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OXIDATIVE STRESS AND LOW-GRADE INFLAMMATORY STATUS AS CARDIOMETABOLIC RISK FACTORS IN ITALIAN OCCUPATIONAL OVERWEIGHT/OBESE SUBJECTS

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Obesity is associated with increased risk of cardiometabolic diseases. Adipocytokines (e.g. leptin), produced by the endocrine function of adipose tissue, can contribute to cardiometabolic risk in overweight and obese people. Oxidative stress, imbalance between oxidants and antioxidants, is considered a cardiovascular risk factor. High serum oxidized LDL (oxLDL) levels, marker of lipid peroxidation, a primary cause of atherosclerosis, can contribute to its progression. The aims of this study are to assess markers of oxidative status and cytokine profile and evaluate their role as cardiometabolic risk factors and possible correlations. In this cross-sectional study, we enrolled 76 occupational overweight-obese adults (46 females, 30 males; aged 46.8±9.5; BMI 33.7±4.8 kg/m^2) without any previous cardiovascular disease. Oxidative status was measured by evaluating serum Reactive Oxygen Species (ROS) levels, Total Antioxidant Capacity (TAC) and oxLDL concentrations. All subjects' soluble cytokine and adhesion molecule levels were evaluated by cytofluorimetric method and compared with 35 controls matched for sex and age. ROS and oxLDL levels were high in 84% and 92% of the study population, respectively, despite adequate TAC (68%). Female ROS levels were significantly higher than those of males (414±99.3 *vs* 318±48.2 UCarr, p<0.0001), while their oxLDL levels were lower (95.3±22 *vs* 105.2±19.4 U/L, p=0.1). Leptin and sICAM-1 (intracellular adhesion molecule involved in leukocyte migration to inflamed area) levels of the study population were significantly higher than those of controls (93.8±89.1 *vs* 25.3±23 ng/mL, p=0.0002 and 505.8±236.7 *vs* 339.2±119.6 ng/mL, p=0.0009, respectively). Overweight/obese

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occupational subjects showed oxidative stress conditions accompanied by low chronic inflammatory status, possibly contributing to increased cardiometabolic risk.

Obesity is a growing phenomenon and in the workplace has repercussion for both workers and their employers as it is a known cause of impaired fitness for work. In fact, it is a medical condition where natural energy reserves, stored in human fatty tissue have drastically increased with the risk of cardiovascular diseases and premature death. Individuals with mid-section adiposity can experience elevated cardiovascular morbidity and mortality, quite independently of any association between obesity and other classical cardiovascular risk factors (i.e. smoking, high low-density lipoprotein cholesterol, hypertension, impaired glucose metabolism) and/or less conventional mechanisms (i.e. oxidative stress, inflammation) (1). Adipose tissue has not only a storage function, but it is an endocrine organ secreting various adipokines, which regulate a wide spectrum of metabolic and immune processes. As recently reported, the production of some pro-inflammatory molecules by adipocytes and macrophages increases together with the growth of the fat mass, therefore obesity is generally characterized by a chronic, low grade inflammatory status (2). In particular, the main cytokines associated with inflammation and secreted by adipocytes are interleukins (i.e. IL-6), monocyte chemotactic protein 1 (MCP-1), leptin and resistin (2). Corrado et al. (3) underlined the importance of assessing acute-phase proteins (high sensitive C Reactive Protein, hsCRP, and fibrinogen), which are independently associated with the incidence of coronary events.

Oxidative stress, an imbalance between reactive oxygen species (ROS) and total antioxidant capacity (TAC), is thought to be a potential pathogenetic mechanism linking obesity to endothelial dysfunction (4). Oxidized LDL (oxLDL) has been shown to play an important role in the pathogenesis of atherosclerosis (5).

The aims of this retrospective observational study are to assess classical cardiometabolic risk factors, such as dyslipidemia and high hsCRP levels, and less conventional markers, such as oxidative stress parameters and pro-inflammatory cytokines, and to evaluate their possible role as early markers of endothelial damage in apparently healthy overweight/obese occupational subjects

MATERIALS AND METHODS

Subjects

Seventy-six consecutive overweight/obese workers (46 women and 30 men, mean age 46.8±9.5, mean Body Mass Index (BMI) 33.7±4.8 kg/m^2, female mean waist circumference (wc) 100.4±12.1 cm and male 109.9±9.2 cm) were enrolled at the "Obesity and Work" outpatient clinic of the Clinica del Lavoro "L. Devoto" of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy. All participants were interviewed regarding general health (no previous cardiovascular events), habitual dietary intake, lifestyle and smoking habits and all provided written informed consent. Obesity was defined when BMI≥30 kg/m^2 while overweight condition when 26<BMI<30 kg/m^2.

In order to avoid lifestyle and nutritional influence all subjects were asked to refrain from physical activity 3 days before and to eat light meals the day before outpatient admittance. Peripheral blood samples were drawn in the morning, after an overnight fast. Two blood specimens from each subject were collected, either without additive for serum ROS concentrations, TAC, lipid panel (total cholesterol, t-Chol; high-density lipoprotein cholesterol, HDL-Chol; low-density lipoprotein cholesterol, LDL-Chol; triglycerides, TGs), inflammatory markers (hsCRP; cytokines) and oxLDL levels; or, with ethylenediaminetetraacetic acid (EDTA) to prevent coagulation, for Complete Blood Count (CBC). Serum samples were frozen and stored at -80°C until analysed. Each subject's parameters were measured using the routine standard hospital methods and procedures: CBC (XE 2100 analyzer, Dasit, Italy), lipid panel, and hsCRP (Modular P analyzer, Roche, Swiss).

A group of 35 healthy normal weight subjects, well-matched for sex and age (20F/15M, mean age 49±10.3, mean BMI 21.6±2 kg/m^2), was enrolled as controls for cytokine pattern evaluation.

This retrospective observational study was carried out according to the Declaration of Helsinki guidelines for Research on Human Subjects and was approved by the Human Ethic Committees of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Registration number: 1370).

Oxidative status evaluation

Serum ROS concentrations and serum TAC were

measured using a spectrophotometric method with commercial kits (d-ROMs test and OXY-Adsorbent test, respectively; Diacron International, Italy) on a F.R.E.E. analyzer (Diacron, Italy) as previously reported (6). Serum oxLDL levels were measured by a commercial Enzyme-Linked-Immunoassorbent Assay (Oxidized LDL Competitive ELISA, Mercodia, Sweden) on an EASIA reader (Medgenix Diagnostics, Belgium) as previously reported (6). Values for the oxidative status analytes were compared with the relevant reference values used in our laboratory.

Cytofluorimetric determination of serum cytokines/ molecules

Soluble cytokines and adhesion molecules were measured by cytofluorimetric method using the Human Obesity 9plex commercial kit (Bender MedSystems, Austria) on FACScan Flow Cytometry Becton Dickinson (BD Biosciences, USA). This bead-based multiplex immunoassay is carried out by using a flow cytometer and is designed for simultaneous quantification of different cytokines, in particular: soluble CD40 Ligand (sCD40L), soluble Intercellular Adhesion Molecule-1 (sICAM-1), Interleukin-6 (IL-6), Leptin, MCP-1, myeoloperoxidase (MPO), Osteoprotegerin (OPG), Resistin, and soluble Tumor Necrosis Factor Receptor (sTNFR). Beads, coated with antibodies specifically reacting with each of the analytes to be detected in the multiplex system, can be differentiated by their size and distinct spectral addresses. A mixture of coated beads for each analyte is incubated with 25 μL of serum sample or standard mixture. The analytes in the sample bind to the antibodies linked to the fluorescent beads. A conjugated second antibody mixture is added, the specific antibodies bind to the analytes captured by the first antibodies. Streptavidin-Phycoerythrin is added and binds to the biotin conjugate emitting fluorescent signals. Analytical sensitivities of sCD40L, sICAM-1, IL-6, Leptin, MCP-1, MPO, OPG, Resistin, sTNF-R were: 24.3 pg/mL, 5.3 ng/mL, 4.4 pg/mL, 1.4 ng/mL, 18.2 pg/mL, 0.02 ng/mL, 7.9 pg/mL, 1.7 pg/mL, 0.08 ng/mL, respectively. Intra-assay variation coefficient (CV) for all parameters was <10%; CV inter-assay of all the analytes except MCP-1 (12.5%) was <10%. Standard curves were obtained by using reference standard beads. Quantitative results were evaluated with FlowCytomix Pro Software (Bender Medsystem, Austria).

Statistical analysis

Data were expressed as mean ± standard deviation. Data distribution was evaluated by using D'Agostino-Pearson test. Comparison between independent data was performed by using Student's *t*-test. To evaluate the degree of association between variables univariate analysis and/or multiple regression were carried out. HsCRP values were log-transformed to normalize their skewed distribution. All analyses were performed with MedCalc statistical software (Belgium).

RESULTS

Table I shows the results of lipid panel, oxidative status and inflammatory markers together with the percentage of subjects with altered values. In particular, the majority of subjects showed an altered lipid panel. The percentages of both TGs and HDL-Chol pathological values of males were higher than those of females (50% *vs* 11%, p=0.0005 and 93% *vs* 65%, p=0.01, respectively). All subjects showed normal haematological status based on the results of the standard CBC panel (data not shown).

Compared with relevant reference cut-off used in our laboratory, the majority of serum ROS and oxLDL concentrations of the subjects were high (84% and 92%, respectively), while 32% of cases had decreased TAC. In particular, female ROS concentrations were significantly higher than those of males (414±99.3 *vs* 318±48.2 UCarr, p<0.0001) and the percentage of females with altered ROS values was significantly higher than that of males (97% *vs* 63%, p=0.0003). Nevertheless, the oxLDL mean levels of females were lower than those of the males, even if not significantly so (95.3±22.0 *vs* 105.2±19.4 U/L; p=0.1), as well as the percentage of female oxLDL altered values (86% vs 100%; p=0.2). ROS concentrations significantly correlated with hsCRP levels (r=0.25; p=0.03). BMI and WC correlated with hsCRP (r=0.53, p<0.0001; r=0.32, p=0.006) but, with multivariate linear regression analysis (considering BMI, WC and ROS as independent variables), only BMI affected hsCRP levels (r=0.56, p=0.0001). As shown in Fig. 1, oxLDL levels correlated significantly with t-Chol and LDL-Chol (r=0.63, p<0.0001 and r=0.63, p<0.0001, respectively) while there was only a trend with TGs (r=0.26, p=0.06) and HDL-Chol (r= -0.24, p=0.09). Table II shows data of soluble cytokines and adhesion molecules of obese and control subjects: only sICAM-1 and leptin levels were significantly higher in obese than in controls. Regarding adipocytokines exclusively secreted by adipose tissue (leptin and resistin), no correlation

Table I. *Lipid Panel, hsCRP levels and Oxidative Status parameters in the whole population.*

Analytes	All Subjects	Female (F)	Male (M)	p (F *vs* M)
Subjects (nr.)	76	46	30	
Age (years)	46.8±9.5	47.7±9.1	45.4±9.9	N.S.
BMI (kg/m^2)	33.7±4.8	34.2±5.4	32.9±3.5	N.S.
Waist circumference (cm)	-	100.4±12.1	109.9±9.2	-
Number of Smokers	18	8	10	N.S.
Mean number of cigarette/day	8.2±2.3	8.6±3	7.8±1.6	N.S.
T-Chol	219.1±35.8	219.5±32.1	218.4±41.3	N.S.
<200 mg/dL	(72)	(70)	(73)	(N.S.)
LDL-Chol	134.7±28.7	132.9±27.2	137.4±31.2	N.S.
<130 mg/dL	(56)	(54)	(57)	(N.S.)
HDL-Chol	-	58.9±12.3	42.1±8.2	-
F>65 mg/dL		(65)	(93)	
M>55 mg/dL				
TGs	144.5±76.5	119.5±50.9	182.9±92.9	0.001
<170 mg/dL	(26)	(11)	(50)	(0.0005)
hsCRP	0.44±0.45	0.48±0.49	0.38±0.39	N.S.
<0.5 mg/dL	(21)	(24)	(16)	(N.S.)
ROS	376.1±95.1	414.0±99.3	318±48.2	<0.0001
<300 U. Carr	(84)	(97)	(63)	(0.0003)
TAC	384.2±67.4	376.2±56.9	396.4±80.5	N.S.
>350 μmolHClO/mL	(32)	(30)	(33)	(N.S.)
oxLDL	99.5±21.3	95.3±22.0	105.2±19.4	N.S.
<70 U/L	(92)	(85)	(100)	(N.S.)

Data are expressed as mean ± standard deviation (SD) and % of altered cases in parentheses.

Table II. *Cytokine concentrations of our population and 35 controls were expressed as mean ± standard deviation (SD).*

Analytes	Obese/Overweight	Controls	p
sCD40L (pg/mL)	2865.4±743.4	2456.5±1503.1	N.S.
sICAM-1 (ng/mL)	505.8±236.7	339.2±119.6	**0.0009**
IL-6 (pg/mL)	71.9±57.1	108.6±112.6	N.S.
Leptin (ng/mL)	93.8±89.1	25.3±23.0	**0.0002**
MCP-1 (pg/mL)	411.1±107.7	423.3±158.7	N.S.
MPO (ng/mL)	335.4±108.7	404.2±209.2	N.S.
Resistin (pg/mL)	13371.5±6536.0	12490.5±5052.1	N.S.
sTNF-R (ng/mL)	2.0±1.1	2.2±1.1	N.S.

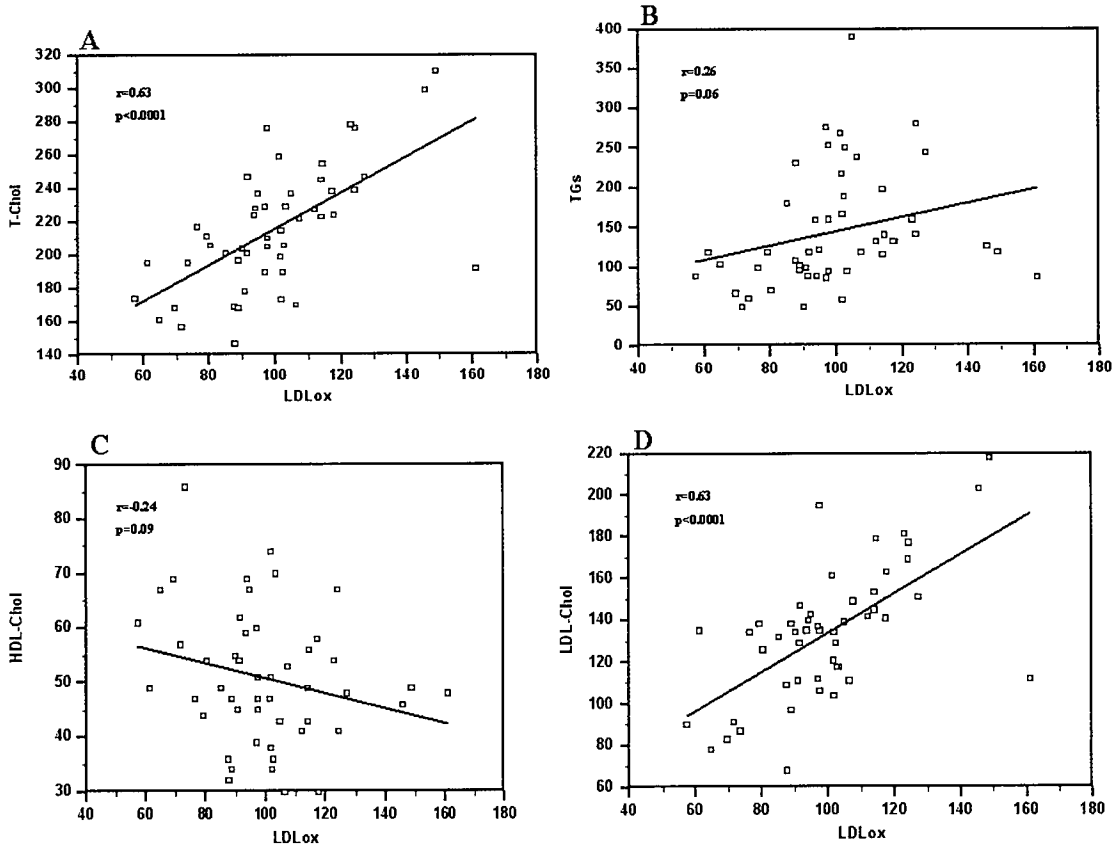

Fig. 1. *Correlations between oxLDL and lipid panel parameters in occupational overweight-obese subjects.*

was found with anthropometric parameters (BMI and WC). Leptin concentrations were significantly higher in overweight/obese females than in males (124.6±90.9 *vs* 35.1±47.9 ng/mL, p=0.0005) while controls did not show such a difference between male and female. With regard to other cytokines, no gender differences were observed. Leptin levels correlated positively with ROS concentrations (r=0.41, p=0.02).

In obese subjects resistin levels were higher than in controls (even if not significantly so) and correlated with hsCRP (r=0.40, p=0.02) and IL-6 (r=0.75, p<0.0001) levels. We did not observe any difference between obese subjects and controls in MCP-1 and IL-6, however the study population, but not controls, showed a significant correlation (r=0.35, p=0.04) between these two molecules.

Approximately 24% of the study subjects smoked and 2 males of the control group were light smokers (<10 cigarettes/day). No correlation was observed between smoking and cytokines in either group. OxLDL levels were higher in smokers than non-smokers (116.0±19.1 vs 96.1±20.5 U/L, p=0.01).

DISCUSSION

Obesity is a rapidly growing health problem also in the workplace, carrying with it an increased risk of morbidity and mortality, especially due to Type 2 diabetes and atherosclerotic cardiovascular disease (1).

Even though it is unclear whether obesity itself or obesity-associated conditions lead to oxidative stress, several mechanisms, such as systemic low-grade inflammatory state, are likely contributors. This study evaluated the oxidative and inflammatory status of healthy overweight/obese subjects.

According to our previous study (7), we evaluated oxidative status by assessing the general pro-oxidant/antioxidant balance instead of measuring a specific marker of molecular or functional damage. ROS are produced by mitochondrial activity in various oxidation pathways and are normally counterbalanced by cellular antioxidant systems. When ROS concentrations increase, they can react with cellular macromolecules and enhance the process of lipid peroxidation, cause DNA damage and/or induce protein and nucleic acid modifications (8). Despite excessive ROS production, increased antioxidant system activation might justify normal TAC.

Increased concentrations of ROS (particularly superoxide and hydrogen peroxide) lead to enhanced oxidation of low-density lipoprotein (LDL), inactivation of endothelium-derived nitric oxide and vascular dysfunction (5). Previous studies have shown that elevated oxLDL concentrations can predict myocardial infarction in elderly people even when the subjects appear to be in good health and after adjusting for age, gender, race, smoking and metabolic syndrome (5). In agreement with other authors (8-9), our findings confirmed an increase in most subjects (92%) of oxLDL concentrations, which indicated lipid peroxidation, more advanced in cases of serious dislipidemia and in smokers.

In particular, in our study population, we found a significant gender-related difference in oxidative status. oxLDL levels of males were higher than those of females; this result may be due to more severe dyslipidemia of the males.

On the contrary, ROS concentrations of the females were significantly higher than those of the males. These findings are in agreement with the study of Vassalle et al. (10) reporting that global Oxidative-INDEX of females was significantly higher than that of males; the authors suggested a role of sex hormone-dependent differences in oxidative stress condition (11, 12).

Several studies have reported that elevated hsCRP concentrations (marker of systemic inflammation) are consistently associated with myocardial infarction and stroke (14). In this study, twenty-one percent of subjects showed hsCRP levels higher than cut-off, highlighting a low-grade systemic inflammation more advanced in subjects with higher BMI, in agreement with Arcari et al. (13).

Inflammation and oxidative stress are closely interrelated: inflammation causes ROS production which promotes the synthesis of pro-inflammatory cytokines (14). This relationship is also confirmed in our study where a positive correlation was found between ROS and hsCRP.

In order to investigate low-grade inflammation associated with obesity, pro-inflammatory cytokines were evaluated together with adipokines (leptin and resistin).

The cytofluorimetric method was chosen to simultaneously quantify several molecules involved. Multiplex immunoassays are promising tools for the protein profiling of complex human body fluids such as serum. Despite the limitations of this new method, some interesting results emerged from evaluation of the cytokine pattern.

Resistin, a cysteine-rich protein secreted by adipose tissue, is involved in the pathogenesis of insulin resistance, adipogenesis and inflammation (2, 15). The serum levels of resistin of our obese subjects were higher than those of the normal weight controls, even if not significantly so. The significant positive correlation found between resistin and inflammatory markers (hsCRP and IL-6), confirm the role of this protein as an inflammatory adipokine (16).

Leptin (encoded by the *Ob* gene, expressed predominantly in adipocytes) is a protein which signals to the central nervous system and peripheral organs of the nutritional status of the body, and plays an important role in regulating body weight and composition (17). In our study, serum leptin concentrations of the obese subjects were significantly higher than those of the controls, probably due to the resistance of hypothalamus to the anorexic effect of this hormone (2).

Several studies have shown a correlation between leptin and sex hormones. According to Chow et al. (17), leptin is involved in the modulation of the hypothalamo-pituitary-gonadal axis during sexual maturation, development and reproduction and its concentrations increase in female but decline in male children. Our results confirmed sexual dimorphism in serum leptin levels probably due to the gender-related differences in sex hormones.

With regard to oxidative status alteration, we found a positive correlation between leptin and ROS, in agreement with Fortuno et al. (18) who reported an association between NADPH oxidase overactivity and leptin concentrations and suggested an involvement of leptin in oxidative status of obese subjects. The association between CC chemokine ligand (MCP-1) and IL-6 in obese subjects but not in controls supported the low-grade inflammatory status associated with obesity.

We underline that vascular wall inflammation is a crucial step in the pathophysiology of atherosclerosis (14). The interactions between inflammatory cells (i.e. macrophages) and structural tissue cells (i.e. endothelial cells) are by means of adhesion molecules, specific cell surface proteins involved in mediating intracellular and extracellular adhesion (19). In particular, sICAM-1 is an intracellular adhesion molecule involved in leukocyte migration to the inflamed area (20). Our obese subjects showed significantly higher sICAM-1 concentrations than controls confirming that obesity *per se* plays a role in systemic inflammation, partially responsible for the elevated risk of cardiovascular complications.

In conclusion, our retrospective observational study, showed that occupational overweight/ obese subjects were characterized by oxidative stress accompanied by an interrelated low-grade chronic inflammatory status, a condition which may contribute to increased cardiovascular risk. Thus, health promotion campaigns in the workplace may be considered for an early intervention on metabolic disorders (e.g. metabolic syndrome, diabetes).

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