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Association of monocyte CCR2 expression with obesity and insulin resistance in postmenopausal women

Abstract

Purpose: Monocytes actively participate in inflammatory mechanisms that contribute to the development of adipose tissue dysfunction and atherogenesis. The aim of this study was to evaluate the association of monocyte CCR2 chemokine receptor expression and intracellular oxidative burst with the metabolic and inflammatory factors related to body weight.

Methods: The study was performed in 67 postmenopausal women with normal, overweight and obese body mass index. Monocyte CCR2 surface expression and intracellular oxidative burst activity (determined using 2',7'-dichlorofluorescein diacetate) were analyzed by flow cytometry. Serum levels of HMW adiponectin, monocyte chemoattractant protein-1 (MCP-1), insulin, glucose, lipids and C-reactive protein were determined.

Results: Subjects with homeostasis model assessment-estimated insulin resistance (HOMA-IR) above the median had significantly higher proportion of CCR2+ monocytes and higher mean fluorescence intensity (MFI) of CCR2 and oxidative burst. The proportion of CCR2+ monocytes and CCR2 MFI were correlated with body weight, body mass index, fat mass, insulin and HOMA-IR. Oxidative burst also correlated with anthropometric measures, fat mass and expression of CCR2. No correlations were found between these markers of monocyte activation and HMW adiponectin or monocyte chemoattractant protein-1. The absolute number of monocytes was associated with insulin and this association remained significant after adjusting for C-reactive protein. In the multiple regression model the monocyte number was determined to be an independent predictor of insulin level.

Conclusion: These results provide support for significant associations of monocyte number and markers involved in monocyte activation with obesity and insulin resistance.

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The contribution of mononuclear-phagocyte cells to the state of chronic low-grade inflammation seems to be crucial for the link between obesity and metabolic disorders like diabetes and atherosclerosis. In obese subjects, macrophages infiltrate adipose tissue, with their proportion rising from about 10% in thin subjects to up to 40% in obese. In addition, 30% to almost 60% of upregulated genes in adipose tissue of obese subjects likely are from a macrophages source [1, 2]. Adipose tissue macrophages (ATM) derive from circulating monocytes. Their recruitment in obesity is similar to the basic inflammatory response seen in the early stages of atherogenesis [3]. The key promigratory signal is the secretion of a chemokine monocyte chemoattractant protein-1 (MCP-1), which binds to the CCR2 receptor on the monocytes and promotes monocyte activation, binding and diapedesis through the endothelium [4]. These CCR2+ blood monocytes, unlike the CCR2- cells, have been shown to be selectively recruited and to differentiate into pro-inflammatory tissue macrophages [5, 6].

The spectrum of adipocytokines in obesity switches to a pro-inflammatory pattern, with higher levels of tumour necrosis factor α , interleukin-6 or MCP-1 and a lower serum concentration of the protective adiponectin. These changes, as part of the adverse metabolic impact of obesity, are enhanced by postmenopausal central body fat redistribution [7]. Adipocytokines, together with insulin, contribute to the circulatory milieu that presumably affects the process of monocyte activation [3]. Monocytes from obese patients were already shown to be in an activated, pro-inflammatory state by the higher level of intracellular cytokines [8]. Postmenopause was also associated with inflammatory alterations in monocytes [9]. To assess the changeable monocyte phenotype, flow cytometric expression of CCR2 receptor was analysed. Reactive oxygen species, as signal molecules, participate in monocyte activation by stimulating expression of adhesion molecules and chemokine receptors so intracellular oxidative burst was also measured [10].

The aim of this study was to evaluate the association of markers participating in monocyte activation with metabolic and inflammatory factors related to body weight. Evidence from experimental research strongly suggests the involvement of monocytes in the development of metabolic disorders. It is still unclear, however, whether this involvement can be observed in a clinical setting, particularly among postmenopausal women who might be susceptible to the increased cardiovascular risk.

Methods

Subjects

Participants were recruited consecutively among postmenopausal women (more than one year without menstrual cycles) from the outpatient endocrine clinic. Exclusion criteria were infections, blood pressure higher than 140/90 mmHg, known cardiovascular disease, diabetes (fasting blood glucose higher than 7 mmol/l or 2 hour oral glucose tolerance test higher than 11.1 mmol/l) or LDL-cholesterol (LDL-c) levels higher than 4 mmol/l. Subjects who smoked and who took hormone replacement therapy or drugs, which might interfere with inflammatory and metabolic factors (non-steroidal anti-inflammatory drugs, corticosteroids, statins, angiotensin-converting inhibitors and angiotensin receptors blockers), were also excluded. Following institutional ethic rules, participants were informed about the study and signed informed consent forms.

Subjects underwent a clinical examination in order to collect demographic and clinical data. Anthropometric measurements were performed according to standard procedures. The bioelectric impedance device, Genius 220 (Jawon Medical, Kyungsan-City, Korea), was used for body composition analysis. Body fat mass was measured in kilograms and the proportion of fat mass relative to total body mass.

The screening period for each patient was up to four days, the recruitment of all patients lasted three months and 67 participants were included in the study. Patients were divided according to body mass index (BMI) into three groups: participants with BMI from 20 to 24.99 kg/m², participants with BMI from 25 to 29.99 kg/m² and participants with BMI greater than 30 kg/m². Participants were also divided in two groups according to the median value of the homeostasis model assessment-estimated insulin resistance (HOMA-IR).

Sample collection and analysis

Laboratory investigations were performed on samples of venous blood after 12 hours of overnight fasting. In each participant all samples were taken on the same occasion, under standardized conditions. Full blood count, glucose, C-reactive protein (CRP), cholesterol, LDL-c, HDL-cholesterol (HDL-c), triglycerides and insulin were determined on the same day by routine laboratory procedures. The blood sample for high molecular weight adiponectin (HMW adiponectin) and MCP-1 was frozen at -80 °C. HMW adiponectin (Bühlmann Laboratories AG, Schönenbuch, Switzerland) and MCP-1 (R&D Systems, Minneapolis, MN, USA) were measured by ELISA

according to the guidelines of the manufacturers. Insulin resistance was assessed by HOMA-IR using the formula:

$\text{HOMA-IR} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin } (\mu\text{U/ml}) / 22.5$ [11].

Flow cytometry

The blood sample for immunophenotype and oxidative burst analysis were used immediately. The antibody CD14 APC (IQ Products, Groningen, Netherlands) was used as a marker of monocytes and expression of CCR2 antigen was determined with the CCR2 PE (R&D Systems) antibody.

Quantification of intracellular oxidative burst was performed by flow cytometry on CD14+ cells using the 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) fluorochrome, which is converted to highly fluorescent 2', 7'-dichlorofluorescein (DCF) using the protocol described by Bass *et al.* with some modifications [12]. For evaluation of oxidative burst of stimulated leukocytes, phorbol-12-myristate-13-acetate (PMA) (Sigma) was used. Assays were performed simultaneously on 100 μl blood samples distributed into three tubes: the first contained 5 μM DCFH-DA, the second also contained DCFH-DA but cells were stimulated by PMA and the third as a negative control. The three tubes were incubated for 15 minutes at 37 °C. In the second tube, 100 ng/ml PMA was then added and the incubation was continued for 15 minutes more at 37 °C. In all three tubes, 2 ml of cold PBS was added and they were kept in refrigerator for 5 minutes at 2 to 8 °C and afterwards centrifuged for 5 minutes at room temperature. The antibody CD14 APC (IQ Products) was added to the pellet in all three tubes and samples incubated in the dark for 15 minutes.

Monocyte gate was determined by forward and side scatter. In this gate, CD14+ cells were selected. Quantification of CCR2 expression was performed by determining the proportion of positive cells (CCR2 %) for respective marker on CD14+ cells inside the monocyte gate and by determining their mean fluorescence intensity (CCR2 MFI). Oxidative burst was measured by the mean fluorescence intensity (MFI) of DCF dye on CD14+ cells inside the monocyte gate on non-stimulated (DCF MFI) and stimulated (DCF PMA MFI) samples.

In order to standardize measurements, MFI values have been converted into molecular equivalents of soluble fluorochrome (MESF), employing calibration beads (DAKO Fluorospheres, Glostrup, Denmark). This procedure enabled the comparison of MFI between different patients during the entire duration of the study. The expression of surface markers and oxidative burst were measured and analysed by Beckman

Coulter Cytomics FC500 flow cytometer and the CXP software package (Beckman Coulter, Brea, USA).

Statistical analyses

Descriptive statistics of the sample were presented as mean $\pm$ standard error of the mean. Parametric and non-parametric methods were used depending on the normality of the variable distribution. A Kolmogorov-Smirnov test was used to confirm the normal distribution of variables. Parametric Student's *t*-tests and non-parametric Mann-Whitney U tests were used for testing the difference between two groups. The difference between several groups was tested by ANOVA and nonparametric Kruskal-Wallis tests. Results of linear correlation are presented by Pearson correlation coefficient (*r*) for normally distributed variables and by Spearman's rank correlation coefficient (ρ) for variables without normal distribution. Multiple linear regression analysis was used to evaluate independent predictive variables and the optimal choice of predictors was conducted by forward stepwise regression procedure. Two-sided value of $P < 0.05$ was considered to be significant. Statistical analysis of data was performed by STATISTICA 7.1 software.

Results

Clinical and laboratory data of the whole sample of 67 patients as well as subgroups according to BMI were presented in Table 1.

Participants were also divided in two groups according to the median value of HOMA-IR (2.04). The difference between the groups with lower and higher insulin resistance was found in the body weight, waist circumference, fat mass and its proportion ($P < 0.05$), but not in BMI. Significantly higher levels of triglycerides, lower levels of HDL-c and higher levels of CRP were found in the group with higher insulin resistance ($P < 0.05$), but there were no differences in the absolute number of monocytes and levels of HMW adiponectin or MCP-1. Significant differences were found in CCR2 expression: proportion of CCR2+cells (CCR2 %) and CCR2 MFI ($P < 0.05$) were higher in subjects with higher HOMA-IR. Oxidative burst in non-stimulated (DCF MFI) and stimulated monocytes (DCF PMA MFI) were also higher in these subjects ($P < 0.05$).

In the correlation analysis, anthropometric parameters and body composition variables were significantly associated with

TABLE 1. Clinical and laboratory data of participants according to BMI groups

	Group 1 (n = 15)	Group 2 (n = 23)	Group 3 (n = 29)	All (n = 67)	Overall <i>P</i>
age (years)	60 (55 – 65.5)	59 (56 – 63)	59 (54 – 67)	59 (55 – 66)	n.s.
BW (kg)	59.1 (53.4 – 60.9) ^{a*}	70.7 (67.9 – 75) ^b	81.2 (77.4 – 91.1) ^c	75.1 (67 – 81)	< 0.001
BMI (kg/m ²)	23.5 (21.1 – 24.3) ^a	26.6 (25.5 – 27.9) ^b	33.5 (31.1 – 35.8) ^c	28.4 (25.4 – 32.6)	< 0.001
waist (cm)	82 (76 – 87.5) ^a	90 (84 – 92) ^b	104 (99 – 112) ^c	91 (84 – 104)	< 0.001
fat mass (kg)	19.1 (15.7 – 21.3) ^a	25.5 (22.9 – 27) ^b	36.6 (32.4 – 39.5) ^c	29.3 (22.5 – 34.4)	< 0.001
fat mass (%)	32 (29.6 – 34.3) ^a	35.9 (34 – 38.1) ^b	42.3 (40.8 – 43.7) ^c	38.1 (34 – 41.9)	< 0.001
cholesterol (mmol/l)	6.1 (5.5 – 6.4)	6.1 (5.6 – 6.5)	5.9 (5.3 – 6.4)	6 (5.6 – 6.4)	n.s.
LDL-c (mmol/l)	3.5 (3.1 – 3.8)	3.4 (3 – 3.7)	3.3 (2.9 – 3.7)	3.4 (3.1 – 3.7)	n.s.
HDL-c (mmol/l)	1.7 (1.3 – 2.2)	1.7 (1.3 – 1.8)	1.4 (1.3 – 1.8)	1.6 (1.3 – 1.8)	n.s.
triglycerides (mmol/l)	0.9 (0.7 – 1.3) ^a	1.3 (1 – 1.4) ^b	1.3 (1.1 – 1.7) ^b	1.2 (1 – 1.4)	0.028
glucose (mmol/l)	5.4 (5 – 5.5)	5 (4.7 – 5.6)	5.4 (5.1 – 5.5)	5.3 (4.9 – 5.5)	n.s.
insulin (μU/ml)	8 (4.6 – 11)	8 (6 – 9) ^a	11 (8 – 14) ^b	9 (7 – 12)	0.042
HOMA-IR	2 (1.1 – 2.8)	1.9 (1.5 – 2.2) ^a	2.5 (1.8 – 3.4) ^b	2 (1.5 – 2.9)	0.031
HMW adiponectin (μg/ml)	4.7 (3.6 – 6.9) ^a	3.5 (2.5 – 4.9)	3.2 (2.2 – 3.9) ^b	3.6 (2.5 – 5)	0.055
MCP-1 (pg/ml)	131.8 (112.1 – 159.5)	119.5 (99.1 – 168.3)	109.8 (105.3 – 142)	119.2 (105 – 147.4)	n.s.
CRP (mg/l)	2.1 (1.4 – 2.7)	1.2 (0.9 – 1.8) ^a	2.8 (1.6 – 3.5) ^b	1.9 (1.1 – 3.1)	0.016

Data are expressed as median and interquartile range.

Group 1 includes participants with body mass index (BMI) from 20 to 24.99 kg/m², group 2: BMI from 25 to 29.99 kg/m², and group 3: BMI higher than 30 kg/m².

BW, body weight; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein; n.s., not significant.

*Different letters (a, b, c) in the same row indicate two groups that are significantly different ($P < 0.05$).

triglycerides, glucose, insulin and HOMA-IR, while HDL-c and HMW adiponectin inversely correlated with them ($P < 0.01$). There was a negative correlation of insulin and HOMA-IR with HDL-c and positive with triglycerides ($P < 0.01$). A positive correlation between HMW adiponectin and HDL-c

($r = 0.69$, $P < 0.01$) and negative between HMW adiponectin and triglycerides ($r = -0.41$, $P = 0.002$) were determined, but the inverse relation between HMW adiponectin and insulin did not reach statistical significance ($r = -0.26$, $P = 0.069$). No

correlations were found for MCP-1, total cholesterol and LDL-c with any of variables.

CRP correlated with all measures of obesity: body weight ($r = 0.3$, $P = 0.023$), waist circumference ($r = 0.33$, $P = 0.012$), BMI ($r = 0.31$, $P = 0.017$) and fat mass ($r = 0.32$, $P = 0.027$). CRP also significantly correlated with insulin ($r = 0.31$, $P = 0.023$) and inversely correlated with HDL-c ($r = -0.29$, $P = 0.026$) and HMW adiponectin ($r = -0.27$, $P = 0.049$).

The absolute number of monocytes correlated with insulin levels ($r = 0.34$, $P = 0.011$), HOMA-IR ($r = 0.29$, $P = 0.031$) and inversely correlated with HDL-c ($r = -0.26$, $P = 0.048$). Considering that there was a correlation between CRP and the number of monocytes ($r = 0.3$, $P = 0.022$), it was important to distinguish whether the association of monocytes with insulin is mediated by CRP. In partial correlation analysis, after adjustment for insulin, the statistically significant relationship between CRP and monocytes ($r = 0.22$, $P > 0.05$) was lost, just as the correlation of CRP with anthropometric variables and body composition measures. When partial correlation analysis was performed with adjustment for the level of CRP, the association of insulin with the absolute number of monocytes weakened only slightly ($r = 0.28$, $P = 0.044$) and remained statistically significant. This result suggested that the relationship between monocytes and insulin was dependent not on CRP but on a different mechanism.

In Table 2, correlations of monocyte activation variables are shown. CCR2 MFI correlated with body weight, BMI, fat mass and proportion, insulin and HOMA-IR. CCR2 % correlated with the mass and the proportion of body fat.

Oxidative burst, determined by MFI, of DCF with and without PMA stimulation correlated with anthropometric measures and the amount of body fat. No significant associations between monocyte activation variables and inflammatory parameters were found. This was expected because patients with acute inflammation were not included in the study.

Finally, there was a statistically significant correlation between MFI and proportion of CCR2 expression with oxidative burst in monocytes.

Multiple linear regression analysis (Table 3), with insulin level as the dependent variable, was performed. The developed model revealed that monocyte number, BMI and triglycerides were independent predictors of insulin. These factors accounted for 38.1% of the insulin level variation and the adjusted coefficient of determination (R^2) was 0.381.

TABLE 2. Correlations of monocyte activation variables

	CCR2 MFI		CCR2 %		DCF MFI	
	r	P	ρ	P	r	P
BW	0.26	0.047	0.27	0.04	0.31	0.019
waist	0.25	n.s.	0.26	n.s.	0.29	0.03
BMI	0.26	0.049	0.28	0.036	0.35	0.007
fat mass (kg)	0.31	0.032	0.33	0.02	0.38	0.007
fat mass (%)	0.38	0.008	0.34	0.016	0.31	0.028
HDL-c	-0.11	n.s.	-0.27	0.04	-0.15	n.s.
triglycerides	0.06	n.s.	0.17	n.s.	0.06	n.s.
glucose	0.13	n.s.	0.19	n.s.	-0.11	n.s.
insulin	0.30	0.028	0.27	n.s.	-0.06	n.s.
HOMA-IR	0.28	0.037	0.29	0.034	-0.07	n.s.
HMW adiponectin	-0.14	n.s.	-0.17	n.s.	-0.22	n.s.
CRP	0.04	n.s.	0.2	n.s.	0.13	n.s.
leukocytes	-0.05	n.s.	0.14	n.s.	-0.10	n.s.
monocytes	0.18	n.s.	0.24	n.s.	-0.04	n.s.

MFI, mean fluorescence intensity; DCF, dichlorofluorescein; PMA, phorbol-12-myristate-13-acetate; CCR2 %, proportion of CCR2 positive CD14 cells; CD11b %, proportion of CD11b positive CD14 cells; BW, body weight; BMI, body mass index; HDL-c, HDL cholesterol; CRP, C-reactive protein; r, Pearson correlation coefficient; ρ , Spearman's rank correlation coefficient; n.s., not significant.

TABLE 3. Multiple linear regression model with insulin level as a dependent variable

	β	P
constant		0.995
triglycerides	0.340	0.004
BMI	0.241	0.039
monocytes	0.221	0.041
HDL-c	-0.166	n.s.

β , standardized regression coefficient; n.s., not significant.

Discussion

The close association of low-grade inflammation with obesity and insulin resistance was evident in our cohort of postmenopausal women. CRP levels typically reflect this phenomenon and among our participants CRP correlated with measures of obesity, insulin and was inversely associated with HMW adiponectin. Dividing study sample into groups according to BMI was ineffective in discriminating inflammatory variables that refer to monocyte activation. Differences in these variables appeared only in two groups with disparate insulin resistance. In the relationship between low-grade inflammation and insulin resistance, one element was highlighted: the number of monocytes. CRP, monocytes and insulin were all related to each other, but the association of monocytes with insulin was independent of CRP. Moreover, monocytes are an independent predictive factor in the multivariate regression model of insulin. Being independent but complementary with CRP, monocyte count reflects a different component of the inflammatory process and specifically the mechanisms by which they are involved [13, 14]. Monocytes and monocyte-derived macrophages have been shown to participate in the pathogenesis of insulin resistance by supporting inflammation. The association of monocytes with insulin has been confirmed in clinical trials [15]. Monocytes are less prone to acute changes than neutrophils or leukocytes and it was observed that, among leukocytes, the biggest cardiovascular risk is brought by monocytes. The link between monocytes and insulin resistance is also supported by the independent association of monocytes with metabolic syndrome components: waist circumference, blood pressure and dyslipidemia [16].

The association of metabolic disorders with phenotype heterogeneity of human monocyte subsets results from the subsequent involvement of chemokines, cytokines and adhesion molecules in monocyte activation [17]. A statistically significant correlation was found between CCR2 expression in monocytes with body weight, fat mass, but also with insulin and HOMA-IR. The association of CCR2 expression with insulin resistance has been previously recognised in subjects having more advanced metabolic disorder: higher monocyte CCR2 expression and higher serum MCP-1 were measured in patients with diabetes compared to non-diabetic patients [18]. In adipose tissue, CCR2 gene expression was elevated in obese patients compared with lean controls in both subcutaneous and visceral depot [19]. Experiments strongly support the hypothesis that CCR2 sustains both chronic inflammation and systemic insulin resistance by mediating the infiltration of inflammatory ATM in adipose tissue [20]. Despite some contrary results [21], CCR2 deficiency also attenuated ATM ac-

cumulation, while pharmacologic CCR2 antagonist reduced ATM content and improved insulin sensitivity in mice [22]. The key to this relationship among CCR2 chemokine receptor, obesity and insulin resistance is thought to be the insulin-sensitizing gene transcription factor, PPAR γ . Although expressed in adipocytes too, PPAR γ has its dominant role in the monocyte-macrophage system [23]. PPAR γ activation in monocytes and macrophages reduces the expression of many inflammatory genes, reduces the oxidative burst and secretion of inflammatory cytokines. Experiments using a ligand demonstrated the entire pathogenetic sequence: PPAR γ activation of circulating monocytes suppressed the expression of CCR2 chemokine receptor, caused the rapid loss of functional CCR2 protein, inhibited the physiological response of monocytes to MCP-1 and inhibited the infiltration of adipose tissue by macrophages [24]. CCR2 seems to be an important tool by which PPAR γ manages the inflammatory process, and monocyte CCR2 expression might, in fact, reflect actions of this key factor in insulin resistance. Although the design of our study does not allow for assumptions about causal relationships, the expression of CCR2 in circulating monocytes in the clinical settings fits into this paradigm.

Evaluation of monocyte activation by measurement of oxidative burst yielded correlations with anthropometric variables and measures of the fat mass in our subjects. Oxidative burst in monocytes is presumed to be one of possible pathophysiologic mediators of chronic inflammation in atherosclerosis and obesity. In the case of obese patients, it was suggested that elevated reactive oxygen species are early markers of monocytic commitment with an inflammatory phenotype [25]. Macrophages were also identified as being responsible for the high levels of free radicals in adipose tissue because of their high capacity of free radical production [26]. In our cohort, oxidative burst correlated with monocyte CCR2 expression, too. The use of antioxidants *in vitro* experiments rapidly reduced the levels of CCR2 in monocytes [27]. Additional insight into the changes of the system was provided by the use of PPAR γ ligands, which demonstrated that oxidative burst in monocytes was mitigated through PPAR γ agonists [28]. Although PPAR γ transcription factor has only indirect impact on free radicals, through its interaction with NF κ B, these findings indicated that it might also take the part in mediating the link between CCR2 and oxidative burst.

The MCP1/CCR2 system is thought to have a direct role in monocyte recruitment to the fat tissue and the arterial wall; however, our data were not able to support the relationship among circulating MCP-1, obesity-related parameters and monocyte activation. Circulating MCP-1 was found to be as-

sociated with obesity in several studies, but this relationship could not be consistently reproduced even in large scale studies [29]. MCP-1 was shown to be very dependent on the impaired metabolic status and our study subjects differed by body weight but they had neither major metabolic disorders nor evident cardiovascular diseases. A large part of MCP-1 variance may also depend on other factors because MCP-1 shows ubiquitous expression in many cell types. For instance, excess adipose tissue seems to be the primary source of MCP-1 in obesity, but in lean individuals osteoblasts are an important source of MCP-1 [30]. Adipose tissue mRNA expression of MCP-1 was determined to be correlated with obesity and this suggests that circulating MCP-1 may not be representative of the local paracrine role of MCP-1 in monocyte activation and adipose tissue infiltration [31].

In conclusion, our study indicated important links between monocytes and obesity and, especially, insulin resistance. The number of monocytes was found to be an independent predictor of insulin level, supporting the hypothesis that monocytes play an important role in chronic inflammation in obesity. The CCR2 marker and oxidative burst in monocytes were significantly associated with insulin resistance, so it remains to be further elucidated how monocyte activation could contribute to the assessment and management of obesity and its comorbidities.

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