta-delta-ct) is sqrt (2*2*0.01) = sqrt (0.04) = 0.2. This SE is determined on 2*(3-1) = 4 degrees of freedom, so

the 95% confidence interval has a half width of t[0.025;4]*0.2 = 2.77*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 (Δct=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 ± standard error of the mean (SEM). Non-normal distributed data was presented as median, 25^{th} and 75^{th} 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.

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 56% (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 [(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/μL (7.1 ± 0.4% of total leucocytes), of which 390 ± 19 cells/μL (89 ± 0.9% of all monocytes) were CD16- and 50 ± 7 cells/μL were CD16+ monocytes (11± 0.9% of all monocytes). Neither the number (Table 5) nor the percentage of CD16+ (11.5±0.9% vs 9.9±1.9%; p=0.410) and of CD16- monocytes (89±0.9% vs 90±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): the 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 PBMC

The pilot study considered PBMC 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_2AR mRNA levels. Indeed, the expression of β_2AR in subjects with CO was less than

half in comparison to those without CO. This finding led us to study β_2AR expression in a larger number of subjects.

We have found lower expression of β_2AR 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_2AR mRNA expression $\geq 2x10^{-6}$[odds ratio (OR) 0.177 with respective confidence interval of 95% (95% CI) (0.040-0.796)] and a higher association for women, relatively to men [≅9:1[OR 8.972 (95% CI) (1.679 – 47.949)]].

To evaluate the clinical relevance of decreased expression of β_2AR, we correlated β_2AR mRNA levels with metabolic/endocrine parameters. When considering all the individuals, the expression of β_2AR in PBMC 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_2AR 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 PBMC in sub-clinical inflammatory obesity. The main findings are fourfold. First, in central obesity, associated with inflammation, as shown by higher plasma levels of leptin and a more inflammatory pattern of monocytes, PBMC under express β_2AR in comparison to PBMC from non-CO subjects. Second, β_2AR expression was inversely correlated with a dyslipidemic lipid profile and with leptin plasma levels. Third, the multiple logistic regression analysis showed a lower and higher association, respectively for β_2AR expression ($\geq 2x10^{-6}$) and female for the occurrence of CO. Fourth, our work suggests a protective role of β_2AR expression in immune cells as well as adrenergic immunomodulation of inflammatory obesity.

β_2ARs are the most expressed ARs 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_2AR modulates cytokine production by activated innate immune cells, primarily inhibiting some 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 PBMC underexpression of β_2AR 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_2AR in PBMC 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_2AR 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_2AR 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_2AR 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, Devêvre 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_2AR 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_2ARs have been pointed out as a promising target in the pharmacotherapy of Multiple Sclerosis (MS) [36]. In MS patients, β_2AR 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_2AR 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_2AR 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_2AR 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_2AR 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_2AR) 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_2AR expression was evaluated in PBMC 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_2AR and it would be relevant to also measure its protein level. Even though, Guereshi et al. (2013) [45] found that β_2AR mRNA levels were higher in naıve T cells than in Treg cells and protein expression confirmed the results for β_2AR 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 β2-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 PBMC in inflammation related to obesity before we can truly apply this knowledge in a rational manner in clinical conditions. In this sense, evaluation of β2-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.

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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Abbreviations

CO: central obesity; *BMI:* body mass index; *WC*: waist circumference; *AT*: adipose tissue; *CA*: catecholamines; *AD*: adrenaline; *NA*: noradrenaline; *PBMC*: peripheral blood mononuclear cells; *AR*: adrenoceptors; *β_2AR*: adrenoceptor β_2; *CD*: cluster of differentiation; *SSC*: sideward light scatter; *SBP*: systolic blood pressure; *DBP*: diastolic blood pressure; *TC*: total cholesterol; *LDL-C*: low-density lipoprotein- cholesterol; *VLDL-C*: very low-density lipoprotein-cholesterol; *HDL-C*: high-density lipoprotein-cholesterol; *TAG*: triacylglycerol; *hsCRP*: high-sensitivity C-reactive protein; *ANOVA*: analysis of variance; *OR*: odds ratio; *EDTA*: ethylenediaminetetraacetic acid; *ELISA*: enzyme-linked immunosorbent assay.

Authors contributions

FL and LR contributed with the conception and study design, acquisition, analysis and interpretation of data, manuscript drafting and approved the final version.

ML contributed with the acquisition and interpretation of data, critical revision of the manuscript and approved the final version.

FM and MC contributed with data acquisition and interpretation, critical revision of the manuscript and approved the final version.

LR as corresponding author takes the responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final manuscript.

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

Abbreviations: *BMI*, Body Mass Index; *WC*, waist circumference; *SB*P, 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, 25^{th} and 75^{th} percentiles.

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

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

	Central obesity			
	No	Yes	*p*	Ratio Yes/No
n	5	10		
F/M	2/3	8/2	0.12	
Age	31 ± 3	40 ± 3	0.10	
BMI	21.1 ± 0.7	30.1 ± 2.4	0.02	
		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 ± standard error of the mean (SEM). F/M, female male ratio; BMI, body mass index; AR, adrenoceptor. For details see Methods.

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

Factor	*F*	*df*	*Mean ± 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 ± 2	37 ± 3	0.194
Weight (Kg)	3.077	1, 55	79 ± 2	72 ± 3	0.085
Height (m)	5.699	1, 55	1.65 ± 0.01	1.71 ± 0.01	**0.020**
BMI (Kgm^{-2})	14.126	1, 55	28.7 ± 0.6	24.4 ± 0.7	**<0.001**
WC (m)	26.869	1, 55	1.00 ± 0.016	0.86 ± 0.016	**<0.001**
SBP (mmHg)	0.000	1, 55	134 ± 2	134 ± 4	0.983
DBP (mmHg)	2.606	1, 55	82 ± 2	77 ± 2	0.112
Glycemia (mg/dL)	0.114	1, 55	85 ± 1	86 ± 1	0.737
HgA1c (%)	1.386	1,55	5.1 ± 0.1	5.2 ± 0	0.244
TC (mg/dL)	4.131	1, 55	200 ± 5	178 ± 9	**0.047**
LDL-C (mg/dL)	2.420	1, 55	127 ± 5	112 ± 8	0.126
HDL-C (mg/dL)	0.065	1, 55	50± 2	51 ± 4	0.800
VLDL-C (mg/dL)	4.596	1, 55	23 ± 2	16 ± 2	**0.036**
TAG (mg/dL)	3.726	1, 55	120 ± 12	79 ± 8	0.059
NA (pmol/L)	2.136	1, 55	1054 ± 129	706± 168	0.150
AD (pmol/L)	0.198	1, 55	167 ± 15	181 ± 30	0.658
Cortisol (μg/dL)	0.024	1, 55	15 ± 0.9	15 ± 1.0	0.877
Leptin (ng/mL)	8.116	1, 38	1.30 ± 0.25	0.15 ± 0.05	**0.007**

Abbreviations: CO, central obesity; *BMI*, body mass index; *WC*, waist circumference; *SBP*, systolic blood pressure; *DBP*, diastolic blood pressure; *HbA1c*, glycated hemoglobin; *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* degree of freedom; *p*, level of significance. *Pearson Chi-Square test was applied for the comparison of 2 categorical variables. Data are presented as mean ± standard error of the mean (SEM).

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

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

Abbreviations: *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 ± standard error of the mean (SEM); *F*, Snedcor's distribution; *df*, degree of freedom; *p*, level of significance. *Data presented as median (25^{th}-75^{th} percentiles) and Mann-Whitney test used for comparison between the two groups.

Comparison of β_2 Adrenoceptors expression in peripheral blood mononuclear cells between groups without and with Central Obesity

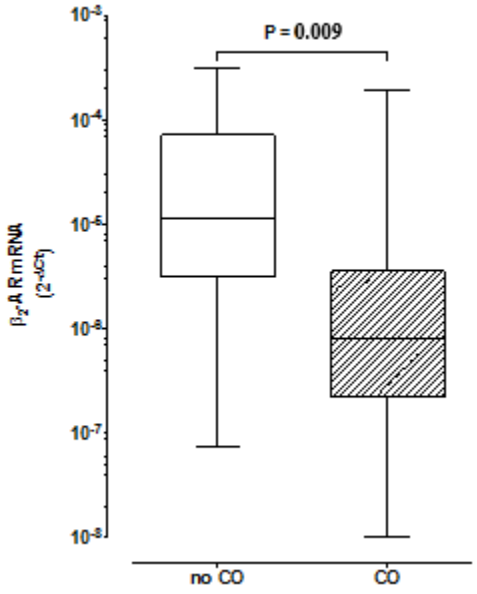


Figure 1 Levels of β2-AR mRNA in PBMC from individuals with and without Central Obesity. Boxes indicate medians with 25th–75th percentiles and whiskers indicate minimum and maximum values.Mann-Whitney test was used for comparison between the two groups. *Abbreviations: CO*, Central Obesity; *non- CO*, without CO; P, level of significance.

Chapter 4: Paper 3- Dopaminergic Receptors and Tyrosine Hydroxylase expression in Peripheral Blood Mononuclear Cells: a distinct pattern in Central Obesity.

4.1 Preface to Paper 3

While several lines of evidence support the involvement of DA in obesity through its role in the central nervous system no information exists so far regarding dopaminergic pathways in peripheral immune cells of obese subjects. We therefore decided to study the expression of dopaminergic receptors and tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of DA, in PBMC from blood donors and their relationship with anthropometric, metabolic and inflammatory parameters in CO. The expression of DR D_2 showed a protective role for the development of CO. We discuss a role for dopaminergic pathways in the crosstalk between immunity and metabolism and whether DR expressed on immune cells could represent pharmacological targets in obesity for better metabolic outcome.

4.2 Paper 3

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Dopaminergic Receptors and Tyrosine Hydroxylase expression in Peripheral Blood Mononuclear Cells: a distinct pattern in Central Obesity

Running title: Peripheral Immune Dopaminergic System in Central Obesity

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Abstract

Background: Dopamine (DA) may be involved in central obesity (CO), an inflammatory condition, through its role in the central nervous system and in periphery, where it may affect immune cell function through five different DA receptors (DR). Whether dopaminergic pathways in peripheral immune cells are implicated in the inflammatory condition linked to CO is however unknown.

Methods: In a cohort of blood donors with and without CO, categorized by waist circumference (WC) (CO: WC ≥0.80 m in women and ≥0.94 /m in men), we studied the expression of DR and tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of DA, in peripheral blood mononuclear cells (PBMC) and their relation with anthropometric and metabolic/endocrine and inflammatory parameters. DR D1-5 and TH expression was assessed by semi quantitative real-time PCR. As inflammatory markers, we investigated the immunophenotype of monocyte subsets by flow cytometry, staining for CD14, CD16, CD11b and CD36.

Results: CO individuals showed higher plasma levels of leptin and higher inflammatory pattern of monocytes compared with non-CO. PBMC expression of DR D2, DR D4 and DR D5 as well as of TH were lower in CO in comparison with non-CO. DR D2, and DR D5 expression correlated with lower WC and weight, and with lower inflammatory pattern of monocytes, and TH expression correlated with lower WC. DR D4 expression correlated with lower plasma levels of glycosylated hemoglobin, and DR D2 expression correlated with lower CO.

Conclusions: Results show that CO is associated with peripheral inflammation and downregulation of dopaminergic pathways in PBMC, possibly suggesting DR expressed on immune cells as pharmacological targets in obesity for better metabolic outcome.

Keywords: central obesity, dopamine, dopaminergic receptors, immune cells

Introduction

The modern obesity epidemic is a major public health issue, with the principal cause of morbidity due to metabolic dysfunction such as insulin resistance, type 2 diabetes, dyslipidemia and cardiovascular disease (1). Although it is widely accepted that a state of chronic low-grade inflammation is responsible for the metabolic dysfunction of obesity, its precise etiology is not completely characterized.

The expression of common receptors and signaling networks in immune cells and adipocytes is the basis of the immunological metabolic cross-talk that explains the inflammatory comorbidities of obesity (2). On the one hand, immune cells play a central role in adipose tissue biology and, on the other hand, adipocytes have been recently proposed as "immune cells" since they express, cytokines, chemokines, multiple receptors and cell molecules involved in the immune response (3). Visceral or central obesity (CO) is indeed regarded as an inflammatory disease (1), and recent research has been focused on the study of peripheral blood mononuclear cells (PBMC) in obesity. Indeed, PBMC are exposed to systemic factors, such nutrients and inflammatory molecules (4), and may constitute potential biomarkers of early homeostatic energy imbalance, and reducing inflammation could be useful in preventing the occurrence of obesity and its consequences (5).

Dopamine (DA), a classical brain neurotransmitter involved in various vital central nervous system functions, including feeding, reward and cognition, has been reported to modulate peripheral immune function (6–8). Indeed, DA-induced immunomodulation is currently the focus of intense experimental research and dopaminergic pathways are increasingly considered a target for drug development in immune disease (9). Immune cells themselves produce endogenous DA, and possibly uptake DA from other sources, using this transmitter

as an autocrine/ paracrine mediator (10–12). The first and rate limiting step of DA biosynthesis is the hydroxylation of the L-tyrosine to L-DOPA via tyrosine hydroxylase (TH). Once released, DA can bind to five distinct G protein-coupled receptors (13). Dopaminergic receptors (DR) are grouped into two families according to their pharmacological profile and main second messenger coupling: the D1-like (D1 and D5) which activate adenylate cyclase and the D2-like (D2, D3 and D4) which inhibit adenylate cyclase (13).

While several lines of evidence support the involvement of DA in obesity through its role in the central nervous system (CNS) (14–16) no information exists so far regarding dopaminergic pathways in peripheral immune cells of obese subjects. We therefore decided to study the expression of DR and TH in PBMC from a cohort of blood donors and their rel ation with anthropometric, metabolic and inflammatory parameters in central obesity.

Methods

Ethics statement

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

Study Population

Thirty blood donors from the blood bank of Clinical Haematology department of Centro Hospitalar of Porto were enrolled in the study. The individuals followed the selection criteria for blood donation and were not under any medicines for at least one month before enrolment. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice and the mean values were used in the analyses; readings were taken on the right arm, with subjects in the supine position after they had rested for 5 min.

Anthropometrics

Height (in m) was based on an identification document and confirmed by medical record. Body weight was measured to the nearest 100 g on electronic weight scales. Body mass index (BMI) was calculated by dividing weight by height squared and expressed in kg x m^{-2} and categorized as described before (17). Waist circumference (WC) was measured with a flexible tape at the level midway between the lowest rib and the iliac crest. CO was defined following International Diabetes Federation criteria by using WC (≥0.80m for women and ≥0.94m for men) (18).

Biochemical analysis

Blood samples were taken under standardized conditions. Fasting plasma glucose, triacylglycerol (TAG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), very low-density lipoprotein-cholesterol (VLDL-C), glycosylated hemoglobin (HbA1c) were measured using standard techniques. Plasma catecholamines (CA) [adrenaline (AD) and noradrenaline (NA)] were assayed by high pressure liquid chromatography with electrochemical detection (HPLC-ED) (CROMSYSTEMS). Leptin was measured in serum by solid phase two-site enzyme immunoassay (Merecodia Leptin ELISA) and high-sensitivity C-reactive protein (hsCRP) by nephelometry (CardioPhase® hsCRP – BnProSpec Siemens).

Flow Cytometry assay of monocytes

Monocytes were analyzed in whole blood samples by means of flow cytometry following a previously described technique (19). The enumeration and the immunophenotypic analysis of monocytes were performed in fresh EDTA-K3 anti-coagulated blood samples. Immunophenotypic studies were done using a whole blood stain-lyse-and-then-wash method and a direct immunofluorescence technique using 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). Information on a minimum of $2x10^5$ events was acquired for each staining and stored as list mode data. For data analysis, the Paint-a-Gate Pro software program (BD) was used. Monocytes were quantified based on the CD14 expression, while CD16 was used to differentiate classical (CD16-) and non-classical (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 scatter (FSC) and side scatter (SSC) of the cell subsets were also measured. In order to overcome inter-individual variations, we calculated the ratio for each parameter between non-classical CD14+CD16+ and classical CD14+CD16- monocytes in each individual.

Real time PCR analysis for the expression of DR and TH in PBMC

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll method) and DR and TH mRNA were assayed by real time PCR as previously described [20]. Briefly, total RNA was extracted from PBMC by PerfectPure™ RNA Cell & Tissue kit (5Prime), and the amount of extracted RNA was estimated by spectrophotometry at 260 nm. Total RNA was then 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). The thermal profile for each gene was: stage 1, 2 min at 50°C; stage 2, 10 min at 95°C; stage 3, 40 cycles including 15 s at 95°C and 1 min at 60°C. Further details about real-time PCR conditions are

shown in **Table 1.** 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 software™. Supporting information (S1 Fig) shows HKG levels (expressed as Ct) for the different genes, and also the comparison between the two groups of subjects. No difference is statistically significant and the range of the individual values is very narrow (about one cycle) and clearly superimposed between groups and throughout genes. In addition, HKG minor variations are not influential and likely due to small variations in the amount of cDNA pipetted for the PCR analysis. Gene expression levels in a given sample were represented as $2^{-\Delta Ct}$ where ΔCt = [Ct (gene) – Ct (18S rRNA)]. The ratio (R) was calculated for DR and TH mRNA expression between individuals with and without CO. R<0.5 was conventionally considered as under expression and R>2.0 as overexpression.

All the parameters were assessed in 30 (17 with and 13 without CO) individuals, except leptin and CD11b, assessed in 21 (11 with and 10 without CO) and 13 (8 with and 5 without CO), respectively.

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. Variables were summarized using relative and absolute frequencies or means±standard error of the mean (SEM), as appropriate. Non-normally distributed data were summarized as median, 25th and 75th percentiles. To compare the quantitative independent variables, we used bivariate ANOVA

or Mann-Whitney tests for normally and non-normally distributed data, respectively. The Pearson Chi-Square test was used to compare qualitative independent variables. Correlations were assessed by non-parametric Spearman rank analysis. Only those correlations with significance lower or equal to 0.01 were considered. The strength of association between variables was estimated by odds ratio (OR) and their respective 95% confidence interval (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).

Results

Characteristics of the study population

In this cohort of blood donors, the prevalence of central obesity was 57.7%, and the mean BMI was 30±1.5 and 24±0.7 kg.m-2, respectively in individuals with and without CO (Table 2). There were no differences between subjects without and with CO, with the exception of plasma leptin which was higher in CO.

Plasma CA levels were similar between WC established groups, but in CO AD correlated with leptin (r = 0.860; P = 0.001). In the whole population, total monocytes were 446±31 cells/μL and no differences were noticed between subjects with and without CO (415±34 vs 487±55, respectively; p = 0.253).

Total monocyte count as well as CD16+ and CD16- monocytes were not different between subjects with and without CO (Tables 2 and 3) however, in comparison to monocytes from subjects without CO, cells from subjects with CO showed lower SSC, CD14, CD11b, and CD36 ratios (Table 3), defining a higher inflammatory phenotype for pro-inflammatory monocytes.

Expression of DR and TH in PBMC

Compared to PBMC from subjects without CO, cells from individuals with CO expressed lower D1-like DR D5 (R = 0.29) and D2-like DR D2 (R = 0.13) and D4 (R = 0.47), as well as lower TH mRNA (R = 0.58) (Fig 1). In centrally obese individuals, the comparison between obese and non-obese BMI defined did not show differences in mRNA levels of TH and DR in PBMC (S1 Table).

Boxes indicate medians with 25^{th}–75^{th} percentiles and whiskers indicate minimum and maximum values. Mann-Whitney test used for comparison between the two groups. Abbreviations: Tyrosine hydroxylase (TH), Central Obesity (CO), DRD1 dopaminergic receptor type1, DRD2 dopaminergic receptor type 2, DRD3 dopaminergic receptor type 3, DRD4 dopaminergic receptor type 4, DRD5 dopaminergic receptor type 5, Significant differences are indicated (P value).

Correlation between DR and TH mRNA expression in PBMC and anthropometric, metabolic/endocrine and inflammatory markers

As shown in Table 4, in the whole cohort, the expression of DR D2 and DR D5 mRNA in PBMC was negatively correlated with weight, BMI, WC and leptin levels, and positively correlated with CD11b ratio in monocytes. Moreover, DR D4 mRNA was negatively correlated with HbA1c and TH mRNA was correlated with lower WC and leptin levels.

In the model of CO, the logistic regression analysis showed a lower association for the development of central obesity for DR D2 mRNA expression ≥0.0000000455 (odds ratio [OR] 0.018 confidence interval [CI] 95% 0002–0.195).

All data are included in the supporting information (S1 Dataset).

Discussion

This is the first work studying the expression of DR and TH in peripheral immune cells in obesity. Our findings show that CO is associated with inflammation, as shown by higher plasma levels of leptin and a more inflammatory pattern of non-classical monocytes, and that in CO PBMC exhibit a distinct pattern of DR and TH expression. In particular: (i) PBMC from subjects with CO show a reduction of the expression of DR D2, DR D4 and DR D5 as well as lower levels of TH mRNA in comparison to cells from the non-obese group; (ii) DR D2 and DR D5 expression in PBMC strongly correlates with lower weight, BMI and WC, lower plasma levels of leptin and with a lower inflammatory pattern, while DR D4 mRNA correlates with less HbA1c and TH mRNA correlates with lower WC and leptin levels; (iii) the expression of DR D2 may have a protective role against the presence of CO.

The relationship between DA and obesity has been subject of extensive research in the nervous system (14-16, 21). Scientific evidence has established an association between obesity and hyposensitivity of dopaminergic systems, both within the CNS (22) and in peripheral tissues (23). While DA brain functions are well known, its peripheral role is an emerging subject of study. An association between the intake of a high fat diet and alterations in brain DA levels (24, 25) has been already described, and Wang et al. (2001) reported that in obese DR D2 availability correlated negatively with BMI (22).

Till now, knowledge about the role of DA in peripheral tissues during obesity has been limited to its influence on pancreatic β cells regulating insulin release (26) and to the modulation of insulin effects on adipocytes (21). Indeed, Borcherding et al (2011) suggested a regulatory role of peripheral DA in adipose tissue functions as human adipocytes cell lines express DR (27).

In our study, we found decreased DR D2, D4 and D5 as well as TH mRNA levels in PBMC from subjects with CO. Decreased expression of DR D2 in PBMC has been already reported in other inflammatory immune mediated diseases such as Crohn's disease (28) and systemic lupus erythematosus (SLE) (29). Indeed, administration of DR D2 agonists reduced disease activity in patients with rheumatoid arthritis (30, 31) and decreased serum immunoglobulin and anti-DNA antibody levels in SLE patients (30). Single-nucleotide polymorphisms of DR D2, associated to lower expression and function, increase inflammation in human renal proximal tubule cells (32). In human lymphocytes, DR D2 agonists increase the secretion of anti-inflammatory cytokines (33). As regards DR D5, their reduction in PBMC has been previously reported in patients suffering from multiple sclerosis (34, 35), an immune-mediated inflammatory disease in which DR D5 expression in PBMC has been also suggested to predict the therapeutic response to immunomodulating treatment with interferon- β (35).

In our study, PBMC expression of DR D2 and D5 (and to a lesser extent also of D4 and TH) were shown to be associated with lower weight, with better metabolic/endocrine parameters and with a less inflammatory pattern. Moreover, logistic regression analysis suggests that DR D2 expression has a protective role for the existence of CO.

The correlations between the expression of DR and TH in PBMC and more favorable metabolic/endocrine and inflammatory parameters suggest a possible role for dopaminergic pathways in the crosstalk between immunity and metabolism. Indeed, adipose tissue express DR and DA regulates adipose tissue functions (27). So far, DA has been shown to have an inhibitory effect on leptin release from human adipocytes through D1-like DR receptors (27) and to modify the secretion of insulin in peripheral tissues (21). Yu et al. (2014) described a

negative effect through DR D4 activation on insulin action via decreasing insulin receptor expression (36). Peripheral blockade of DR could therefore result in increased insulin release and sensitivity, thus promoting adipogenesis, weight gain, insulin resistance, and ultimately type 2 diabetes (21), adverse metabolic effects also described for neuroleptics (37). DA inhibitory effects on the release and action of insulin may explain the inverse correlation between DR D4 expression in PBMC and HbA1c plasma levels found in the present work. The inhibitory effect of DA on leptin release mediated by DR is in agreement with the relationship found between DR expression and leptin, considering PBMC as early sensors for metabolic dysfunction. The major sources of DA in peripheral blood are neuronal fibers, adrenal medulla and neuroendocrine cells (38), and although the type of food ingested could affect blood DA levels (21, 39-41) and putatively the expression and function of DR, in our study blood samples were collected in a fasted state.

Our work has some limitations. First, we did not analyze DR at protein level. Even though, Araki et al (2007) showed that DR mRNA and membrane-associated protein changes correspond with each other; moreover, the same authors demonstrated that DR mRNA expression or receptor protein are valid surrogates for receptor function (42) and our group previously showed that DR responsiveness in human lymphocytes is better predicted by mRNA rather than membrane receptor expression (12).

Secondly, we do not know if the different pattern of DR and TH expression on PBMC associated with CO is a cause or consequence of inflammatory obesity. This open question should be answered prospectively with further studies addressing the effect of weight loss on DR expression in peripheral immune cells. Finally, DR expression was assessed in PBMC as a whole, and future studies should address the possible differential expression on distinct

mononuclear cell subsets (e.g. lymphocytes vs monocytes, T helper (TH) 1 vs Th2, regulatory T cells vs Th17).

Conclusions

To our knowledge, this is the first work studying the expression of DR and TH in peripheral immune cells in obesity. CO is associated with under expression of DR D2, DR D4 and DR D5, lower levels of TH mRNA in PBMC and with a higher innate inflammatory pattern. The expression of DR correlated with lower weight, with better metabolic profile and lower inflammatory pattern. The expression of DR D2 showed a protective role for the development of CO. Results thus indicate that CO is associated with peripheral inflammation and downregulation of dopaminergic pathways in PBMC, and warrant further research to establish whether DR expressed on immune cells could represent pharmacological targets in obesity for better metabolic outcome.

Supporting Information

S1 Dataset. Main study data.

(XLSX)

S1 Fig. Comparison of HKG levels (expressed as Ct) for the different DRD and TH genes between subjects with and without central obesity (CO).

(DOCX)

S1 Table. Comparison in central obesity (CO) of TH and DR expression in PBMC between individuals with and without obesity BMI defined.

(DOCX)

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Author Contributions

Conceived and designed the experiments: FL LR. Performed the experiments: FL ML FM. Analyzed the data: FL MC FM LR. Contributed reagents/materials/analysis tools: FL ML FM MC LR. Wrote the paper: FL MC LR.

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Competing Interests

The authors have declared that no competing interests exist.

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Table 1 Real-Time PCR gene expression. Data are from RefSeq - NCBI Reference Sequence Database (http://www.ncbi.nlm.nih.gov/refseq/)

Gene	UniGene ID	Interrogated Sequence	Translated Protein	Exon Boundary	Assay Location	Amplicon Length
TH	Hs.435609	NM_199292.2	NP_954986.2	3-4	424-422	63
DRD_1	Hs.2624	NM_000794.3	NP_000785.1	1-2	462-1620	110
DRD_2	Hs.73893	NM_000795.3	NP_000786.1	2-3	524	64
DRD_3	Hs.121478	NM_033663.3	NP_387512.3	3-4	809-725	73
DRD_4	Hs.99922	NM_000797.3	NP_000788.2	1-2	285-283	99
DRD_5	Hs.380681	NM_000798.4	NP_000789.1	1-1	1092-744	88
18SrRNA	X03205.1	N/A	N/A	N/A	N/A	187

Abbreviations:TH tyrosine hydroxylase, *DRD_1* dopaminergic receptor type1, *DRD_2* dopaminergic receptor type *2*, *DRD_3* dopaminergic receptor type *3*, *DRD_4* dopaminergic receptor type *4*, *DRD_5* dopaminergic receptor type *5*.

Table 2 Characteristics of overall the participants (n=30) and comparison between groups defined by Waist Circumference

		overall	*Central Obesity*				
Parameter	Reference values (range)	mean± SEM/∗	*F*	*df*	*No CO* (N=13)	*CO* (N=17)	*P*
Age (years)		41±2	0.795	1, 28	39±3	42±2	0.380
Weight (Kg)		76±3	3.302	1, 28	70±2	80±4	0.080
Height (m)		1.67±0.02	5.780	1, 28	1.71±0.01	1.64±0.02	0.023
BMI (kgm^{-2})		27.2±1.0	10.448	1, 28	24±0.7	30±1.5	0.003
WC (m)		0.95±0.03	13.492	1, 28	0.86±0.02	1.02±0.04	0.001
SBP (mmHg)	<130	134±3	0.120	1, 28	133±4	135±5	0.732
DBP (mmHg)	<85	79±2	0.362	1, 28	77±2	80±3	0.552
Glycemia (mg/dL)	70 - 105	85±1	0.200	1, 28	86±1	84±2	0.659
HgA1c (%)	3.8 - 5.6	5.2±0.06	2.070	1, 28	5.1±0.08	5.3±0.07	0.161
TC (mg/dL)	0 - 200	188±7	0.807	1, 28	181±10	193±9	0.377
LDL-C (mg/dL)	0 - 130	117±6	0.084	1, 28	115±9	118±8	0.774
HDL-C (mg/dL)	35 - 55	51±3	0.080	1, 28	50±4	52±4	0.780
VLDL-C (mg/dL)	3 - 56	20±2	2.901	1, 28	16±2	23±3	0.100
TAG (mg/dL)	40 - 160	99±10	2.855	1, 28	80±9	113±16	0.102
hsCRP* (mg/L)	< 1.0 low risk 1.0 <3.0 intermediate risk >3.0 high risk	1.575 (0.571-3.020)	-	-	0.801 (0.571-2.090)	1.840(0.672-3.560)	0.408
NA (pmol/L)	709 - 4019	879±131	0.444	1, 28	778±185	956±185	0.511
AD (pmol/L)	<328	167±17	0.172	1, 28	175±31	160±18	0.682

Cortisol (μg/dL)	6.2 – 19.4	15±0.8		0.439		1, 28	16±1	15±1	0.513
Leptin (ng/mL)	2.0 – 5.6	0.9±0.3		6.779		1, 19	0.138±0.047	1.626±0.542	0.017
Leucocytes (cells/μL)	4500 - 13000		6403±280	1.823	1, 28	5977±483	6729±319	0.188	
Monocytes (cells/μL)	400 - 500		446±31	1.361	1, 28	487±55	415±34	0.253	
Lymphocytes (cells/μL)	1000 - 4800		1951±96	3.729	1, 28	1748±123	2106±131	0.064	

Abbreviations: 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; *NA*, noradrenaline; *AD*, adrenaline; *F* Snedcor's distribution; *df* default freedom; *p* level of significance. Data are presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed by ANOVA. *Data presented as median, 25th and 75th percentiles and statistical analysis performed with Mann-Whitney U test for the differences between the 2 groups.

Table 3 Immunophenotypic characteristics of CD16+ and CD16- monocytes in overall of the study participants and categorized by CO

		overall			without CO		With CO		
		Mean±SEM	Ratio CD16+/CD16-	p*	Mean±SEM	Ratio CD16+/CD16-	Mean±SEM	Ratio CD16+/CD16	p-
cells/μL	CD16+	54±12	0.14±0.02	<0.001	57±21	0.12±0.03	53±13	0.15±0.03	ns
	CD16-	392±25			430±43		362±28		
cells%	CD16+	11.4±1.4	0.14±0.02	<0.001	10±2	0.13±0.03	12±2	0.15±0.03	ns
	CD16-	88.6±1.4			90±2		88±2		
FSC	CD16+	557±11	1±0.005	ns	546±18	1.00±0.01	560±13	1.00±0.01	ns
	CD16-	558±11			548±17		565±13		
SSC	CD16+	418±10	0.86±0.01	<0.001	435±17	0.89±0.02	405±11	0.84±0.01	0.022
	CD16-	486±8			488±13		483±10		
CD14	CD16+	875±109	0.41±0.03	<0.001	893±163	0.51±0.05	860±151	0.34±0.03	0.009
	CD16-	2340±322			1874±353		2561±476		
CD36	CD16+	307±30	0.43±0.03	<0.001	332±59	0.51±0.05	282±28	0.38±0.03	0.023
	CD16-	733±43			671±78		781±45		
CD11b	CD16+	106±25	0.49±0.05	0.050	90±35	0.64±0.05	115±35	0.40±0.06	0.015
	CD16-	304±112			143±54		405±174		

Abbreviations: *CO* central obesity; *FSC* forward scatter; *SSC* side scatter; *ns* not significant. Values of CD14, CD36 and CD11b expressed as fluorescence arbitrary units (AU). Data are presented as mean ± standard error of the mean (SEM). Differences between groups analyzed by ANOVA p, level of significance; p* significance between CD16+ and CD16- monocytes subsets.

Table 4 Correlations between dopaminergic receptors and tyrosine hydroxylase expression and anthropometric, metabolic/endocrine and inflammatory parameters

	TH		DRD_2		DRD_3		DRD_4		DRD_5	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Weight	ns		-0.502	0.005	ns		ns		-0.483	0.007
BMI	ns		-0.577	0.001	ns		ns		-0.557	0.001
WC	-0.529	0.003	-0.681	<0.001	ns		ns		-0.621	<0.001
HbA1c	ns		ns		ns		-0.605	<0.001	ns	
Leptin	-0.555	0.009	-0.739	<0.001	ns		ns		-0.618	0.003
Ratio CD11b	ns		0.927	<0.001	ns		ns		0.748	0.003

Abbreviations:*TH* tyrosine hydroxylase, *DRD_2* dopaminergic receptor type *2*, *DRD_3* dopaminergic receptor type *3*, *DRD_4* dopaminergic receptor type *4*, *DRD_5* dopaminergic receptor type *5*, *BMI*, Body mass index; *WC* waist circumference**; *HbA1c* glycosylated hemoglobin; *Ratio CD11b*, ratio between the expression of CD11b in CD16+ monocytes and the expression of CD11b in CD16- monocytes and t**he median fluorescence intensity of CD11b was assessed in each subset and expressed as fluorescence arbitrary units; ***r* correlation coefficients, calculated by Spearman's rho test; *P* level of significance; *ns* not significant if *P>0.01*.**

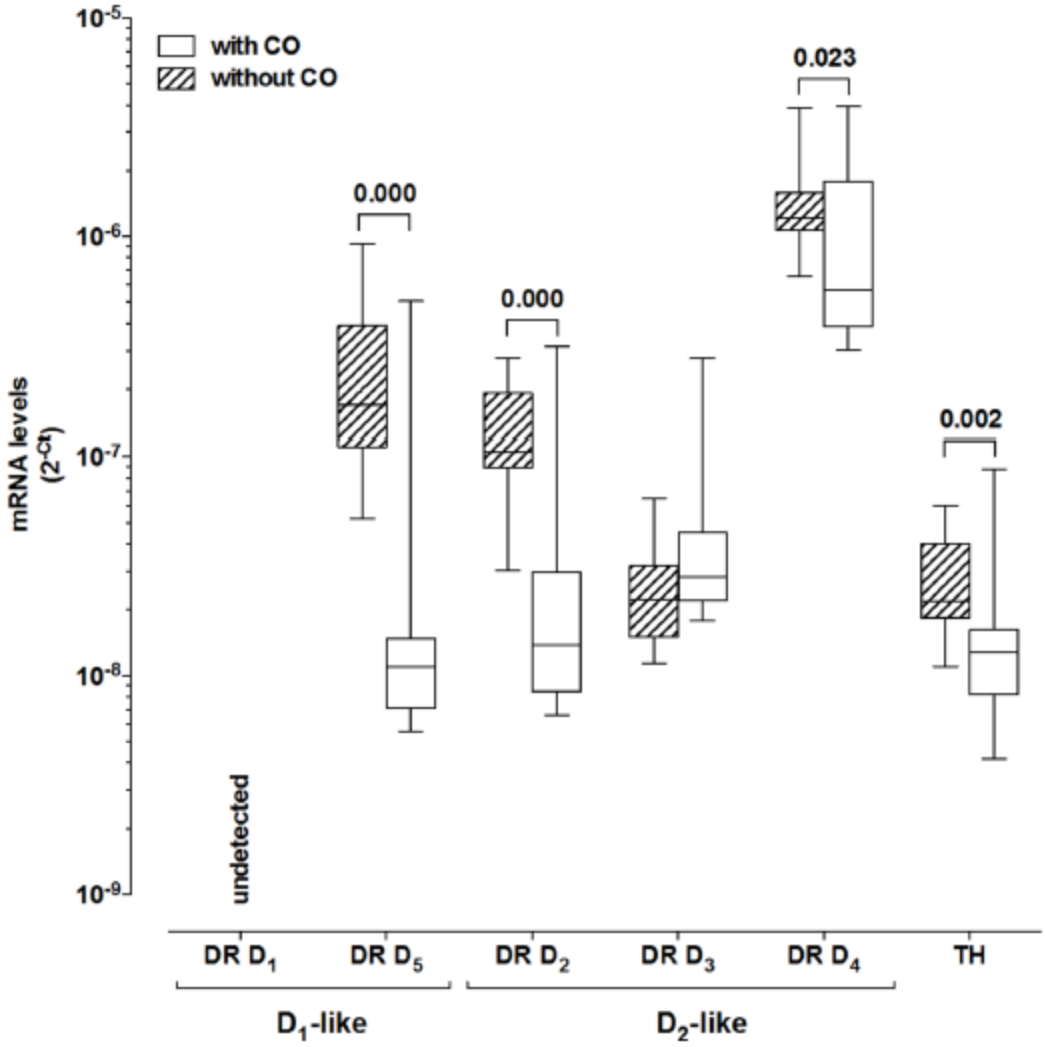


Fig 1. Comparison of the expression of Dopaminergic receptors in Peripheral Blood Mononuclear Cells between Groups with and without Central Obesity.

doi:10.1371/journal.pone.0147483.g001

Chapter 5: Paper 4 (Letter to Editor)- The effect of blood donor characteristics on transfusion outcomes: should obesity be assessed in future clinical trials?

5.1 Preface to Paper 4

Our results showed differences in peripheral immune cells concerning catecholaminergic receptors and tyrosine hydroxylase transcripts and phenotype between blood donors with and without inflammatory obesity. We also found different plasma levels of molecules with an immumodulating role such lipids and leptin. Taking into account the association between blood donor characteristics (BDC) and transfusion outcomes, we hypothesize that obesity is one characteristic that may affect transfusion recipient outcomes. Moreover, we performed a preliminary study in 63 BD to investigate the associations between BMI, WC, leptin and high-sensitivity C-reactive protein plasma levels as donor characteristics and red blood cells transfusion recipient mortality (data not published). We discuss the clinical and biological rationale for this hypothesis.

5.2 Paper 4

Leite F, Leite Â, Ribeiro L. The effect of blood donor characteristics on transfusion outcomes: should obesity be assessed in future clinical trials? *JAMA Intern Med*. 2017;177(4):599. doi:10.1001/jamainternmed.2017.0236.

The effect of blood donor characteristics on transfusion outcomes: should obesity be assessed in future clinical trials?

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The effect of blood donor characteristics on transfusion outcomes: should obesity be assessed in future clinical trials?

To the Editor: The work of Chassé and colleagues[1] in a recent issue of *JAMA Internal Medicine* reported that blood donor (BD) characteristics may affect red blood cell (RBC) transfusion outcomes and describe a higher risk of death for recipients of RBC transfusions from young donors and female donors independently of recipient age, sex, and comorbidities. The authors discuss these results mentioning the healthy donor phenomenon and the blood composition in female gender, not ruling out unknown biological/environmental factors.

Considered healthier than the general population, BD present cardiovascular risk factors, particularly, obesity. The health consequences for recipients of blood transfusion or bone marrow transplantation from donors with obesity are unrevealed notwithstanding being expected. The clinical and biological rationale for this hypothesis is based on scientific evidences widely accepted: i) obesity is an inflammatory disease and "inflamed donors", apart from showing altered immune cells at genomic, phenotype and function levels, also have in circulation immunoregulators such as leptin, lipids, extracellular vesicles, microRNAs signalling molecules implicated in adaptive and innate immune activation with a role in obesity and cardiovascular comorbidities[2]; ii) the signalling of leptin, a proinflammatory cytokine primarily produced by adipocytes proportionally to adiposity and present in higher levels in women, is associated with the occurrence, progression and prognosis of hematologic malignancies[3]; iii) it has been described low CD34+ cells harvest in overweight or obese mothers, candidates for stem cell donation of umbilical cord blood units, and in new-borns weighing above the average[4]. Although the composition of immune

cells in the allograft may affect the clinical outcomes of patients, the impact of donor weight/BMI on CD34+ cells remains controversial. Central obesity, a more sensitive obesity measurement for inflammation than body mass index (BMI), could, partially, explain the contradictory results in the association between BMI/weight and immune cell composition of allograft. Moreover, even leukodepleted, RBC have some residual leukocytes with stem cell function.

A population of BD with CO prevalence of 57.7%, showed higher inflammatory phenotype pattern of monocytes in CO compared with their counterpart[5]. The existence of "inflamed" donors is even more important when a significant part of the recipients is immunocompromised. Future clinical trials with vein-to-vein methodology are needed to evaluate if inflammatory obesity matters as a characteristic of donor on clinical outcomes of recipients of blood transfusion/hematopoietic stem cell transplantation.

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5. Leite F, Lima M, Marino F, et al. Dopaminergic receptors and tyrosine hydroxylase expression in peripheral blood mononuclear cells: A distinct pattern in central obesity. *PLoS ONE 2*016;11(1): e0147483.

Invited Commentary,[1] we need to leverage behavioral science and information technology to develop tools that discourage such inappropriate antibiotic use.

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Conflict of Interest Disclosures: None reported.

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Blood Donor Characteristics on Transfusion Outcomes—Should Obesity Be Assessed In Future Clinical Trials?

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Considered healthier than the general population, BDs present cardiovascular risk factors, particularly, obesity. The health consequences for recipients of blood transfusions or bone marrow transplantation from donors with obesity are unrevealed notwithstanding being expected. The clinical and biological rationale for this hypothesis is based on scientific evidences widely accepted: (1) obesity is an inflammatory disease and "inflamed donors," apart from showing altered immune cells at genomic, phenotype, and function levels, also have in circulation immunoregulators such as leptin, lipids, extracellular vesicles, microRNAs signaling molecules implicated in adaptive and innate immune activation with a role in obesity and cardiovascular comorbidities[2]; (2) the signaling of leptin, a proinflammatory cytokine primarily produced by adipocytes proportionally to adiposity and present in higher levels in women, is associated with the occurrence, progression, and prognosis of hematologic malignancies[3]; (3) low CD34+ cell count has been described in blood harvested from overweight or obese mothers, candidates for stem cell donation of umbilical cord blood units, and in newborns weighing above average.[4] Although the composition of immune cells in the allograft may affect the clinical outcomes of patients, the impact of donor weight and/or body mass index on CD34+ cells remains controversial. Central obesity, a more sensitive obesity measurement for inflammation than body mass index, could, partially, explain the contradictory results in the association between body mass index and/or weight and immune cell composition of allograft. Moreover, even leukodepleted, RBC have some residual leukocytes with stem cell function.

A population of BD with central obesity prevalence of 57.7%, showed higher inflammatory phenotype pattern of monocytes in central obesity compared with their counterpart.[5] The existence of "inflamed" donors is even more important when a significant part of the recipients is immunocompromised. Future clinical trials with vein-to-vein methodology are needed to evaluate if inflammatory obesity matters as a characteristic of donor on clinical outcomes of recipients of blood transfusion and/or hematopoietic stem cell transplantation.

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Conflict of Interest Disclosures: None reported.

1. Chassé M, Tinmouth A, English SW, et al. Association of blood donor age and sex with recipient survival after red blood cell transfusion. *JAMA Intern Med*. 2016;176(8):1307-1314.

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In Reply Leite and colleagues raise the hypothesis that part of the observed effect in the cohort in our study[1] may be due to donor obesity. This hypothesis is interesting and potentially important at a population level. Although the reporting of body mass index is currently not available in our donor cohort, we obtained the prevalence of obesity estimates from Statistics Canada[2] to evaluate how obesity could influence our results.

The overall prevalence of overweight and obesity in Canada in 2014 was 61.8% for men and 46.2% for women. This excess of obesity in men compared with women was observed across all age groups. As for age, the prevalence of obesity was 26% in adults younger than 20 years, 42% in adults between the ages 20 to 34 years, 54.5% in adults between the ages 35 to 44 years, and stabilized around 60% in adults older than 45 years. If we assume that the national distribution of obesity is similar in our donor cohort,[1] it

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would imply that young donors and female donors are less likely to be obese. Inferring from this indirect observation, obesity would therefore have to be protective for mortality to completely explain the observation.

We need to be extremely cautious of using indirect evidence and cannot rule out that a greater proportion of deaths occurred in recipients that received a greater number of units from obese donors. To do so would require individual-level as opposed to group-level obesity data. However, we agree fully with Leitz and colleagues that future investigations of the donor-recipient continuum would be strengthened by the collection of additional patient risk factors such as body mass index, which may influence the clinical outcome of transfusion recipients.

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Conflict of Interest Disclosures: None reported.

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CORRECTION

Missing Conflict of Interest Disclosure: In the article titled "Health and Health Care Use Among Individuals at Risk to Lose Health Insurance With Repeal of the Affordable Care Act," a conflict of interest disclosure was missing for Dr Kanzaria-Mandic. This article has been corrected online.

1. Kanzaria-Mandic P, Jena AB, Ross JS. Health and health care use among individuals at risk to lose health insurance with repeal of the Affordable Care Act [published online January 20, 2017]. *JAMA Intern Med*. doi:10.1001/jamainternmed.2016.9541

Incorrect Conflict of Interest Disclosure and Incorrect Author Affiliation: In the article titled "Effect of Testosterone Treatment on Volumetric Bone Density and Strength in Older Men With Low Testosterone: A Controlled Clinical Trial," the conflict of interest disclosures and author affiliation were incorrect for Dr Kearney. This article has been corrected online.

1. Snyder PJ, Kopperdahl DL, Stephens-Shield AJ. Effect of testosterone treatment on volumetric bone density and strength in older men with low testosterone: a controlled clinical trial [published online February 21, 2017]. *JAMA Intern Med*. doi:10.1001/jamainternmed.2016.9539

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Chapter 6: Discussion

6.1. Summary of Findings

In this thesis project, our first drive was to find early markers for inflammatory obesity in order to precociously intervene therapeutically and contribute for prevention of comorbidities obesity-associated that threaten human life span.

Considered healthier than the general population due to donation eligibility requirements, blood donors were deliberately chosen as a low CV risk subjects. In our sample, the central adiposity measure, contrary to general, was sensitive to metabolic risk factors and to a more pro-inflammatory pattern of CD16+ monocytes (reflecting a higher activation of the innate immune system) with no expansion of this monocyte subset. In CO, lower CD14 expression on CD16+ cells as well lower cellular complexity, in comparison to their counterpart, describe a more mature monocyte population claimed with higher capacity of migration and recruitment into the adipose tissue (AT).

According to our results, studies in human chronic low-grade pathologies associated with inflammation did not find changes in CD16+monocytes counting (1-3). Several works aimed at functional characterization of the monocyte subsets have been undertaken by different research groups (4-7) with contradictory results. This fact can be explained by lack of a standardized gating strategy for immunophenotyping by flow cytometry and by the use of different protocols to isolate purified monocytes (8). We performed the experiments in whole blood, avoiding purification protocols that can contribute to significant changes in receptor expression.

Multiple linear regression analysis was performed to identify the variables that best predict CO after controlling for potential confounders. A model, including leptin, ratio CD14,

VLDL-C, was found to be predictor of more than one third of CO. It is well-established that leptin promotes activation and proliferation of circulating monocytes, their differentiation into macrophages and inducing the secretion of proinflammatory cytokines (such as IL-1, TNF or IL-6 in synergy with lipopolysaccharide (LPS)), stimulates the oxidative burst and can enhance chemotactic responses by acting as a chemoattractant in monocyte and/or macrophages. Despite its immune regulatory role on human monocytes (9), leptin could not be the only factor explaining the CD16+ inflammatory pattern found in centrally obese, as its levels were also elevated in general obese. On the other hand, centrally obese individuals showed significantly higher levels of TAG, TC and VLDL-C comparing to non-centrally obese and these differences were not noticed between BMI groups. Circulating lipids may modify immune cells function (10, 11) being responsible for the production of intercellular signaling molecules. The hepatic overproduction of VLDL seems to be the primary and crucial defect in obesity (12). VLDL particles, exposed to lipoprotein lipase in the peripheral circulation which hydrolyses the triglyceride in VLDL particles, generate FFAs. Saturated FFAs, originated from hypertrophied AT, constitute a chemoattractant factor with a key role in AT monocytes recruitment. These lipids may directly induce inflammatory pathways in adipocytes through the activation of cell receptors producing inflammatory cytokines. Thus, in centrally obese, dyslipidemia, resulting from metabolic and visceral AT dysfunction, and leptin may contribute to a more inflammatory phenotype of circulatory CD16+, an early event of inflammatory obesity. Our work suggests that in subclinical inflammatory obesity, dyslipidemia and leptin could induce monocytes maturation process rather than an independent arise of CD16+ monocytes.

CD16+ monocytes seem to reside in a marginal pool mobilized by a catecholamine-dependent pathway (13). In CO, but not in BMI-defined obese, AD is associated with the expansion of CD16+ monocyte subset, but with a less inflammatory phenotype.

In line with the results of Thorand *et al*. (14), our study describes a stronger association of inflammation and obesity in female. Indeed, women showed higher levels of leptin, that, as previously mentioned, is an important immunomodulator usually associated to inflammatory immune responses (9). Moreover, CA behave differently by gender (14). AD plasma levels were associated with a lower inflammatory CD16+monocytes phenotype in women and with a higher level of inflammation in man. In opposite, NA was associated with a higher inflammatory phenotype only in female. These findings could be explained by higher percentage of adiposity in female and differences in endogenous hormones.

In obesity, the identity of immunoreceptors sensing metabolic-stress signals and triggering inflammation is largely unknown. PBMC, our model of immunity, are modulated by CA, being able to produce and utilize DA, NA and AD as mediators acting through activation of AR and DR expressed in immune cells (15). Our first work suggested that CA (AD and NA) have an immunomodulatory role in obesity. In the second part of our work, we hypothesized the existence of a different pattern of the expression of catecholaminergic receptors in PBMC in inflammatory obesity and their association with a different metabolic profile and inflammatory sate.

The preliminary results obtained through a pilot study considering PBMC expression of all the 9 AR (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3) in a subgroup of subjects with and without CO revealed no differences between groups except for β_2AR mRNA levels. The expression of β_2AR in the subjects with CO was less than half in comparison to that in individuals without CO. This finding led us to study β_2AR expression in whole population

showing that β_2AR in PBMC were under expressed in CO individuals in comparison to non-CO subjects. The multiple logistic regression analysis showed a lower and higher association, respectively for β_2AR expression ($\geq 2x10^{-6}$) and female gender for the occurrence of CO.

β_2AR transcripts correlated with HDL-C and inversely with TAG, VLDL-C and leptin plasma levels. AD and NA plasma levels were associated with dyslipidemia. As stated before, dyslipidemia in obesity is characterized by an increase of FFAs, ligands for TLR4. The activation of TLR4 induces downstream signaling of the nuclear factor-kappa B (NF-kB) and consequently the expression of pro-inflammatory cytokines with down regulation of β_2AR turning the immune cells responsiveness (10). Our results are in line with the influence of cellular lipid homeostasis on the levels, phenotype and function of immune cells (16) since in centrally obese HDL-C levels were positively related with β_2AR mRNA and inversely with AD. On the other hand, NA, an independent factor for the development of metabolic syndrome (17), correlated not only with TC and LDL-C, but also with the number of "pro-inflammatory monocytes" and hsCRP, findings that corroborate an inflammatory role for CA in inflammation obesity-associated (18). β_2AR expression in PBMC might have a protective role for the presence of CO being associated with a better metabolic lipid profile.

A possible explanation for PBMC β_2AR under expression in CO could be desensitization and down-regulation resultant from the action of circulating or endogenously produced CA, leptin and other cytokines and lipids. β_2ARs are the most expressed ARs on immune cells and considered the main mediators of CA immune effects; their activation on human monocytes is usually antiinflammatory and immunosuppressive (19, 20).

Our work supports the immunomodulatory role of β_2AR in central obesity since β_2AR mRNA inversely correlated with the plasma level of leptin, a pro-inflammatory adipokine (9) and leptinemia correlated with hsCRP levels and with a more inflammatory phenotype of monocytes in the high hsCRP cardiovascular risk group. Leptin receptors, found in monocytes and lymphocytes (21) mediate the production of proinflammatory cytokines by these cells (22). In centrally obese, leptin plasma values were also correlated with the hsCRP, also synthesized by adipocytes (23) and described as able to affect β_2AR function (24).

Further studies are needed to elucidate whether the altered pattern of β_2AR and monocytes observed in an early phase of CO is a cause or consequence of inflammatory obesity. The lower expression of β_2AR in PBMC of CO individuals is in accordance with the inflammatory pattern of visceral obesity and as a result of the effects of leptin and lipids on immune cells. Our findings are in line with a decreased number of β_2AR in PBMC from patients with inflammatory immune mediated diseases (25-29) and with critical heart disease, a chronic low-intensity inflammation condition, also associated with reduced β-AR on lymphocytes due to a non-regulated increased release of proinflammatory cytokines (29).

While several lines of evidence support the involvement of DA in obesity through its role in the central nervous system no information existed regarding dopaminergic pathways in peripheral immune cells of obese subjects. We therefore decided, in a third experimental work, to study the expression of DR and TH, the rate-limiting enzyme in the synthesis of DA, in PBMC.

PBMC from subjects with CO showed a reduction of the expression of DR D2, DR D4 and DR D5 as well as lower levels of TH mRNA in comparison to cells from the non-obese group. Decreased expression of DR D2 and/or DR D5 in PBMC has been already reported

in other inflammatory immune mediated diseases (30, 31). In human lymphocytes, DR D2 agonists increase the secretion of anti-inflammatory cytokines (32).

DR D2 and DR D5 expression in PBMC strongly correlates with lower weight, BMI and WC, lower plasma levels of leptin and with a lower inflammatory pattern, while DR D4 mRNA correlates with less HbA1c and TH mRNA correlates with lower WC and leptin levels.

That is, PBMC expression of DR D2 and D5 (and to a lesser extent also of D4 and TH) were shown to be associated with lower weight, with better metabolic/endocrine parameters and with a less inflammatory pattern. Moreover, logistic regression analysis suggests that DR D2 expression has a protective role for the existence of CO.

The correlations found between the expression of DR and TH in PBMC and more favorable metabolic/endocrine and inflammatory parameters suggest a possible role for dopaminergic pathways in the crosstalk between immunity and metabolism. Indeed, AT express DR and DA regulates AT functions (33). So far, DA has been shown to have an inhibitory effect on leptin release from human adipocytes through D1-like DR receptors (33) and to modify the secretion of insulin in peripheral tissues (34). Peripheral blockade of DR could therefore result in increased insulin release, thus promoting adipogenesis, weight gain, insulin resistance, and ultimately T2D (34), adverse metabolic effects also described for neuroleptics (35). DA inhibitory effects on the release and action of insulin may explain the inverse correlation between DR D4 expression in PBMC and HbA1c plasma levels. The inhibitory effect of DA on leptin release mediated by DR is in agreement with the relationship found between DR expression and leptin, considering PBMC as early sensors for metabolic dysfunction.

The results of this experimental work indicate that CO, associated with peripheral inflammation and downregulation of dopaminergic pathways in PBMC, warrant further research to establish whether DR expressed on immune cells could represent pharmacological targets in obesity for better metabolic outcome. We have commented that dopaminergic pathways could represent a promising approach clarifying the biological basis of obesity, claimed by Hotamisligil (36, 37), regulating the immune response in metabolic disease clusters. Targeting DA might allow simultaneously target obesity and inflammation.

It has been approached the issue of transfusion outcomes of blood recipients, already immunocompromised, from "inflamed blood donors". Since blood donor's characteristics have implications on transfusion outcomes (38) and we found differences between centrally obese and non-centrally obese considering immune cells characteristics and circulating immunomodulators molecules, we questioned whether obesity should be included in future clinical trials. The health consequences for recipients of blood transfusions or bone marrow transplantation from donors with obesity are unrevealed notwithstanding being expected. The clinical and biological rationale for this hypothesis is based on scientific evidences widely accepted: (i) obesity is an inflammatory disease and "inflamed donors," apart from showing altered immune cells at genomic, phenotype, and function levels, also have in circulation immunoregulators such as leptin, lipids, extracellular vesicles, microRNAs signaling molecules implicated in adaptive and innate immune activation with a role in obesity and cardiovascular comorbidities (39); (ii) the signaling of leptin, a proinflammatory cytokine primarily produced by adipocytes proportionally to adiposity and present in higher levels in women, is associated with the occurrence, progression, and prognosis of hematologic malignancies (40); (iii) low CD34+ cell count has been described in blood harvested from overweight or obese mothers, candidates for stem cell donation of umbilical

cord blood units, and in newborns weighing above average (41); (iv) although the composition of immune cells in the allograft may affect the clinical outcomes of patients, the impact of donor weight and/or BMI on CD34+ cells remains controversial. CO, a more sensitive obesity measurement for inflammation than BMI, could, partially, explain the contradictory results in the association between BMI /weight and immune cell composition of allograft. Moreover, even leukodepleted, red blood cells have some residual leukocytes with stem cell function. Inflammatory obesity, concerning the effect of donor's characteristics on transfusion outcomes, should be considered in blood donor's selection process. If vein-to-vein database will be able to be collected and confirms this hypothesis, it is expectable to have consequences in Transfusion Medicine policies concerning effectiveness and safety issues.

6.2 Potential impact

This PhD thesis significantly contributes to the discussion on two major public issues. The findings of a predictor model and the protective role of β_2AR and DR D2 expression in peripheral immune cells for inflammatory obesity are expected to impact, firstly on early diagnosis with implementation of WC measurement at primary care settings to define inflammatory obesity, and secondly, on therapeutic interventions in inflammation obesity-associated.

β_2AR agonists are currently used as bronchodilating agents in asthma, however the relative contribution of any eventual immunomodulating activity to their overall therapeutic effects remains to be established. Hence, evaluation of these drugs as potential antiinflammatory drugs is strongly warranted.

Evidence also suggests the opportunity to apply a pharmacogenetics approach to better understand adrenergic modulation of the immune response and in particular of innate immunity. β_2AR has mutant forms (42), nonetheless, the present knowledge about the adrenergic modulation of innate immunity supports relevant therapeutic applications. Moreover, as adrenergic pathways also provide a link between stressful events and chronic inflammatory disease such as obesity and atherosclerosis (43), investigating neuroimmune adrenergic mechanisms will likely provide several opportunities to repurpose the wide array of sympathoadrenergic agents currently used in medical therapy for various non-immune indications for novel and previously unanticipated indications.

6.3 Limitations

The expression of adrenergic and dopaminergic receptors was evaluated in PBMC and 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 studied AR and DR transcripts and it would be relevant to also measure their protein levels. Nonetheless, Guereshi *et al*. (44) found that β_2AR mRNA levels were higher in naıve T cells than in Treg cells and protein expression confirmed the results for β_2AR transcripts. Studies examining the distribution of cells expressing DR mRNA and DR binding sites have shown a good agreement between distributions (45). It has been postulated that DA receptors in peripheral blood lymphocytes reflect the status of homologous brain receptors (46, 47). In addition, DR mRNA and membrane-associated protein changes correspond with each other considering DR mRNA expression or receptor protein valid surrogates for receptor function (48). Our group also

showed in human lymphocytes that DR responsiveness is better predicted by mRNA rather than membrane receptor expression (49).

Secondly, we could not answer whether the different pattern of AR, DR and TH expression on PBMC associated with CO is a cause or consequence of inflammatory obesity. This open question should be answered prospectively with further studies addressing the effect of weight loss on AR and DR expression in peripheral immune cells.

6.4 Conclusions

This thesis has the novelty of identification and prediction of subclinical inflammatory obesity. Determination of plasma leptin and VLDL-C levels and CD14 expression in monocytes subsets predict CO in more than 33%. The study herein described supports in subclinical inflammatory obesity a process of monocytes maturation rather than an independent arise of CD16+ cells induced by dyslipidemia and leptin. Moreover, our work provides important insights explaining the stronger association of CO measures with cardiometabolic risk in comparison with general obesity assessed by BMI. In addition, the implementation of WC, in a public health setting, should be a priority tool for inflammatory obesity screening in low cardiovascular risk populations.

Monocytes of centrally obese sense metabolic/inflammatory circulating factors and these signaling molecules induce a maturation process, rather than an expansion, revealed by a macrophage-like phenotype with enhanced antigen-presenting capacities, higher endothelial affinity, production of proinflammatory cytokines and probable more prone to migrate into AT where they could differentiate into macrophages.

This is the first work studying the expression of AR, DR and TH in peripheral immune cells in obesity, suggesting immunomodulation by CA in this inflammatory condition involving the expression of catecholaminergic receptors.

The expression of DR D_2 and β_2AR in immune cells may have a protective role for the development of CO. In obesity, the increased of inflammatory regulators molecules such lipids, leptin and other inflammatory cytokines induce changes at genetic, phenotype and consequently at functional levels of peripheral immune cells. This inflammatory systemic environment downregulates catecholaminergic receptors in peripheral immune cells, mainly those receptors usually associated to anti-inflammatory responses. Both processes perpetuate a vicious cycle of inflammatory cell recruitment and secretion of deleterious adipokines and FFAs by AT that predispose to metabolic dysfunction.

The expression of catecholaminergic receptors in PBMC correlated with lower weight, with better metabolic profile and lower inflammatory pattern. More studies are needed to know whether AR and DR expressed in immune cells could represent pharmacological targets in obesity to promote a lower inflammatory state and reverse impaired metabolism.

6.5 Future Directions

Our findings seem very challenging to further investigate in an early phase of CO the phenotypic pattern of circulating monocytes, and its relationship with migratory and inflammatory functions.

Experimental strategies to investigate the immunomodulatory role of endogenous CA can be performed interfering with synthesis/degradation, intracellular storage/release/uptake,

through effect of receptor blockade and/or use of non-pharmacological approaches, e.g., suppression of expression of key proteins by means of gene silencing techniques.

Further studies are needed to elucidate whether the altered pattern of catecholaminergic receptors expression in immune cells observed in an early phase of CO is a cause or consequence of inflammatory obesity. It would be interesting to study prospectively the effect of weight loss on the expression of the catecholaminergic receptors in immune cells whose transcripts were decreased in inflammatory obesity.

Results thus indicate that CO is associated with peripheral inflammation and downregulation of adrenergic and dopaminergic pathways in PBMC, and warrant further research to establish whether AR and DR expressed on immune cells could represent pharmacological targets in obesity for better metabolic outcome. An important challenge now is to understand how AR and DR function on PBMC in inflammatory mechanisms 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 and DR D2 agonists as potential anti-inflammatory drugs is strongly warranted.

The results obtained in this thesis raised a very pertinent question regarding the characteristics of blood donors which may affect transfusion outcomes in patients. Blood donors with CO showed differences in circulating immune cells and signaling molecules traducing a more inflammatory state. Immune transfusion reactions may affect recipient outcomes and are likely to be related to donor characteristics. Until the present, the main exposures that have been studied included donor age and donor sex, with primary outcome recipient survival analysis post-red blood cells transfusion. Our work adds grounds for future clinical trials to include the assessment of obesity (50), namely central, to prospectively study transfusion outcomes covering a clinically representative range of

adverse short and long-term events after transfusion (mortality, cardiovascular, oncology, mortality, infections, renal). Answering the question of donors obesity effects on patients outcomes may improve blood recipient´s health and will impact on decision makers in transfusion policy.

6.6 References

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Appendix - Prizes, Grants and Awards

Prize- Boston, USA – American Association of Blood Banks- 2012- TOP POSTER ABSTRACT-entitled "Does Obesity Matter in a Blood Donor Population?"

Honorable Mention of Basic Investigation with the work: Fernanda Leite, Ana Santos, Margarida Lima, Maximiliano Legnaro, Franca Marino, Marco Cosentino, Laura Ribeiro: "Immune cells dopaminergic receptors and tyrosine hydroxylase expression in obesity" (24th November 2013- by the Portuguese Society for the Study of Obesity SPEO)

Investigator´s Grant (2014) by the European and the International Societies of Hypertension together with the Scientific Committee of the Joint Meeting ESH-ISH Hypertension, Athens, Greece.

Travel awards by the American Society of Cell Biology in 2012, 2013 and 2014. http://ascb.org/meetings/program/web/files/assets/basic-html/page28.html

Travel & accommodation grant by the Center for Research in Medical Pharmacology, University of Insubria, Varese – Italy, November 2013.



