Faculdade de Medicina da Universidade do Porto

Serviço de Higiene e Epidemiologia

Obesity and Inflammation: associated polymorphisms

Mestrado em Medicina e Oncologia Molecular

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BEST REGARDS

Ao Professor Henrique Barros, obrigada pela oportunidade, pelos sábios conselhos e pela orientação da tese.

A todos os colegas do Serviço de Higiene e Epidemiologia, obrigada por me receberem, pelas opiniões e pelo contributo que deram a este trabalho.

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Joana Barroso

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ABBREVIATIONS LIST

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BMI – Body Mass Index

CRP – C-reactive protein

Fc - Fragment crystallizable

GPIIb/IIIa – Glycoprotein IIb/IIIa

GTPases - Guanine triphosphatases

IKK - I Kappa B Kinase

IL1β – Interleukin 1 beta

IL-6 – Interleukin 6

ICAM-1 - Intercellular cell adhesion molecule-1

MCP-1 - Chemotactic factor monocyte chemoattractant protein-1

(NF)-κβ - Nuclear factor

TNFα – Tumor Necrosis Factor alpha

VCAM-1 - Vascular cell adhesion molecule-1

WC – Waist Circumference

WHO – World Health Organization

WHR – Waist-hip ratio

ABSTRACT

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Objective: Obesity has been characterized by a state of chronic low-grade inflammation, given that increased levels of the inflammatory markers have been related with adiposity. The assumption is that adipokines, cytokines, and other factors produced and released by fat are responsible for the chronic inflammatory state of obesity. Once that IL-6, IL-1 and TNF are increased in adipocytes in the obese state and are early-acting inducers of inflammatory cascades, genetically determined subsets of the population may have altered acute phase responses to certain stimuli. Therefore, we studied the influence of fat distribution in the inflammatory outcome phenotype of specific polymorphisms affecting genes enconding pro-inflammatory cytokines.

Design: Cross-sectional study.

Subjects: 411 non-institutionalized inhabitants of Porto, Portugal.

Measurements: Participants answered a structured questionnaire and were genotyped for the following polymorphisms: IL-6 -174 G/C, IL1β -511C/T, TNFα -308G/A. Analytical and anthropometrics measurements were obtained after 12 h fasting. CRP, fibrinogen, leukocytes and uric acid levels were measured.

Results: Genotyping of the IL-6 -174 G/C polymorphism was performed in 322 people. There were 144 (44.7%) participants with GG genotype, 132 (41.0%) GC heterozygotes, and 46 (14.3%) CC homozygotes. It was found a significant association between waist circumference and C carriers – GC (β=0.039, p<0.001) and CC (β=0.037, p=0.006), within C-reactive protein. No interaction was found between waist circumference and C carriers, in relation to leukocytes, but this association became statistically significant after adjustment for gender, age and smoking habits when comparing GG homozigotes with heterozigotes GC (β=0.022, *p*=0.018) and with homozigotes CC (β=0.045, *p*=0.020). There is a significant association between waist circumference and C carriers in relation to uric acid levels – GC (β=0.392, *p*<0.001) and CC (β=0.485, *p*=0.007). In relation to

fibrinogen, it was found a significant association between waist circumference and homozigotes GG (β=-0.002, *p*=0.015) and GC genotype (β=0.016, *p*=0.006).

Genotyping of the IL1β -511 C/T polymorphism was performed in 254 subjects. There were 110(43.3%) participants with CC genotype, 106(41.7%) heterozygotes CT, and 38(15.0%) homozygotes TT. It was found no interaction between waist circumference and homozigotes TT, in relation to CRP concentrations. The interaction of homozigotes CC (β=0.027, *p*<0.001) and heterozigotes CT (β= -0.027, *p*<0.001) with WC showed an effect on CRP concentrations, even after adjustment for gender, age and smoking habits. In relation to leukocytes, there is no interaction between waist circumference and C carriers, but once adjusted for gender, age and smoking habits, the interaction between waist circumference and CC homozigotes (β=0.028, *p*=0.009) and heterozigotes CT (β= -0.026, *p*=0.018) affected leukocyte levels. No interaction was seen between waist circumference and homozigotes TT, in relation to uric acid concentrations. The interaction of homozigotes CC (β=0.586, *p*<0.001) and heterozigotes CT (β= -0.543, *p*<0.001) with WC showed an effect on uric acid levels, even after adjustment for gender, age and smoking habits. The interaction of homozigotes CC (β=0.016, *p*=0.038) and heterozigotes CT (β=-0.018, *p*=0.021) with WC showed an effect on fibrinogen concentrations, and no interaction was found between waist circumference and homozigotes TT.

Genotyping of the TNF-α -308 G/A polymorphism was performed in 308 subjects. There were 228(74.0%) participants with GG genotype, 76(24.7%) heterozygotes GA, and 4(1.3%) homozygotes AA. It was found no interaction between waist circumference and homozigotes GG and AA, in relation to CRP concentrations. The interaction of heterozigotes GA with WC showed an effect on CRP concentrations (β= 0.038, *p*<0.001), even after adjustment for gender, age and smoking habits. There is also no interaction between waist circumference and GG, GA and AA genotypes, in relation to leukocytes concentrations. The interaction of homozigotes GG and GA with WC showed an effect on uric acid concentrations (β=0.056, *p*<0.001 and β=0.410, *p*=0.003, respectively), even after adjustment for gender, age and smoking habits. It was found no interaction between

waist circumference and genotypes GG, GA and AA, in relation to fibrinogen concentrations. After adjustment for gender, age and smoking habits, the interaction of homozigotes GG (β=-0.002, *p*=0.034) and heterozigotes GA (β= -0.017, *p*=0.001) with WC showed an effect on fibrinogen concentrations.

Conclusions: For the analysed polymorphisms, there is an interaction with waist circumference in relation to at least one inflammatory marker level.

INTRODUCTION

INTRODUCTION

An important recent development in our understanding of obesity is the emergence of the concept of a state of chronic low-grade inflammation [1], as indicated by increased levels of inflammatory markers as C-reactive protein. The current working hypothesis is that adipokines, cytokines, and other factors produced and released by fat induce a chronic inflammatory state. Since IL-6, IL-1 and TNF are early-acting inducers of inflammatory cascades, genetically determined subsets of the population may have altered acute phase responses to certain stimuli.

INFLAMMATION

The word inflammation comes from the latin “inflammare” (to set on fire). The cardinal signs of acute inflammation were described centuries ago as redness, heat, swelling and pain [2]. Thus, in its origin, inflammation was defined by a combination of clinical signs and symptoms, not by specific pathophysiology. This definition according to clinical signs and symptoms had limitations, as in most cases the cellular processes and signals that underlie the cardinal signs occur at a subclinical level and do not give rise to heat, redness, swelling, or pain. In the 19th century new definitions arose for inflammation as a non-specific complex stereotypical cellular response that follows trauma [2]. Advances in molecular biology placed additional complexity on this model, disclosing that tissue may be influenced by proinflammatory signalling molecules, even in the absence of inflammatory cell invasion and that aspects of both inflammation and repair can be triggered and modulated by primary events occurring outside the vasculature, such as vibration, hypoxia, and mechanical loading [2].

Inflammation is categorized as acute or chronic. Basically, acute inflammation that has lasted longer than a few weeks is considered chronic. At the cellular level there is a difference in the nature of the tissue lesions, secreted effector molecules and cell types. In acute inflammation there is an abundance of phagocytic cells (principally neutrophils and macrophages) whereas in chronic inflammation lymphocytes and monocytes predominate. It is clear that each type of inflammation is not a simple linear cascade, but rather a complex, highly orchestrated and fine-tuned process, involving interactions between many different types of cells, soluble mediators and tissue matrix [3]. Inflammation causes the immediate and sequential release of signalling factors including chemokines, cytokines, eicosanoids, that bring leucocytes (polymorphonuclears, eosinophils) from the microvasculature to the site of inflammation to neutralize the injurious agent [4]. After leucocyte trafficking, peripheral blood monocytes accumulate at the inflammatory site and differentiate locally in to larger more granular phagocytosing macrophages [4]. Once the inflammatory cells have neutralized the injurious agent they must be disposed of in a controlled and effective manner [4]. Apoptotic polymorphonuclear leucocytes or eosinophils are phagocytosed by macrophages, which in turn are cleared from the site of inflammation either by dying locally or by programmed cell death or by clearing to the draining lymphatics [4] (figure 1-A). Given a favourable genetic predisposition, failure of acute inflammation to resolve adequately could result in a predisposition to chronic inflammation, collateral tissue injury or auto-immunity typified by the accumulation of inflammatory leucocytes fibrosis and auto-antibodies to endogenous cellular and tissue antigens [4] (figure 1-B).

Inflammation is regulated by cytokines, chemokines, and growth factors, many of which may be active in the chronic inflammation [5]. Inflammation triggers the production of primary proinflammatory cytokines such as IL-1β and TNFα. IL-1 and TNF are primarily produced by monocytes and macrophages but can also be generated by a variety of resident cells in tissues [6]. These cytokines stimulate the production of chemoattractant

cytokines (chemokines), which may play a major role in atherogenesis, and stimulate the production of IL-6, a secondary proinflammatory cytokine, which in turn stimulates the production of acute-phase proteins by the liver [7]. Examples of these proteins include C-reactive protein and fibrinogen [7].

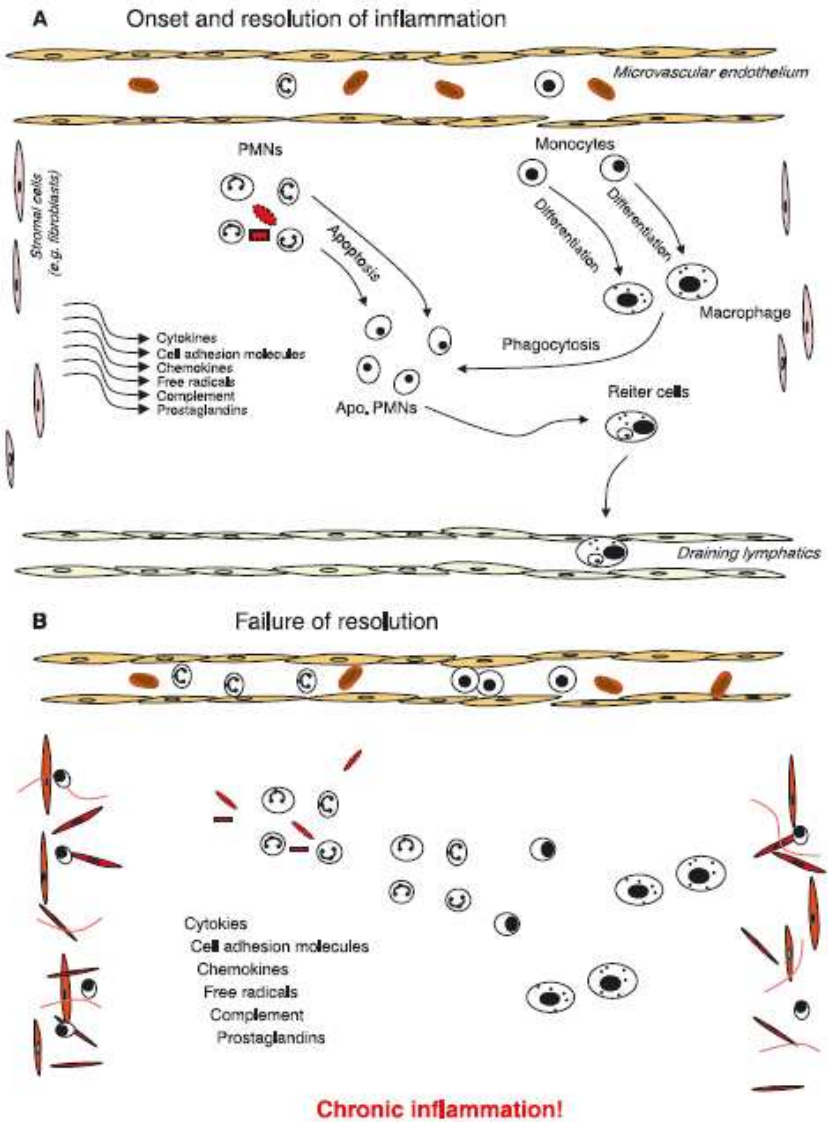

Figure 1. Illustration of the cellular kinetics and sequential release of mediators during the evolution of the inflammatory response. (Adapted from Lawrence, T. and Gilroy, D.W., 2007)

IL-1 and TNF are also crucially important in mediating the infiltration of tissue by leukocytes, via the initial induction of leukocyte adhesion molecules such vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and E-selectin on endothelial cells [8]. The induction of adhesion molecules by these cytokines

allows for the adhesion of leukocytes to endothelial cells [9]. The endothelium retracts, allowing for the migration of the transiently adhered leukocytes into the inflamed tissue in response to chemoattractant cytokines that are also induced by IL-1 and TNF [9].

Interleukin 1

Interleukin-1 is a glycoprotein that exists in two major biologically active forms, IL-1α and IL-1β [10]. These two functionally similar molecules, IL-1α and IL-1β, are encoded by separate genes (respectively, IL1A and IL1B) [10]. Of these, IL-1β is the predominant circulating isoform in humans. IL-1α and IL-1β have undistinguishable functions and their pro-inflammatory effects are numerous on most cell types. However, IL-1β is a secreted protein, while IL-1α is mainly a cell-associated molecule [11]. The main sources of IL-1 are stimulated blood monocytes and tissue macrophages, and IL-1β is also present in the hypothalamus [6]. A recent study demonstrates that expression of IL1β is increased in both obese rodents and humans [12].

Interleukin-1 plays an important role in the regulation of the inflammatory response. Indeed, this primary inflammatory cytokine has been implicated in mediating both acute and chronic pathological inflammatory diseases [13].The actions of IL-1 appear to be mediated by a relatively well-known biochemical pathway. IL-1 binds to the type1 IL-1 receptor-associated protein, then binds to the complex, initiating intracellular signalling pathways, such as the κβ kinase pathway, or those involving various small GTPases [14]. This results in the activation of transcription factors that in turn increases the expression of proinflammatory genes encoding chemokines, cytokines, acute-phase proteins, cell adhesion molecules, degradative metalloproteinases, and other enzymes [14]. Indeed, administration of IL-1 to humans induces the release of secondary cytokines such as IL-6 and IL-8 [15].

A polymorphism (C/T) was described at the position -511 in the promoter region of the human IL-1β gene [16], and T allele showed a modest increase in transcriptional activity when compared with C allele [17 18 19].

Tumor necrosis factor

Originally described by its antitumor activity, tumor necrosis factor alpha (TNFα) is now recognized as a cytokine with multiple biological capacities. The cytokine TNFα is a non-glycosylated protein acting as modulator of gene expression in adipocytes and is implicated in the development of insulin resistance and obesity [20]. Fat tissue is a significant source of endogenous TNFα production, and the expression of this cytokine is elevated in human obesity in adipose tissue [20]. An earlier study [21] demonstrated that adipocytes constitutively express the proinflammatory cytokine TNF and that TNF expression in adipocytes of obese animals (ob/ob mouse, db/db mouse and fa/fa Zucker rat) is markedly increased. These observations provided the link between an increase in the expression and the plasma concentration of a proinflammatory cytokine and insulin resistance. Monocytes and macrophages are the main producers of TNFα, but other cells such as T-lymphocytes, natural killer cells, smooth muscle cells, endothelial cells and some tumour cells also produce TNFα [22].

TNFα is a powerful local regulator within adipose tissue, acting in both an autocrine and a paracrine manner to influence a range of processes, including apoptosis [23 24]. There appears to be a hierarchy of cytokines within fat, with TNF playing a pivotal role in relation to the production of several cytokines and other adipokines [24]. TNFα stimulates cellular kinase complex known as I Kappa B Kinase (IKK), which activates nuclear factor (NF)-κβ, a transcription factor that, in turn, drives the production of proinflammatory cytokines including IL-1β, IL-6, TNF and interferon [25].

Linkage analysis has shown that a marker near the TNFα region on chromosome 6 was significantly linked with obesity in Pima Indians [26]. The gene for human TNFα is located on the short arm of chromosome 6 and a G-A substitution at position -308 upstream from the transcription initiation site in the promoter region of the gene has been identified [27]. In vitro experiments have demonstrated that this substitution increases transcriptional activation of the TNFα gene [28]. Although controversial, the majority of the data support a direct role for this biallelic polymorphism in the elevated TNFα levels observed in homozygotes for the -308A allele [29].

Interleukin 6

Interleukin-6 (IL-6) is an acute-phase response cytokine produced by many different cell types, including immune and endothelial cells, fibroblasts, myocytes, and adipocytes [30]. Fat mass has been implied as a major source for circulating IL-6, with visceral fat producing higher levels of IL-6 compared with subcutaneous fat [31]. In obese subjects with high waist-to-hip ratio, the participation is even greater [32.]

It regulates humoral and cellular responses and plays a central role in inflammation and tissue injury [33]. IL-6 is one of the main inducers of the hepatic synthesis and secretion of C - reactive protein (CRP) in response to infection or inflammation [34]. It has been proposed that IL-6 has direct central actions on the control of fat mass, as IL-6 receptors were found in hypothalamus in mice [32 35], and as it was found a negative correlation between the concentration of IL-6 in the cerebrospinal fluid and the fat mass, in obese subjects [36].

A polymorphism (G/C) at the position -174 in the promoter region of the human IL-6 gene was described [37] and suppression of IL-6 transcription [37 38] resulted from this single nucleotide change from G to C.

C-reactive protein

C-reactive protein is an ancient, highly conserved molecule and consists of five identical, non-glycosylated peptide subunits linked to form a cyclic polymerase [39]. CRP is an acute phase reactant synthetized and secreted by the liver in response to a variety of inflammatory cytokines, increases rapidly in response to trauma, inflammation, and infection and decreases just as rapidly with the resolution of the condition [40]. Thus, the measurement of CRP can be used to monitor inflammatory states. The development of high sensitivity assays for CRP has enabled the detection of mild elevation of CRP within the normal range [41]. The application of these assays during the last years has made it possible to study CRP in a wide variety of inflammatory diseases, being the most commonly used and best standardized inflammatory marker of cardiovascular and metabolic disorders [41 42].

CRP has a role in the function of the innate immune system. It activates complement, binds to Fc receptors, and acts as an opsonin for various pathogens [43]. Binding of CRP to Fc receptors leads to generation of proinflammatory cytokines [43]. CRP can recognize altered self and foreign molecules based on pattern recognition [43]. Thus, enhanced levels of CRP can be used as a marker of inflammation.

Fibrinogen

Fibrinogen, a glycoprotein dimmer composed of three pairs of non-identical polypeptide chains (alpha, beta and gamma) [44] linked to each other by disulphide bonds, is a key coagulation factor and acute phase reactant exclusively synthesized by the liver [39] and is inducible by IL-6 as part of the acute phase reaction [45]. Fibrinogen has a plasma half-life of 3–5 days and is a key plasma protein [44]. At the final step of the coagulation cascade, it is transformed into fibrin under the action of thrombin [44]. Fibrinogen binding to the

GPIIb/IIIa receptor in activated platelets is the key step in platelet aggregation [46]. Furthermore, fibrinogen is the major determinant of plasma viscosity and erythrocyte aggregation and, therefore, affects blood viscosity both at high shear rate (primarily relevant for flow in arteries, arterioles, and capillaries) and low shear rate (relevant for flow in veins and under stasis) [47].

Fibrinogen is a ligand for ICAM-1, that behaves as a cell surface ligand for a few integrins, and enhances monocyte-endothelial cell interaction [48]. Fibrinogen upregulates and increases the concentration of ICAM-1 proteins on the surface of endothelial cells, resulting in increased adhesion of leukocytes, platelets and macrophages on the surface of endothelial cells [49].

Leucocytes

Leukocytes, also called white blood cells, can be categorized into three main groups, neutrophils, monocytes/macrophages, and lymphocytes [50]. Leukocyte recruitment is necessary for host defense against infection and for normal wound healing [51]. Neutrophils, or polymorphonuclear leukocytes (PMNs), are the most common leukocyte in humans, numbering ~5 × 10^6 per milliliter of blood [51]. Their recruitment and subsequent transmigration into inflamed tissue is the earliest cell adhesion event following tissue insult, and this occurs in virtually every organ [51].

Molecular specificity in the targeting of leukocytes at sites of inflammation [52] is mediated by selectins, integrins, and the immunolglobulin gene superfamily. In the surrounding tissue of the inflammation site, the chemoattractants released trigger a complicated cascade, which results in the migration of circulating leukocytes towards the site of inflammation (chemotaxis) [53]. This is a well coordinated process involving first the attraction of polymorphmononuclear leukocytes, followed by the activation and adhesion

of these cells to the endothelium of the blood vessel (margination) and finally, diapedesis (infiltration) into the extravascular space and migration to the site of inflammation [53].

Uric Acid

Uric acid is the main end product of metabolism of purines, which in turn are derived mostly from diet, *de novo* biosynthesis, and breakdown of nucleic acids [54]. Serum uric acid levels, therefore, increase with higher protein intake, increased endogenous production of urate, or decreased excretion of monosodium urate by kidneys [54]. In most mammals, uric acid is degraded by the hepatic enzyme uricase to allantoin [55]. However, mutations in the uricase gene occurred during primate development, with the consequence that humans have relatively higher levels of serum uric acid [56]. Elevated levels of uric acid correlate with aging, male gender, hyperlipidemia, obesity, hyperinsulinemia, diabetes mellitus, and glucose intolerance [57 58].

Uric acid activates the complement system [59], and in soluble form induces the development of oxidative stress and LDL oxidation [59]. It is proinflammatory in rat vascular smooth muscle cells and stimulates human mononuclear cells to produce cytokines [60 61]. It also stimulates the inflammatory response by increasing the production of the chemotactic factor monocyte chemoattractant protein-1 (MCP-1) in vascular smooth muscle cells and CRP synthesis in human vascular endothelial and smooth muscle cells [62 63]. Hyperuricemic rats have a significant increase in macrophage infiltration in their kidneys independent of crystal deposition [55].

OBESITY

The World Health Organisation (WHO) defines obesity as “an abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired” [64]. The currently accepted classification of adult obesity for epidemiological purposes defines overweight at body mass index (BMI) levels greater than 25 kg/m^2 and obesity beginning at BMI of 30 kg/m^2 [64]. Worldwide 1.1 billion people are currently estimated to be overweight, at least 300 million of them obese [64]. Obesity is an epidemic, affecting all races, with high incidence, mainly, in the western societies [65] and with increasing prevalence among Portuguese people [66]. It is associated with the incidence of several adverse health problems, including diabetes mellitus, cardiovascular disease, hypertension and cancer [67]. It was previously reported that obesity is more strongly linked to chronic diseases than living in poverty, smoking, or drinking [68].

Obesity is not a single disorder but a heterogeneous group of conditions with multiple causes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure [69].

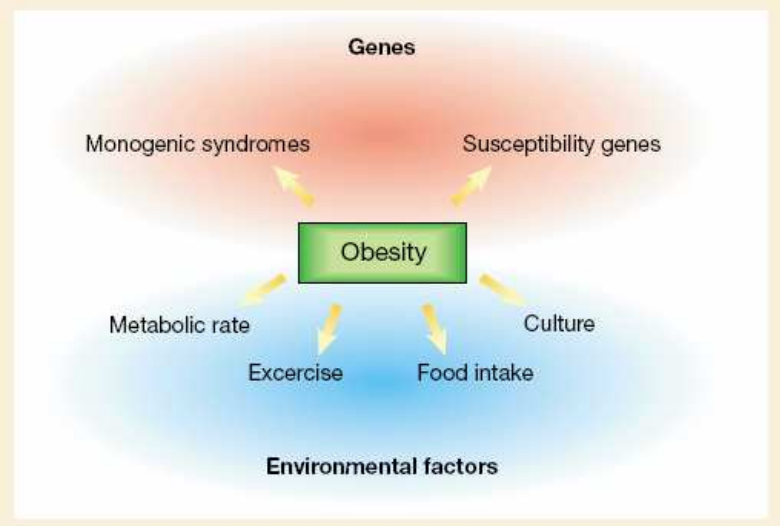

Figure 2. Factors influencing the development of obesity (Kopelman, P.G., 2000)

Obesity is characterized by an expanded fat mass that, in the past, was mainly seen as a storage organ [70], being recently recognized as functionally comparable to an endocrine organ, producing and secreting various adipokines, such as leptin and adiponectin, and cytokines such as tumor necrosis factor α (TNF-α), interleukin-6 and interleukin 1 [11]. TNF-α, IL-6 and IL-1 are classical pro-inflammatory cytokines inducing both acute and chronic inflammatory responses [11], with a potential role in obesity and obesity complications [21]. In recent years, evidence indicates that chronic low-grade activation of the immune system plays an important role in the aetiology of obesity and related metabolic dysfunctions [12].

The different anatomic location of adipose tissue accumulation plays an important role in the development of obesity related co-morbidities. Upper body fat includes the visceral and abdominal subcutaneous depots [71]. The visceral fat depot is contained within the body cavity, surrounding the internal organs, and is composed of the mesenteric and the greater and lesser omental depots [71]. Visceral depots represent 20 and 6% of total body fat in men and women, respectively [72]. The abdominal subcutaneous fat depot is situated immediately below the skin in the abdominal region [71]. In the lower body, all adipose depots are subcutaneous with the two larger sites of storage in the gluteal and femoral regions [71].

Not all fat is created equal: cells in some parts of the body may pump out more of the molecular signals that promote obesity-related disease [73]. Visceral fat, which can affect both the lean and obese, seems to be particularly problematic because it dumps signalling molecules directly into blood heading for the liver, the main site where glucose and fat are converted from one to another [73]. Subcutaneous fat, seems to be less metabolically active and therefore may produce less of these molecules [73]. Individuals with comparable amounts of fat stored in the femoral or gluteal depots (lower body obesity) have a much lower risk of morbidity from metabolic disturbances [71].

OBESITY AND INFLAMMATION

The origin of systemic inflammation in metabolic obesity has been subject of debate in recent years and evidence is accumulating that adipose mass plays a major role in production of cytokines like interleukin-1, interleukin-6 and tumour necrosis factor alpha [74]. Over the past decade it has become clear that fat cells send out distress signals that can promote insulin resistance and trigger inflammation [21], which may, in turn, cause type 2 diabetes, cardiovascular disease, increased cancer risk and other obesity-associated problems [73].

It was described that adipocyte precursors have potent phagocytic capacity and can be transformed into macrophage-like cells in response to appropriate stimuli [75]. Experiments in mice bone marrow chimeras have demonstrated that adipose mass macrophages are bone marrow derived, indicating that macrophages present in adipose tissue do not derive *in situ* from differentiation of preadipocytes but rather from circulating monocytes infiltrating fat mass[76]. Despite the different results, it appears that obesity is associated with a low-grade inflammation characterized by increased macrophage infiltration [76]. This infiltration increases in proportion to BMI and to adipocyte hypertrophy [31]. Activated macrophages contribute to a downward spiral of inflammation, releasing cytokines and biologically active molecules such as TNFα, IL-6, and IL-1 [30]. In turn, these molecules increase production of acute-phase proteins.

These data may suggest that once inflammatory trigger is established in adipose tissue with increasing macrophage infiltration and increased cytokines, a self perpetuating mechanism develops. However, remains unclear what triggers macrophage infiltration. One recent study reports that >90% of macrophages in white adipose tissue of obese mice and humans are localized to dead adipocytes [77]. It is postulated that adipocyte

hypertrophy promotes adipocyte death, macrophage aggregation and adipose tissue inflammation.

Several inflammatory cytokines are now recognized to be expressed in, and secreted by, white adipocytes. The first to be identified was TNFα, whose expression was initially demonstrated in rodents and found to be markedly increased in obese models [21]. As well IL6 and IL1 were found to be present in fat mass [1 31 32]. The production of a cytokine is influenced by single base changes (single nucleotide polymorphisms), usually in the promoter region of its gene [78]. Therefore, individuals may have a genetically determined propensity for raised amount of cytokine production and, consequently, for higher production of acute phase proteins. The possibility of an inter-individual and genetically determined difference in basal and post-stimulus of IL1, IL6 and TNF levels suggested that these polymorphisms may play a role in the regulation of inflammatory processes and synthesis of acute-phase reactants. Given that cytokine gene polymorphisms have been shown to be involved in the susceptibility, clinical performance, and outcome in a variety of diseases [79], exploration of the genetic relationship between proinflammatory cytokine polymorphisms and adiposity has significant implications for understanding the pathogenesis of obesity. To observe this effect on inflammation, we used four inflammatory markers, C-reactive protein, leukocytes, fibrinogen and uric acid. The basal values of CRP and white blood cell appear to be significantly heritable (≈ 40%) [80], and therefore it is very likely that polymorphisms in genes controlling inflammatory markers expression may influence their levels, as well as for fibrinogen and uric acid.

AIMS

AIMS

In the sense of studying the influence of fat distribution in the inflammatory outcome phenotype of specific polymorphisms affecting genes enconding pro-inflammatory cytokines, the aims of this study are:

- To identify the prevalence of the polymorphisms in the study group;
- To analyse if there is an association between the polymorphisms and obesity;
- To assess whether polymorphisms in genes coding for IL-6, TNFα and IL-1β influence inflammatory markers levels;
- To evaluate the interaction between the study polymorphisms and fat distribution in relation to inflammatory markers concentrations.

PARTICIPANTS AND METHODS

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Participants

Participants were selected as part of a population based health and nutrition survey previously described in detail [81]. Non-institutionalized inhabitants of Porto, Portugal, were selected using random digit dialing. After the identification of a household, permanent residents were characterized according to age and sex, and one adult was selected by simple random sampling and invited to visit our department for interview and examination. If there was a refusal, replacement was not allowed. The participation rate was 70% [81]. As part of the ongoing cohort study, we revaluated a convenient sample of 359 individuals.

The local institutional ethics committee approved the study and all participants gave written informed consent.

Trained interviewers collected information using a structured questionnaire. Data on social, demographic, personal and family medical history and behavioral characteristics were obtained as self-reported.

Anthropometric measurements

Anthropometrics were obtained after 12 h fasting, the participant in light clothing and no footwear. Body weight was measured to the nearest 0.1 kg using a digital scale, and height to the nearest centimeter in the standing position using a wall stadiometer. Body mass index (BMI) was calculated as weight in kilograms divided by square height in meters.

Waist and hip circumferences were measured to the nearest centimeter, with the subject standing, with a flexible and non-distensible tape, avoiding exertion of pressure on the tissues. Waist circumference (WC) was measured midway between the lower limit of the

rib cage and the iliac crest, hip circumference was the maximal circumference over the femoral trochanters, and the waist to hip circumference ratio (WHR) was calculated.

Waist circumference and WHR were used to analyze the effect of the interaction between the IL6 -174G/C polymorphism and abdominal adiposity in CRP levels. Fat mass was obtained by bioelectrical impedance analysis.

Measurement of CRP plasma levels

Blood was drawn after a 12 hour overnight fast. High sensitivity C-reactive protein levels were determined by means of particle-enhanced immunonephelometry using a BN™ II nephelometer (Dade Bhering). For the purpose of this study, we evaluated 359 participants, of whom we excluded 37 (10.3%) for CRP analysis because they presented CRP levels above 10mg/L, which might indicate clinically relevant inflammatory conditions[41 82].

Uric acid and fibrinogen concentration was assessed by a standard colorimetric enzymatic assay.

Leukocytes count was measured by flux citometry, using an automatic hematologic counter Sysmex® XE-2100.

Genotyping

Genomic DNA was retrieved from blood samples using standard proteinase K digestion and phenol/chloroform extraction. The G/C single nucleotide polymorphism at position -174 of the interleukin-6 gene and the C/T single nucleotide polymorphism at position -511 of the interleukin 1 β were performed by polymerase chain reaction (PCR) amplification, using the following primer pairs:

IL6 Primer forward	5' GCCTCAATGACGACCTAAGC 3'
IL6 Primer reverse	5' AATGTGGGATTTTCCCATGA 3'
IL1 β Primer forward	5' GCCTGAACCCTGCATACCGT 3'
IL1β Primer reverse	5' GCCAATAGCCCTCCCTGTCT 3'

The reaction was carried out in a final volume of 25µL, containing 200µmol/L each dNTP, 20pmol each primer, 50mmol/L of KCl, 10mmol/L Tris-HCL (pH 9.0), 1.5mmol/L of $MgCl_2$ and 1U Taq polymerase (Amershan Biosciences, New Jersey). DNA was amplified during 35 cycles with an initial denaturation of 30 seconds at 94ºC, a 30 seconds annealing at 58ºC and an extension of 30 seconds at 72ºC.

PCR products were digested with 5U restriction enzyme *Aval* (MBI, Fermentas) and buffer Y+/Tango 1x (33mM Tris-acetate, 10mM magnesium acetate, 66mM potassium acetate, 0.1mg/mg BSA) at 37ºC overnight and separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products were sized relative to a 1-kilobase ladder. The IL6 alleles were designated as follows: G allele with 2 bands of 110 and 49 bp, C allele with a single band of 164 bp, and the C/G allele with 3 bands of 164, 110 and 49 bp.

The IL1 alleles were designated as follows: C allele with 2 bands of 90 and 65 bp, T allele with a single band of 155 bp, and the C/T allele with 3 bands of 155, 90 and 65 bp.

The G/A single nucleotide polymorphism at position -308 of the tumour necrosis factor-α gene was performed by TaqMan system (ABI Prism 7000 Sequence Detection System, Applied Biosystems, using assays-on-demand, from Applied Biosystems (C_7514879, C_11918223_10, respectively). For each genotyped individual we used TaqMan Universal Master Mix 1x and assay-on-demand 1x, with a total volume of 11µL. DNA was amplified during 40 cycles of 15 seconds at 95ºC and 60 seconds at 60ºC. Allelic discrimination was performed during 60 seconds at 60ºC.

Statistical Methods

Chi-square analysis was used to determine if genotype frequencies were in Hardy-Weinberg equilibrium. Differences in sex distribution between genotypes were evaluated by Pearson Chi-square. Characteristics were tested for differences between genotypes using ANOVA for continuous variables (age, body weight, BMI, waist, hip, WHR, fat mass, free fat mass).

Linear regression was used to adjust outcome for age and gender, according to allele.

Due to CRP non-normally distributed data, we used Box Cox transformation to convert it to a normal distribution. To assess the effect of polymorphisms in inflammatory markers levels and to observe if there was an interaction between the polymorphisms and WC and WHR with any effect in inflammatory markers levels, we used univariate analysis of variance. Spearman's rho was used to evaluate the correlation between CRP plasma levels and body weight, BMI, waist and hip circumferences, WHR, fat mass, free fat mass. Pearson correlation was used to evaluate the correlation between leukocyte, fibrinogen and uric acid levels and obesity indices.

To evaluate the correlation between inflammatory markers we used spearman's ρ and pearson correlation.

Levels of statistical significance were set at $p < 0.05$.

Data were analyzed using SPSS software (version 14.0.0) and R software (version 2.6.0).

CHAPTER I

IL-6 -174G/C POLYMORPHISM INTERACTS WITH ABDOMINAL ADIPOSITY TO INCREASE C-REACTIVE PROTEIN

RESULTS

RESULTS

Genotyping of the IL-6 -174 G/C polymorphism was performed for 322 subjects (215 women and 107 men). There were 144 (44.7%) participants with GG genotype, 132 (41.0%) GC heterozygotes, and 46 (14.3%) CC homozygotes. Genotype and allele proportions were in Hardy Weinberg equilibrium (X^2= 2.98; p=0.225).

The relative frequency of -174C allele was 0.35. Main characteristics of the study population, according to genotype are presented in table 1.

Table 1. Subjects' characteristics according to the -174G/C genotype

	G/G	G/C	C/C	*P*[2]
n (%)	144(44.7)	132 (41.0)	46 (14.3)	---
Gender				
Male n(%)	49 (34.0)	39 (29.5)	19 (41.3)	0.333
Female n(%)	95 (66.0)	93 (70.5)	27 (58.7)	
Age (years)[1]	56.9±15.20	56.4±14.76	57.4±17.21	0.920
Body weight (kg)[1]	69.9±15.32	72.1±13.70	73.2±13.02	0.279
BMI (kg/m^2)[1]	27.1±5.15	28.9±5.87	28.2±4.49	0.028
Waist (cm)[1]	91.6±13.59	93.8±13.19	95.4±11.17	0.156
Hip (cm)[1]	101.4±9.03	104.4±10.78	103.7±8.20	0.034
WHR[1]	0.9±0.08	0.9±0.08	0.9±0.06	0.283
Fat Mass (kg)[1]	21.8±9.43	24.3±10.28	23.2±8.31	0.095

[1] Results are expressed as mean ± SD.
[2] ANOVA
BMI, body mass index; WHR, waist-hip ratio.

When we evaluated markers of obesity, according to -174 G/C genotype, we found significant differences for BMI (p=0.028) and hip circumference (p=0.034), with people carrying the rare C allele presenting higher mean values than GG homozygotes.

When comparing GG homozygotes with C carriers, after adjusting for age and gender, C carriers' BMI (β=1.572, p=0.006) and hip circumference (β=2.825, p=0.007) mean values

remained significantly higher as was the case for mean waist circumference (β=2.795, p=0.036) and fat mass (β=2.195, p=0.030) (table 2).

Table 2. Subjects' characteristics according to allele, adjusted for age and gender

	C carrier[1]		G carrier[2]	
	β[3]	*P*[4]	β[3]	*P*[4]
Body weight (kg)	2.661	0.079	-1.217	0.574
BMI (kg/m^2)	1.572	0.006	-0.293	0.720
Waist (cm)	2.795	0.036	-2.071	0.277
Hip (cm)	2.825	0.007	-1.065	0.478
WHR	0.004	0.620	-0.011	0.284
Fat Mass (kg)	2.195	0.030	-0.655	0.654

BMI, body mass index; WHR, waist-hip ratio.
[1] Reference class - homozygotes G/G
[2] Reference class - homozygotes C/C
[3] Values were calculated by linear regression.
[4] *P* value adjusted for age and gender.

CRP was significantly correlated with body weight (Spearman's rho=0.150, p<0.001), BMI (Spearman's rho=0.303, p<0.001), waist (Spearman's rho=0.242, p<0.001) and hip circumference (Spearman's rho=0.259, p<0.001), WHR (Spearman's rho=0.102, p<0.001) and fat mass (Spearman's rho=0.316, p<0.001).

CRP plasma levels were not significantly different according to genotypes, when comparing homozigotes GG with heterozigotes GC (β=0.055, p=0.673) and homozigotes CC (β=-0.066, p=0.718).

To evaluate if there was an interaction between IL6 -174C/G polymorphism and abdominal fat in relation to CRP levels, individuals were evaluated according to WC and WHR. It was found a significant association between waist circumference and C carriers – GC (β=0.039, p<0.001) and CC (β=0.037, p=0.006), meaning that for people with equal values of waist circumference, C carriers have higher CRP levels than homozigotes GG (table 3). This association remained statistically significant even after adjustment for gender, age and smoking habits (table 3).

In relation to WHR, significant association was found with heterozigotes GC, which showed higher CRP levels than homozigotes GG (table 3). No significant difference was seen between CRP levels for homozigotes CC and homozigotes GG (table 3).

Table 3. The effect of IL6 -174G/C polymorphism in the association between waist circumference and waist-to-hip ratio with CRP

		Crude			Adjusted*		
IL6 -174 G/C		**WC**	**WCxGC**	**WCxCC**	**WC**	**WCxGC**	**WCxCC**
	β	-0.001	0.039	0.037	-0.001	0.038	0.033
	p	0.382	<0.001	0.006	0.478	<0.001	0.015
IL6 -174 G/C		**WHR**	**WHRxGC**	**WHRxCC**	**WHR**	**WHRxGC**	**WHRxCC**
	β	0.499	4.151	1.828	1.193	4.502	1.506
	p	0.635	0.010	0.502	0.306	0.004	0.578

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

DISCUSSION

DISCUSSION

It is well recognized that genetic polymorphisms in susceptibility genes may modulate the response to an inflammatory stimulus and that CRP is subjected to genetic modulation[83].

Considering the association between IL-6 genetic variants and abdominal fat, as well as the association between the latter and C-reactive protein plasma levels, we might speculate that a possible interaction between the -174G/C polymorphism and abdominal fat could be one of the reasons for the controversial results observed in studies that evaluated the effect of the polymorphism on CRP levels.

Therefore, we genotyped 322 individuals, of whom 44.7% were GG homozygotes, 41.0% were heterozygotes and 14.3% were CC homozygotes. The frequency of the -174C allele was 0.35, which is close to other European Caucasian populations[37 84].

Once we compared subjects' characteristics according to IL-6 -174G/C genotype, significant differences were shown for BMI, with C carriers presenting higher mean values, as previously seen in a study with two populations, one consisting of hypertensive individuals and other consisting of 20 year younger nonobese healthy females[85]. The -174C allele seems to be associated with lower IL-6 transcription[37 86] and there is clear evidence from experimental studies that endogenous IL-6 suppresses body fat mass and prevents late-onset obesity[35]. Recent studies indicate that -174C allele is associated with decrease energy expenditure[87]. Taken together, these results indicate that -174C allele decreases IL-6 production, which in turn results in decreased energy expenditure and accumulation of body fat.

Furthermore, we evaluated subjects' characteristics according to genotype in a dominant (GG vs CG+CC) and recessive (CC vs CG+GG) models. C carriers were associated with higher values of obesity markers, such as BMI, waist and hip circumference and fat mass. A study of 270 non-smoking men free from metabolic disorders, showed a significant effect of the IL-6 polymorphism on obesity, the -174G allele being more common among lean subjects (low BMI)[88]. Also, GG homozygotes presented a significantly smaller waist circumference and -174C allele carriers a larger waist line[88]. These results fit well with the finding of a lower basal metabolic rate in CC homozygotes[87], associating this genotype with higher indices of obesity and obesity markers. Waist to hip ratio and body weight were the only obesity parameters that showed no significant association with IL-6 -174G/C.

We confirmed the known positive association between CRP levels and BMI[89 90], also present with other obesity markers[91 92]. CRP levels were strongly positive correlated with waist circumference, demonstrating that as larger is the waist line, the higher are CRP levels. WC is a strong correlate of visceral fat[93], and elevated CRP concentration may reflect cytokine production by visceral adipocytes, because IL-6 and CRP levels are closely related with visceral fat[94]. A prior study with 190 overweight subjects has also shown association between waist circumference and CRP levels[95].

Even though we cannot discard the possibility that the IL-6 -174G/C polymorphism alone could influence CRP plasmatic levels, we found no significant differences among the three genotypes. Other studies also found no statistically significant differences in CRP levels between genotypes[96 97]. It is possible that this IL-6 polymorphism has none or only modest effects on CRP induction when expressed alone. As shown in a study using IL-6-deficient mice, it was demonstrated that injection of IL-6 is not sufficient for induced expression of CRP gene[98]. In fact, these effects might be expressed only in the presence of other factors[99]. Combined results have established that IL-6 is the principal inducer of the CRP

gene, while IL-1, glucocorticoids and other factors, including complement activation products, act synergistically with IL-6 [43]. In this sense, we might hypothesize that the interaction with WC could be responsible for the different results described on the impact IL6 -174G/C polymorphism on CRP in humans.

Möhlig et al. demonstrated a similar interaction between -174G/C polymorphism and BMI, in relation to IL6, an inflammatory marker as well [100]. In their study, increased BMI was correlated with higher IL-6 concentrations for the CC genotype than for GG genotype[100]. Effect modification between a genotype and an environmental factor is a scientifically important model. Therefore, we assessed a possible interaction between the polymorphism and abdominal adiposity within CRP levels. For the GC and CC genotype, WC is associated with higher CRP levels, when compared with homozigotes GG. These data demonstrate a gene-environment interaction that may help explain the controversial results described in the literature concerning the effect of the polymorphism in CRP concentrations. The mechanisms by which the C allele and WC could cause an increased in CRP levels are unknown, but this allele might act as a triggering agent of the dose-dependent lipolytic effect of IL6 on peripheral storages [101] driving to fat mobilization toward the abdominal compartment, which in turn is a source of pro-inflammatory cytokines[11] that stimulate the hepatic secretion of CRP. On the other hand, a putative synergistic effect between abdominal fat and IL6 polymorphism regarding CRP levels is possible, as both factors have been described as influencing IL6 concentrations[31 37 86] and consequently CRP levels, given that IL6 is the main inducer of hepatic secretion of this acute-phase protein[34].

To the best of our knowledge, no other study tried to evaluate the interaction between IL-6 polymorphism and abdominal adiposity within CRP levels and although we observed an interaction, in epidemiological studies, like this one, we cannot elucidate the mechanisms responsible for the interactions described. Thus, which factors linked to WC and IL6 gene

expression dependent on the -174G/C polymorphism differentially regulate CRP remain to be evaluated.

Our sample is neither ethnically diverse nor nationally representative, and is uncertain how our results would apply to other ethnic groups. However, in genetics studies, sample homogeneity is beneficial in order to reduce population stratification. Not having a direct measure of visceral adiposity is also a limitation of this study, although waist circumference was shown as a good surrogate for visceral adiposity[102]. Further investigation using other techniques to measure fat distribution may provide new insights to understand that association.

CHAPTER II

IL6, IL1BETA AND TNFALFA GENOTYPE AND FAT DISTRIBUTION: EFFECT ON INFLAMMATORY MARKERS

RESULTS

RESULTS

We evaluated three polymorphisms (IL6 -174G/C; IL1β -511C/T; TNFα -308G/A) encoding for transcription of pro-inflammatory proteins and their association with four inflammatory markers (C-reactive protein; uric acid; leukocytes and fibrinogen).

We found a strong positive correlation between the four inflammatory markers (table 1), with the exception of uric acid and fibrinogen.

Table 1. Correlation between the four inflammatory markers

	C-reactive Protein (mg/L)		**Leukocytes** ($x10^9$/L)		**Uric Acid** (mg/L)	
	Spearman's ρ	*p*	Pearson Correlation	*p*	Pearson Correlation	*p*
Leukocytes ($x10^9$/L)	0.240	<0.001	------	------		
Uric Acid (mg/L)	0.103	<0.001	0.110	<0.001	------	------
Fibrinogen (g/L)	0.441	<0.001	0.181	<0.001	-0.010	0.742

C-reactive protein showed strong correlation with body weight (Spearman's rho=0.150, $p<0.001$), BMI (Spearman's rho=0.303, $p<0.001$), waist (Spearman's rho=0.242, $p<0.001$) and hip circumference (Spearman's rho=0.259, $p<0.001$), waist-hip ratio (Spearman's rho=0.102, $p<0.001$) and fat mass (Spearman's rho=0.316, $p<0.001$) (table 2).

Leukocytes showed correlation with body weight (Pearson correlation=0.063, $p=0.022$), waist circumference (Pearson correlation=0.084, $p=0.002$), waist-hip ratio (Pearson correlation=0.117, $p<0.001$) and free fat mass (Pearson correlation=0.058, $p=0.037$) (table 2).

Uric acid was correlated with all obesity markers, body weight (Pearson correlation=0.441, $p<0.001$), BMI (Pearson correlation=0.283, $p<0.001$), waist (Pearson correlation=0.434, $p<0.001$) and hip circumference (Pearson correlation=0.201, $p<0.001$), waist-hip ratio

(Pearson correlation=0.461, *p*<0.001), fat mass (Pearson correlation=0.205, *p*<0.001) and free fat mass (Pearson correlation=0.458, *p*<0.001) (table 2).

Fibrinogen demonstrated correlation with BMI (Pearson correlation=0.112, *p*<0.001), hip circumference (Pearson correlation=0.093, *p*=0.003), fat mass (Pearson correlation=0.123, *p*<0.001) and free fat mass (Pearson correlation=-0.188, *p*<0.001) (table 2).

Table 2. Association between inflammatory markers and obesity indices

	C-reactive Protein (mg/L)		**Leukocytes** ($x10^9$/L)		**Uric Acid** (mg/L)		**Fibrinogen** (g/L)	
	Spearman' s ρ	*p*	Pearson Correlation	*p*	Pearson Correlation	*p*	Pearson Correlation	*p*
Body weight (kg)	0.150	<0.001	0.063	0.022	0.441	<0.001	-0.052	0.100
BMI (kg/m^2)	0.303	<0.001	0.053	0.052	0.283	<0.001	0.112	<0.001
Waist (cm)	0.242	<0.001	0.084	0.002	0.434	<0.001	0.050	0.113
Hip (cm)	0.259	<0.001	0.014	0.619	0.201	<0.001	0.093	0.003
WHR	0.102	<0.001	0.117	<0.001	0.461	<0.001	-0.024	0.451
Fat Mass (kg)	0.316	<0.001	0.033	0.238	0.205	<0.001	0.123	<0.001
Free Fat Mass (kg)	-0.047	0.091	0.058	0.037	0.458	<0.001	-0.188	<0.001

BMI, body mass index; WHR, waist-hip ratio.

In relation to IL6 -174G/C polymorphism, we previously saw that people carrying the C allele had higher BMI and hip circumference, and after adjustment for age and gender, they showed higher waist circumference and fat mass. We also noticed that CRP plasma levels were not significantly different according to genotypes, when comparing homozigotes GG with heterozigotes GC, but when we evaluated the interaction between WC and IL6 polymorphism, there was a significant association between waist circumference and C carriers, with heterozigotes GC and homozigotes CC presenting higher CRP levels than homozigotes GG.

Leukocytes levels revealed no significant differences when comparing homozigotes GG with heterozigotes GC (β=0.271, *p*=0.138) and homozigotes CC (β=0.228, *p*=0.382).

It was found no interaction between waist circumference and C carriers. This association became statistically significant after adjustment for gender, age and smoking habits when comparing GG homozigotes with heterozigotes GC (β=0.022, *p*=0.018) and with homozigotes CC (β=0.045, *p*=0.020) (tabela 3).

In relation to WHR, no significant association was found (table 3).

Table 3. The effect of IL6 -174G/C polymorphism in the association between waist circumference and waist-to-hip ratio with leukocytes

		Crude			Adjusted*		
IL6 -174 G/C		**WC**	**WCxGC**	**WCxCC**	**WC**	**WCxGC**	**WCxCC**
	β	0.001	0.012	0.037	0.002	0.022	0.045
	p	0.400	0.225	0.062	0.178	0.018	0.020
IL6 -174 G/C		**WHR**	**WHRxGC**	**WHRxCC**	**WHR**	**WHRxGC**	**WHRxCC**
	β	-0.187	2.552	4.239	1.310	2.184	5.034
	p	0.903	0.264	0.285	0.438	0.321	0.197

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Uric acid levels revealed no significant differences when comparing homozigotes GG with heterozigotes GC (β=0.739, *p*=0.675) and homozigotes CC (β=1.430, *p*=0.569).

It was found a significant association between waist circumference and C carriers – GC (β=0.392, *p*<0.001) and CC (β=0.485, *p*=0.007), meaning that for people with equal values of waist circumference, C carriers have higher CRP levels than homozigotes GG (table 4). This association remained statistically significant even after adjustment for gender, age and smoking habits (table 4).

In relation to WHR, no significant association was found (table 4).

Table 4. The effect of IL6 -174G/C polymorphism in the association between waist circumference and waist-to-hip ratio with uric acid

		Crude			Adjusted*		
IL6 -174 G/C		**WC**	**WCxGC**	**WCxCC**	**WC**	**WCxGC**	**WCxCC**
	β	0.048	0.392	0.485	0.031	0.349	0.398
	p	0.003	<0.001	0.007	0.027	<0.001	0.014
IL6 -174 G/C		**WHR**	**WHRxGC**	**WHRxCC**	**WHR**	**WHRxGC**	**WHRxCC**
	β	96.550	-21.560	19.700	58.795	-15.792	19.031
	p	<0.001	0.270	0.560	<0.001	0.398	0.564

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Fibrinogen levels revealed no significant differences when comparing homozigotes GG with heterozigotes GC (β=0.169, *p*=0.109) and homozigotes CC (β=0.137, *p*=0.334).

It was found a significant association between waist circumference and homozigotes GG (β=-0.002, *p*=0.015) and GC genotype (β=0.016, *p*=0.006) (table 5). This association remained statistically significant even after adjustment for gender, age and smoking habits (table 5).

In relation to WHR, no significant association was found (table 5).

Table 5. The effect of IL6 -174G/C polymorphism in the association between waist circumference and waist-to-hip ratio with fibrinogen

		Crude			Adjusted*		
IL6 -174 G/C		**WC**	**WCxGC**	**WCxCC**	**WC**	**WCxGC**	**WCxCC**
	β	-0.002	0.016	0.012	-0.002	0.015	0.012
	p	0.015	0.006	0.270	0.013	0.013	0.244
IL6 -174 G/C		**WHR**	**WHRxGC**	**WHRxCC**	**WHR**	**WHRxGC**	**WHRxCC**
	β	-0.117	2.356	1.984	-0.259	2.504	1.488
	p	0.903	0.090	0.373	0.807	0.069	0.509

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Genotyping of the IL1β -511 C/T polymorphism was performed in 254 subjects (168 women and 86 men). The main characteristics of the study population, according to

genotype are in table 6. There were 110(43.3%) participants with CC genotype, 106(41.7%) heterozygotes CT, and 38(15.0%) homozygotes TT. Genotype and allele proportions were in Hardy Weinberg equilibrium (X^2= 2.17; p=0.338).

The relative frequency of the -511T allele was 0.36.

Table 6. Subjects' characteristics according to the IL1β -511C/T genotype

	C/C	C/T	T/T	*p*
N (%)	110(43.3)	106(41.7)	38(15.0)	---
Gender				
Male n(%)	34(30.9)	36(34.0)	16(42.1)	0.453
Female n(%)	76(69.1)	70(66.0)	22(57.9)	
Age (years)[a]	55.3±15.78	55.4±16.83	56.0±15.93	0.970
Body weight (kg)[a]	71.8±15.78	67.9±13.31	70.8±15.59	0.145
BMI (kg/m^2)[a]	28. ±5.54	26.5±5.14	27.6±5.11	0.085
Waist (cm)[a]	93.5±13.79	89.4±12.93	92.7±14.68	0.081
Hip (cm)[a]	103.0±9.49	100.7±9.22	101.7±8.99	0.187
WHR[a]	0.9±0.08	0.9±0.08	0.9±0.09	0.186
Fat Mass (kg)[a]	23.8±9.92	20.5±9.26	21.6±9.74	0.045
Free Fat Mass (kg)[a]	47.9±9.64	47.3±8.73	48.5±10.84	0.776

[a] Results are expressed as mean ± SD.
BMI, body mass index; WHR, waist-hip ratio.

When we evaluated markers of obesity, according to IL1β -511C/T genotype, we found significant differences for fat mass (*p*=0.045) (table 6).

When comparing homozygotes CC with T carriers, significant associations remained for fat mass (β=-2.905, *p*=0.011) and additionally for body weight (β=-3.834, *p*=0.026), BMI (β=-1.327, *p*=0.037), waist circumference (β=-3.624, *p*=0.018) and waist-hip ratio (β=-0.018, *p*=0.036), when adjusted for age and gender (table 7).

Table 7. Subjects' characteristics according to IL1β -511C/T allele, adjusted for age and gender

	T carrier[a]		C carrier[b]	
	β	***p****	**β**	***p****
Body weight (kg)	-3.834	0.026	0.400	0.868
BMI (kg/m^2)	-1.327	0.037	-0.243	0.785
Waist (cm)	-3.624	0.018	-0.273	0.898
Hip (cm)	-2.037	0.075	0.174	0.913
WHR	-0.018	0.036	-0.003	0.831
Fat Mass (kg)	-2.905	0.011	0.294	0.855
Free Fat Mass (kg)	-0.943	0.205	0.355	0.733

BMI, body mass index; WHR, waist-hip ratio.
[a] Reference class - homozygotes C/C
[b] Reference class - homozygotes T/T
*p**, *p* value adjusted for age and gender.

CRP levels revealed no significant differences when comparing homozigotes CC with heterozigotes CT (β=-0.165, *p*=0.271) and homozigotes TT (β=-0.394, *p*=0.058).

It was found no interaction between waist circumference and homozigotes TT, in relation to CRP concentrations. The interaction of homozigotes CC (β=0.027, *p*<0.001) and heterozigotes CT (β= -0.027, *p*<0.001) with WC showed an effect on CRP concentrations, even after adjustment for gender, age and smoking habits (table 8).

In relation to WHR, there was an interaction with homozigotes CC, increasing CRP levels (β= 3.078, *p*=0.013) (table 8).

Table 8. The effect of IL1β -511C/T polymorphism in the association between waist circumference and waist-to-hip ratio with CRP levels.

		Crude			Adjusted*		
IL1β -511C/T		**WC**	**WCxCT**	**WCxTT**	**WC**	**WCxCT**	**WCxTT**
	β	0.027	-0.027	-0.012	0.026	-0.026	-0.006
	p	<0.001	<0.001	0.402	<0.001	<0.001	0.630
IL1β -511C/T		**WHR**	**WHRxCT**	**WHRxTT**	**WHR**	**WHRxCC**	**WHRxTT**
	β	3.078	0.676	-1.517	4.249	0.040	-1.260
	p	0.013	0.701	0.524	0.002	0.981	0.588

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Leukocytes levels revealed no significant differences when comparing homozigotes CC with heterozigotes CT (β=0.017, *p*=0.934) and homozigotes TT (β=0.018, *p*=0.951).

It was found no interaction between waist circumference and C carriers. Once adjusted for gender, age and smoking habits, the interaction between waist circumference and CC homozigotes (β=0.028, *p*=0.009) and heterozigotes CT (β= -0.026, *p*=0.018) affected leukocyte levels (table 9).

In relation to WHR, no significant association was found (table 9).

Table 9. The effect of IL1β -511C/T polymorphism in the association between waist circumference and waist-to-hip ratio with leukocytes

		Crude			Adjusted*		
IL1β -511C/T		**WC**	**WCxCT**	**WCxTT**	**WC**	**WCxCT**	**WCxTT**
	β	0.020	-0.018	-0.024	0.028	-0.026	-0.020
	p	0.063	0.097	0.246	0.009	0.018	0.338
IL1β -511C/T		**WHR**	**WHRxCT**	**WHRxTT**	**WHR**	**WHRxCC**	**WHRxTT**
	β	1.631	0.841	-1.846	3.279	0.556	-1.744
	p	0.348	0.738	0.598	0.092	0.823	0.612

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Uric acid levels revealed no significant differences when comparing homozigotes CC with heterozigotes CT (β=-1.078, *p*=0.591) and homozigotes TT (β=-2.589, *p*=0.359).

It was found no interaction between waist circumference and homozigotes TT, in relation to uric acid concentrations. The interaction of homozigotes CC (β=0.586, *p*<0.001) and heterozigotes CT (β= -0.543, *p*<0.001) with WC showed an effect on uric acid levels, even after adjustment for gender, age and smoking habits (table 10).

In relation to WHR, there was an interaction with homozigotes CC, increasing uric acid levels (β= 90.87, *p*<0.001) (table 10).

Table 10. The effect of IL1β -511C/T polymorphism in the association between waist circumference and waist-to-hip ratio with uric acid

		Crude			Adjusted*		
IL1β -511C/T		**WC**	**WCxCT**	**WCxTT**	**WC**	**WCxCT**	**WCxTT**
	β	0.586	-0.543	-0.271	0.467	-0.437	-0.258
	p	<0.001	<0.001	0.145	<0.001	<0.001	0.131
IL1β -511C/T		**WHR**	**WHRxCT**	**WHRxTT**	**WHR**	**WHRxCC**	**WHRxTT**
	β	90.870	-16.550	-38.930	57.033	-17.377	-26.822
	p	<0.001	0.446	0.199	<0.001	0.413	0.363

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Fibrinogen levels revealed no significant differences when comparing homozigotes CC with heterozigotes CT (β=0.151, *p*=0.280) and homozigotes TT (β=-0.068, *p*=0.723).

It was found no interaction between waist circumference and homozigotes TT, in relation to fibrinogen concentrations. The interaction of homozigotes CC (β=0.016, *p*=0.038) and heterozigotes CT (β= -0.018, *p*=0.021) with WC showed an effect on fibrinogen concentrations. After adjustment for gender, age and smoking habits, the interactions (WCxCC, WCxCT) effect on fibrinogen levels remained statistically significant (table 11).

In relation to WHR, after adjustment for gender, age and smoking habits, there was an interaction with homozigotes CC, increasing fibrinogen levels (β=2.927, *p*=0.029) (table 11).

Table 11. The effect of IL1β -511C/T polymorphism in the association between waist circumference and waist-to-hip ratio with fibrinogen

		Crude			Adjusted*		
IL1β -511C/T		**WC**	**WCxCT**	**WCxTT**	**WC**	**WCxCT**	**WCxTT**
	β	0.016	-0.018	-0.020	0.015	-0.017	-0.017
	p	0.038	0.021	0.136	0.053	0.028	0.185
IL1β -511C/T		**WHR**	**WHRxCT**	**WHRxTT**	**WHR**	**WHRxCC**	**WHRxTT**
	β	2.397	0.130	-2.060	2.927	-0.103	-1.822
	p	0.051	0.943	0.366	0.029	0.954	0.414

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Genotyping of the TNF-α -308 G/A polymorphism was performed in 308 subjects (205 women and 103 men). The main characteristics of the study population, according to genotype are in table 12. There were 228(74.0%) participants with GG genotype, 76(24.7%) heterozygotes GA, and 4(1.3%) homozygotes AA. Genotype and allele proportions were in Hardy Weinberg equilibrium (X^2= 0.711; p=0.701).

The relative frequency of the -308A allele was 0.14.

Table12. Subjects' characteristics according to the TNF-α -308 G/A genotype

	G/G	**G/A**	**A/A**	***p***
N (%)	228(74)	76(24.7)	4(1.3)	---
Gender				
Male n(%)	82(36.0)	21(27.6)	0(0.0)	0.148
Female n(%)	146(64.0)	55(72.4)	4(100.0)	
Age (years)[a]	55.0±16.11	55.8±14.66	51.0±19.58	0.814
Body weight (kg)[a]	69.8±14.63	68.4±12.14	64.78±4.83	0.612
BMI (kg/m^2)[a]	27.1±5.37	27.3±4.63	25.3±3.42	0.740
Waist (cm)[a]	90.8±13.14	91.1±12.25	89.8±6.69	0.967
Hip (cm)[a]	101.5±9.10	101.4±8.95	99.9±4.31	0.946
WHR[a]	0.9±0.08	0.9±0.08	0.9±0.05	0.934
Fat Mass (kg)[a]	21.6±9.78	22.2±8.80	23.0±4.74	0.865
Free Fat Mass (kg)[a]	47.9±9.40	46.2±7.69	41.5±3.15	0.155

[a] Results are expressed as mean ± SD.
BMI, body mass index; WHR, waist-hip ratio.

We didn't see any statistical significant difference between genotypes for the obesity indices (table 12), even when comparing between alleles (table 13).

Table 13. Subjects' characteristics according to TNF-α -308 G/A allele, adjusted for age and gender

	A carrier[a]		G carrier[b]	
	β	***p****	**β**	***p****
Body weight (kg)	-0.681	0.691	0.790	0.905
BMI (kg/m^2)	-0.050	0.937	1.801	0.464
Waist (cm)	0.519	0.731	-1.903	0.745
Hip (cm)	-0.428	0.706	1.838	0.676
WHR	0.009	0.271	-0.037	0.253
Fat Mass (kg)	0.115	0.920	-0.416	0.924
Free Fat Mass (kg)	-0.501	0.502	1.256	0.659

BMI, body mass index; WHR, waist-hip ratio.
[a] Reference class - homozygotes G/G
[b] Reference class - homozygotes A/A
*p**, *p* value adjusted for age and gender.

CRP levels revealed no significant differences when comparing homozigotes GG with heterozigotes GA (β=0.162, *p*=0.256) and homozigotes AA (β=0.737, *p*=0.175).

It was found no interaction between waist circumference and homozigotes GG and AA, in relation to CRP concentrations. The interaction of heterozigotes GA with WC showed an effect on CRP concentrations (β= 0.038, *p*<0.001), even after adjustment for gender, age and smoking habits (table 14).

In relation to WHR, there was an interaction with homozigotes GG, increasing CRP levels (β= 2.141, *p*=0.014) (table 14).

Table 14. The effect of TNF-α -308 G/A polymorphism in the association between waist circumference and waist-to-hip ratio with CRP levels.

		Crude			Adjusted*		
TNF-α -308 G/A		**WC**	**WCxGA**	**WCxAA**	**WC**	**WCxGA**	**WCxAA**
	β	0.000	0.038	0.091	0.000	0.034	0.102
	p	0.801	<0.001	0.320	0.926	<0.001	0.257
TNF-α -308 G/A		**WHR**	**WHRxGA**	**WHRxAA**	**WHR**	**WHRxGA**	**WHRxAA**
	β	2.141	1.735	-4.594	3.137	0.873	-4.180
	p	0.014	0.336	0.706	0.002	0.624	0.725

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Leukocytes levels revealed no significant differences when comparing homozigotes GG with heterozigotes GA (β=-0.332, *p*=0.123) and homozigotes AA (β=-0.594, *p*=0.485).

It was found no interaction between waist circumference and GG, GA and AA genotypes, in relation to leukocytes concentrations (table 15).

In relation to WHR, no significant association was found (table 15).

Table 15. The effect of TNF-α -308 G/A polymorphism in the association between waist circumference and waist-to-hip ratio with leukocytes.

		Crude			Adjusted*		
TNF-α -308 G/A		**WC**	**WCxGA**	**WCxAA**	**WC**	**WCxGA**	**WCxAA**
	β	0.002	0.017	0.187	0.003	0.026	0.278
	p	0.234	0.271	0.199	0.121	0.092	0.050
TNF-α -308 G/A		**WHR**	**WHRxGA**	**WHRxAA**	**WHR**	**WHRxGA**	**WHRxAA**
	β	1.998	1.830	27.935	2.975	1.191	36.542
	p	0.113	0.505	0.148	0.049	0.657	0.051

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Uric acid levels revealed no significant differences when comparing homozigotes GG with heterozigotes GA (β=-1.080, *p*=0.580) and homozigotes AA (β=-4.766, *p*=0.536).

The interaction of homozigotes GG and GA with WC showed an effect on uric acid concentrations (β=0.056, *p*<0.001 and β=0.410, *p*=0.003, respectively), even after adjustment for gender, age and smoking habits (table 16). It was found no interaction between waist circumference and homozigotes AA, in relation to uric acid concentrations.

In relation to WHR, there was an interaction with homozigotes GG, increasing uric acid levels (β= 82.08, *p*<0.001) (table 16).

Table 16. The effect of TNF-α -308 G/A polymorphism in the association between waist circumference and waist-to-hip ratio with uric acid

		Crude			Adjusted*		
TNF-α -308 G/A		**WC**	**WCxGA**	**WCxAA**	**WC**	**WCxGA**	**WCxAA**
	β	0.056	0.410	0.420	0.038	0.336	0.095
	p	<0.001	0.003	0.743	0.008	0.009	0.934
TNF-α -308 G/A		**WHR**	**WHRxGA**	**WHRxAA**	**WHR**	**WHRxGA**	**WHRxAA**
	β	82.085	-11.035	-81.076	46.687	-3.420	-73.913
	p	<0.001	0.628	0.610	<0.001	0.876	0.625

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

Fibrinogen levels revealed no significant differences when comparing homozigotes GG with heterozigotes GA (β=-0.041, *p*=0.770) and homozigotes AA (β=-0.117, *p*=0.806).

It was found no interaction between waist circumference and genotypes GG, GA and AA, in relation to fibrinogen concentrations. After adjustment for gender, age and smoking habits, the interaction of homozigotes GG (β=-0.002, *p*=0.034) and heterozigotes GA (β= 0.017, *p*=0.001) with WC showed an effect on fibrinogen concentrations (table 17).

In relation to WHR, after adjustment for gender, age and smoking habits, there was an interaction with homozigotes GG, increasing fibrinogen levels (β=1.958, *p*=0.046) (table 17).

Table17. The effect of TNF-α -308 G/A polymorphism in the association between waist circumference and waist-to-hip ratio with fibrinogen

		Crude			Adjusted*		
TNF-α -308 G/A		**WC**	**WCxGA**	**WCxAA**	**WC**	**WCxGA**	**WCxAA**
	β	-0.002	0.020	-0.058	-0.002	0.017	0.018
	p	0.053	0.120	0.845	0.034	0.001	0.162
TNF-α -308 G/A		**WHR**	**WHRxGA**	**WHRxAA**	**WHR**	**WHRxGA**	**WHRxAA**
	β	1.602	1.880	-3.951	1.958	1.030	-3.575
	p	0.054	0.368	0.770	0.046	0.618	0.786

WC, waist circumference; WHR, waist to hip ratio.
*Adjusted for age, gender, smoking habits and the main effect of the polymorphism.

DISCUSSION

DISCUSSION

In common multifactorial diseases, the interaction between genes and the environment is subtle and complex: susceptibility genes modulate the effect of environmental risk factors, making the initial pathologic event more or less likely [103]. Whether this pathologic event triggers the development of a clinically detectable disease, and how fast the disease develops, is influenced by genetic modifiers that exaggerate or suppress disease progression [103]. One example in which genes may act as both susceptibility factors and modifiers for some disease states can be found in the cytokine system [103]. It is possible to postulate that genetic variation affecting the activity of certain cytokine genes may produce individuals with a more exaggerated or prolonged inflammatory response [103]. Therefore, we analysed three polymorphisms encoding for IL6, IL1β and TNF-α genes, and their relation to fat, regarding four inflammatory markers levels.

As expected [62 104 105], we saw a significant correlation between the four inflammatory markers, since they are all involved in the complexity of inflammatory process. Obesity has been associated with higher levels of CRP [89], and in our study we found a positive correlation between CRP levels and obesity indices. Other studies presented the same results [92 95 106], indicating a potential role of this acute-phase protein in the obesity inflammatory process. Uric acid, leukocytes, and fibrinogen also showed positive correlation with a few obesity indices, as it was previously seen [94 107 108].

Interleukin 6

For IL6 -174G/C polymorphism, we genotyped 322 individuals, of whom 44.7% were GG homozygotes, 41.0% were heterozygotes and 14.3% were CC homozygotes. The

frequency of the -174C allele was 0.35, which is close to other European Caucasian populations [37 84].

In our formerly study (unpublished data) we noticed that CRP plasma levels were not significantly different according to genotypes, when comparing homozigotes GG with heterozigotes GC, but when evaluated the interaction between WC and IL6 polymorphism, there was a significant association between waist circumference and C carriers, with heterozigotes GC and homozigotes CC presenting higher CRP levels than homozigotes GG.

In this study we observe similar results in two other inflammatory markers, uric acid and leukocytes (after adjustment). Given that IL-6 is involved in hematopoiesis [109], we suggest that these associations might in part be due to adipocytes sitmulation of IL6 production and different fat IL-6 levels attributed to the IL-6 polymorphism at position –174. We couldn't probably saw the same results for fibrinogen because of the small number of people with CC genotype and measured levels of fibrinogen, but there is a nonsignificant trend toward elevated levels in C carriers. In a study with 598 adult participants, plasma fibrinogen levels did not differ among patients with the different genotypes of IL6 -174G/C polymorphism [110], although some *in vitro* studies showed that IL6 has a direct effect on transcription of the fibrinogen α, β and γ genes [111 112].

Interleukin 1β

For IL1β -511C/T we genotyped 254 individuals, of whom 43.3% were CC, 41.7% were heterozygotes CT, and 15.0% were homozygotes TT. The relative frequency of the -511T allele was 0.36, which is close to other previous studies [19 113 114].

In our study, there were only differences between genotypes for fat mass, but when we compared T carriers with CC homozigotes, they presented lower levels of body weight, BMI, WC, WHR and fat mass. Although we cannot discard other interactions and haplotypes effect, it seems that T allele carriers are more prone to have lower obesity indices, than homozigotes CC.

Since that IL1β -511 T allele has been related to higher levels of the cytokine [17-19], and given that IL1 plays a key role in autoimmune and inflammatory diseases, we expected to see it related with higher inflammatory levels, given that inflammation itself proceeds like a cascade, and therefore only minor adjustments at the beginning of this process could have a major outcome at the end of the process. Even though, we found no differences between the three genotypes, regarding inflammatory markers levels. IL1 acts early in the cascade of inflammatory response, inducing the reaction, and it could be that at intermediate steps of the inflammatory process, other inflammatory mediators interact with IL1 inducing a protective effect from excessively strong inflammatory reactions by this genotype.

When we evaluated the interaction between abdominal fat and IL1β -511C/T polymorphism, within inflammatory markers levels showed a statistically significant interaction for heterozigotes and a nonsignificant trend toward lower inflammatory levels for TT homozigotes. As IL1β -511C/T polymorphism alone has no effect on inflammatory markers levels, and since the T allele is related with lower obesity indices, we could hypothesized that the interaction of T allele with abdominal fat originates lower indices of adiposity, and therefore lower inflammation levels, since that increased adipose mass contributes directly toward an increase in systemic inflammation [115].

Tumor necrosis factor- α

Genotyping of the TNF-α -308 G/A polymorphism was performed in 308 subjects, 74.0% with GG genotype, 24.7% heterozygotes GA, and 1.3% homozygotes AA. The relative frequency of the -308A allele was 0.14. The allelic frequency is in accordance with allelic frequencies observed in other studies in Caucasian populations [116 117].

Fat tissue is a significant source of endogenous TNFα production and the expression of this cytokine is elevated in human obesity in adipose tissue [20 118], thus TNFα was considered as a candidate gene for obesity. Although, as it was previously shown [119], there were no significant differences between the genotype groups with respect to estimates of obesity and body fat distribution. In a study with 284 participants, no significant differences were found between TNFα -308G/A genotypes and BMI and waist-hip ratio [119]. Results from other studies, investigating TNF-α gene effects on obesity, lipid metabolism and anthropometric parameters, also found no association between genotypes and these parameters [120 121]. Higher production of TNFα linked to -308A variant may induce adipose tissue development by increasing the total number of stromal-vascular and/or uncommitted cells within the tissue, given that Kras et al. [122] have reported that these cells may be recruited to become preadipocytes or may serve alternatively as infrastructure to support adiposity growth. Even though, it is noteworthy that the patogenesis of obesity is complex, probably involving several genes and environmental factors, and therefore, we couldn't saw any differences between genotypes.

When we evaluated inflammatory markers levels according to genotype, there were no statistically significant differences. Further analysis of interaction between waist circumference and TNFα -308G/A polymorphism, within inflammatory markers levels,

showed a statistically significant interaction for heterozigotes and a nonsignificant trend toward higher inflammatory levels for AA homozigotes. This nonsignificant trend was probably caused by the lower number of people with AA genotype, since the elevation in inflammatory markers levels is quite notorious when compared with GG homozigotes. Provided that T allele is related to higher TNFα levels [29], and that has been demonstrated that adipocytes are responsive to TNFα, with a downstream activation of inflammatory signalling cascades [123], we postulate that this could be one possible interaction between abdominal fat and TNFα -308G/A polymorphism that gives rise to higher inflammatory levels.

This study has limitations. We couldn't perform genotyping for the three polymorphism, in all participants, nonetheless we presented allele frequencies similar to those previously reported. We were also not able to genotype other potentially functional variations in the genes locus so that such interference could be ruled out. The finding of a relationship between the interaction of some polymorphisms and fat distribution, regarding inflammatory markers, is a statistical finding, which does not clarify causality, but we can hypothesized that polymorphisms show phenotipically expression only in combination with other risk factors. To the best of our knowledge, no other study tried to evaluate the interaction between polymorphisms and abdominal adiposity within inflammatory markers levels and although we observed an interaction, we cannot elucidate the mechanisms responsible for the interactions described. Thus, which factors linked to WC and cytokine genes expressions dependent on the respective analyzed polymorphisms differentially regulate inflammatory levels remains to be evaluated.

CONCLUSION

CONCLUSION

In the study we tried to evaluate the interaction between obesity, especially abdominal adiposity and few polymorphisms in genes enconding pro-inflammatory cytokines in relation to four inflammatory markers levels. For each one of the analysed polymorphisms, there is an interaction with waist circumference in relation to at least one inflammatory marker level. We found that this interaction has a similar effect on inflammatory markers, even though not always statistically significant, what could indicate that the genetic effect is broadcasted for all the inflammatory process. As we cannot assess the mechanisms responsible for the interactions, further studies are necessary to better evaluate these interactions and the possible causes

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