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Serum Proinflammatory Chemokines in Healthy Elderly Taking or not Taking Simvastatin – Data from the Brisighella Heart Study**

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Recent preclinical evidence and data from adult subjects suggests that statins could improve the proinflammatory profile of hypercholesterolemic subjects.
Objectives. We aim to compare the serum levels of a set of proinflammatory chemokines in elderly statistical twins taking or not taking statins.
Material and Methods. Among the historical cohort of the Brisighella Heart Study, we chose 40 healthy elderly subjects continuously treated with statins for at least 1 year and 40 cross-matched subjects not treated with statins (M : F = 1 : 1) characterized by similar age, body mass index (BMI), leisure-time and working activity, smoking habits, history of cardiovascular disease, systolic and diastolic blood pressure, fasting plasma glucose, plasma lipids, uric acid, and creatinine.
Results. The proinflammatory chemokine serum level is similar in statin untreated and treated statistical twins. The OR to have a serum level of monocyte chemoattractant protein (MCP-1) lower than the 50th percentile of the distribution in statin-treated subjects compared to the statin untreated subjects is 0.669 (95% CI 0.193; 2.327), the OR for interleukin-8 (IL-8) = 0.818 (95% CI 0.236; 2.835), the OR for γ-interferon inducible protein-10 (IP-10) = 1.361 (95% CI 0.358; 5.175), and for interleukin-18 (IL-18) = 0.545 (95% CI 0.155; 1.914).
Conclusions. In relatively healthy, elderly subjects selected from a randomized general population sample, we did not observe differences in the serum levels of the selected set of proinflammatory chemokines in statin treated and untreated subjects with similar LDL-C level, suggesting that cholesterol reduction per se could be a main determinant of statin anti-inflammatory effects (**Adv Clin Exp Med 2014, 23, 5, 723–728**).

Key words: chemokines, interleukin-8, interleukin-18, monocyte chemoattractant protein, statins.

Atherosclerosis is a predominantly age-related and multifactorial disease that appears to be caused by lipid retention and LDL enzyme-mediated modification and oxidation, which provoke chronic inflammation at susceptible sites in the walls of all major conduit arteries [1]. In particular, activated endothelial cells lead to the up-regulation of cell adhesion molecules and chemokines, mediating the recruitment of circulating monocytes, whose accumulation together with monocyte-derived phagocytes in the wall of large arteries stimulates chronic inflammation and the progression of atherosclerosis [2]. Monocyte accumulation and activation appears to play a key role in the loss of collagen in the plaque fibrous cap, a prelude to fibrous cap rupture through the release of collagen degrading enzymes. It is also associated with the death of collagen synthesizing smooth muscle cells, which further contributes to the loss of fibrous cap integrity [3]. From the earliest stages of the disease, the involvement of

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the immune system is impressive, with the presence of T- and B-lymphocyte aggregates in the adventitia and with particularly aggressive T-cell activity, leading to a consequent pleiotropic proinflammatory imbalance with systemic damaging effects [4]. Most of these processes are mediated by proinflammatory chemokines that could be involved in atherogenesis since the earliest phases of disease development. In fact, proinflammatory chemokines have been associated, in specific categories of patients, with some well-known cardiovascular risk factors such as cigarette smoking [5], arterial hypertension [6], obesity and dyslipidemia [7], insulin-resistance and diabetes [8]. In particular, monocyte chemoattractant protein (MCP-1) is strongly related to atherosclerosis development [9] and its plasma levels are significantly linked to coronary artery disease, starting to increase as early as 3 h after the onset of chest pain [10], while MCP-1 and interleukin 8 (IL-8) are mostly associated with the components of the metabolic syndrome [11]. Interleukin 18 (IL-18), either directly or through metalloproteinase expression induction, has the capacity to alter endothelial function and even to induce endothelial cell death or vascular smooth muscle cell migration and/or proliferation [12]. Conversely, a healthy lifestyle, and in particular physical activity, is linked to the reduction of MCP-1 and IL-8 serum levels [13]. Similarly, MCP-1 serum level is reduced in humans assuming inhibitors of the 3-Hydroxy-3-Metil-Glutaryl-Coenzime A reductase (statins) [14, 15]. The aim of our study was to evaluate the serum level of a set of proinflammatory chemokines in elderly statistical twins taking or not taking simvastatin.

Material and Methods

Brisighella Heart Study

The Brisighella Heart Study is a prospective, population-based longitudinal epidemiological cohort study carried out continuously since 1972 in accordance with the principles of the Helsinki Declaration and after the approval of the local Ethics Committee. It started in 1972, involving 2939 randomly selected participants, aged 14 to 84 years, residents in the northern Italian rural village of Brisighella. The requirement to be included was the absence of cardiovascular disease at enrollment. Each participant was first clinically examined at the beginning of the study and then every 4 years, together with the collection of fasting blood samples, the registration of his or her nutritional habits and the assessment of morbidity and all-cause mortality [16].

Subject Selection

In this study we selected from the cohort database 40 overall-healthy, elderly subjects, continuously treated with simvastatin 20 mg for at least 1 year, and 40 statistical twins not treated with statins, characterized by similar age, body mass index (BMI), leisure-time and working activity, smoking habits, history of cardiovascular disease, systolic and diastolic blood pressure, fasting plasma glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, uric acid, and creatinine. The sex distribution was also balanced (M : F = 1 : 1). We selected patients taking simvastatin 20 mg, because it was the most used statin in subjects with the defined inclusion and exclusion criteria.

Heavy smokers (> 10 cigarette/day), diabetics, patients affected by cardiovascular disease, cancer or rheumatologic/autoimmune disorders or taking drugs potentially affecting proinflammatory chemokine serum level (anti-inflammatory drugs, corticosteroids, angiotensin II receptor blockers) were excluded from subject selection. The main characteristics of the considered sample are presented in Table 1. The enrolled subjects were overall representative of the historical cohort of the Brisighella Heart Study.

Laboratory Data

Standard biochemical exams were carried out with standardized methods, as largely reported in previous literature [16, 17].

The serum level (100 μL) of IL-8, MCP-1, IP-10 and IL-18 were measured by the use of a custom 4-plex proteomics platform following the manufacture's protocol (Bio-Rad Laboratories, Inc. Life Science Research Group, Hercules, CA, USA). Multiplex proteomics is the newest technique employed to measure multiple biomarkers in various samples. This technology utilizes microspheres as the solid support for a standard sandwich immunoassay. Each microsphere is labeled with a different fluorophore and the combination of different beads makes it possible to simultaneously measure protein markers [18].

Statistical Analysis

A complete descriptive analysis was carried out for all the available variables. Since the normality distribution tests showed that most of the available continuous variables were not normally distributed, non-parametric tests were used (the Mann-Whitney *U*-test and Kendall correlation) for measures. Moreover, a Pearson chi square, Fisher exact test and odd

Table 1. Clinical characteristics of the elderly subjects selected for the study

Variables		Cases (statin treated subjects; n = 40)	Controls (statin untreated subjects; n = 40)	Mann-Whitney-*U*-test	P
Age (years)	50°P (25°P–75°P)	72 (69.25–77.00)	73 (67.25–75.75)	186	0.718
BMI (kg/m^2)	50°P (25°P–75°P)	26.035 (23.9–29.2)	26.85 (24.5–29.6)	180	0.588
SBP (mm Hg)	50°P (25°P–75°P)	142 (130–158)	149 (136–161)	168	0.386
DBP (mm Hg)	50°P (25°P–75°P)	83 (74–90)	80 (71.5–89)	87	0.586
Fasting Plasma Glucose (mg/dL)	50°P (25°P–75°P)	93 (87.5–103.5)	89.5 (79.7–96.7)	124	0.299
Total cholesterol (mg/dL)	50°P (25°P–75°P)	213.5 (189–230)	206 (182–232.7)	191	0.808
Triglycerides (mg/dL)	50°P (25°P–75°P)	112 (73–148.7)	112.5 (73.2–180.5)	195	0.903
HDL-cholesterol (mg/dL)	50°P (25°P–75°P)	48.5 (42–63.2)	48 (41.5–55.7)	172	0.461
LDL-cholesterol (mg/dL)	50°P (25°P–75°P)	141.1 (121.2–150.7)	137.2 (120.3–158.2)	195	0.892
Uric acid (mg/dL)	50°P (25°P–75°P)	4.65 (2.7–5.6)	4.25 (2.7–5.6)	196	0.914
Creatinine (mg/dL)	50°P (25°P–75°P)	0.93 (0.81–1.08)	1.02 (0.91–1.10)	151	0.185

BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, HDL – high density lipoprotein, LDL – low density lipoprotein.

ratios were calculated for qualitative parameters or for measures split over median value. A first type error $p < 0.05$ was accepted. All statistical analyses were carried out with SPSS.17.0 for Windows.

Results

As reported in Table 1, the simvastatin treated and statin untreated subjects were matched by age, BMI, systolic and diastolic blood pressure, fasting plasma glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, uric acid, and creatinine. Moreover, they were also matched as regards physical activity in leisure-time (chi-square = 3.782, p = 0.286) because all except one in every group were pensioners (Fisher test p = 1), smoking habits (chi-square = 1.222, p = 0.543), and history of cardiovascular disease (Fisher test p = 0.695). In particular, while 3 subjects per group were mild smokers (< 10 cigarette/day), there were 3 ex-smokers in the statin-treated group and 4 in the statin-untreated one.

The proinflammatory chemokine serum level was similar in statin untreated and treated statistical twins (Fig. 1). The OR to have a serum level of MCP-1 lower than the 50th percentile of the distribution in simvastatin-treated subjects compared to the statin-untreated subjects was 0.669 (95% CI 0.193; 2.327), the OR for IL-8 = 0.818 (95% CI 0.236; 2.835), the OR for IP-10 = 1.361 (95% CI 0.358; 5.175), and for IL-18 = 0.545 (95% CI 0.155; 1.914).

Taking into consideration the levels of LDL-C, they were normal in all except one of our subjects. We split the subjects over the median in higher and lower LDL-C level groups. No statistically significant difference was revealed in chemokine levels between the 2 groups, even after correction for statins treatment.

The OR_{MH} for lower LDL-C/higher LDL-C corrected for statin treatment were: for MCP-1 = 0.839 (95% CI 0.247; 2.854), the OR for IL-8 = 0.700 (95% CI 0.203; 2.410), the OR for IP-10 = 0.341 (95% CI 0.088; 1.320), and for IL-18 = 0.429 (95%

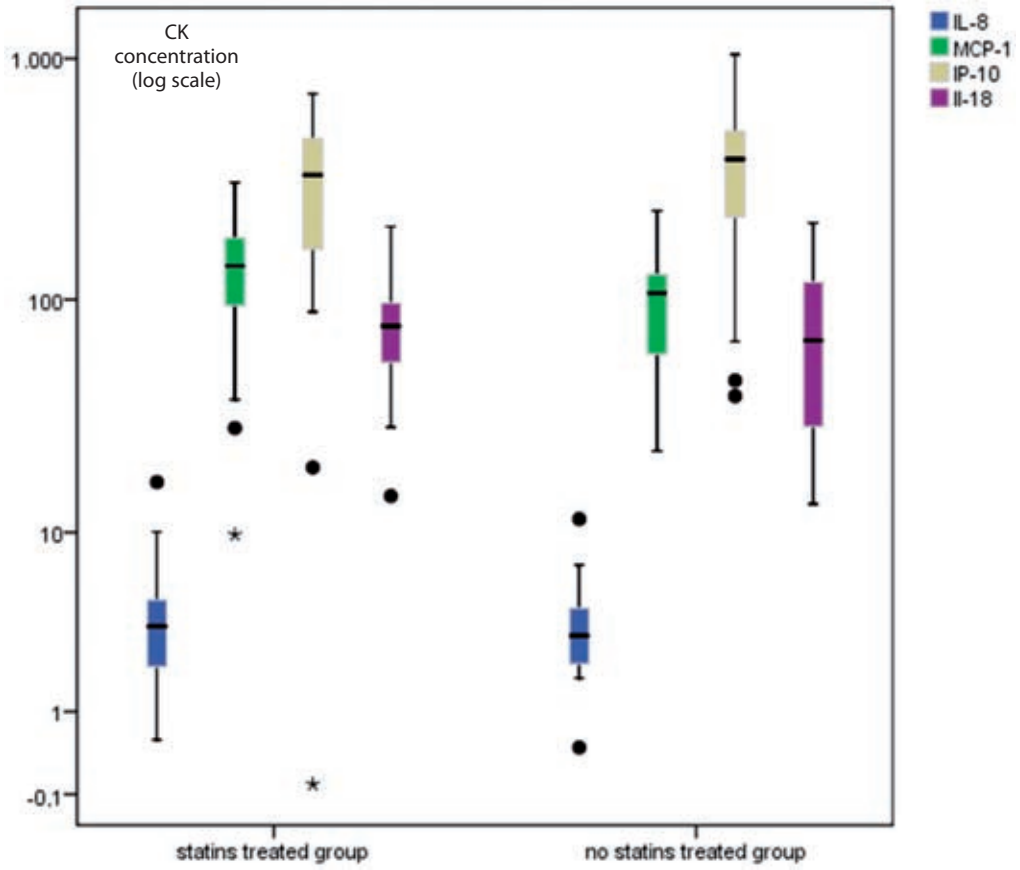

Fig. 1. Boxplot of chemokine concentration in statins treated and untreated, overall healthy, elderly patients (black points represent the extremes, stars represent the outliers). MCP-1 – monocyte chemoattractant protein; IL-8 – interleukin 8; IP-10 – γ-interferon inducible protein-10; IL-18 – interleukin 18

CI 0.117; 1.571). Consequently, no statistically significant correlation was observed between LDL-C and chemokine serum level in simvastatin treated and untreated subjects.

Discussion

Systemic inflammation is a typical characteristic of ageing humans, and commonly it seems to be independent of BMI, blood pressure and lipid levels [19], except for the result of a study carried out on a sample of young people in which IL-8 appeared to be associated with systolic blood pressure [20]. Inflammation biomarkers; however, have only a small impact on increasing the role of traditional risk factors to predict coronary events in elderly patients [21]. A large body of evidences suggests that the preventive action of statins against cardiovascular diseases is related not only to their cholesterol-synthesis inhibition, but also to a range of non-lipid effects, named "pleiotropic effects", and in particular to their anti-inflammatory action [22, 23]. Recently, it has also been proposed that the anti-inflammatory action of statins has a role in improving lung diseases too [24]. On the other hand, some authors support the hypothesis that the anti-inflammatory effect of statins is directly mediated by their reducing action on plasma LDL cholesterol [25]. In this context, we tried to observe if, at the same level of LDL-cholesterol, subjects treated with simvastatin have or do not have the same level of proinflammatory chemokines as the untreated subjects. Selecting very similar subjects for a relatively large number of clinical characteristics, what we called "statistical twins", we observed that no difference exists in proinflammatory chemokine levels between the groups studied. Our results suggest that having a similar LDL-cholesterol level is per se responsible for the lack of difference in the proinflammatory chemokine level. This result puts in perspective the potential relevance of the statin pleiotropic effects, suggesting that statins play a role in reducing the chemokine levels only through their well-known cholesterol-lowering effect.

Some studies show that MCP-1, IL-18 and other chemokines serum level is also significantly related to patient age [19]. We were not able to observe this difference, probably because of the relatively small sample size of our study and because of the narrow age interval of the elderly patients we

selected. Moreover, beyond clinical variables, the serum concentration of MCP-1 (and presumably of other chemokines) is also strongly related to individual genetic pattern [26]. On the other hand, in contrast to some authors who analyzed a sample of post myocardial infarction patients [27], our data agrees with those of Njemini et al., who observed no difference in chemokine levels between genders [28]. A number of studies have reported that statins' anti-inflammatory effects are mediated by inhibition of isoprenylation, due to blockage of isoprenoid synthesis. These intermediary metabolites of cholesterol synthesis play a role in protein attachment to cellular membranes, which determines their biological activity. The best characterized targets of statins' inhibition of isoprenylation are small GTPases which mediate signaling involved in inflammatory cell activation and chemokine secretion: these effects appear to be directly related to the statin antihypercholesterolemic power [29]. However, the observation of our data in elderly subjects taking low doses of moderately powerful statins suggests that cholesterolemia per se could be a main determinant of serum level of proinflammatory chemokines.

Our study, therefore, has several limitations. The first one is the relatively small sample size. However, subjects were matched according to a very large number of anagraphical and clinical data, so that the groups could be considered statistical twins. Moreover, we excluded those subjects whose proinflammatory chemokine level could have been significantly modified by diseases or pharmacological therapy and we focused our attention on elderly patients. On the other hand, this probably limits the extension of our results to only specific subjects, and not to a general elderly population, which is usually affected by more diseases at the same time. In addition, we limited our study to subjects taking simvastatin, which was; however, the most prescribed statin in our cohort of subjects. Furthermore, as per the study design, we had information on the drug assumption by the volunteers, but we had no direct way to check their compliance to the treatment, apart from verifying the continuity of prescription by the general physician and the congruity of the actual LDL-C level vs. the basal one. Another limitation is that we measured only a few chemokines, while many others could be evaluated. However, this is a preliminary report and majority of clinical studies showing a modulating effect of statins on proinflammatory chemokines measured only MPC-1 [14]. Moreover, the strong selection of relatively healthy, elderly subjects has reduced the representativity of the sample when compared to the general population. On the other hand, to the best of our knowledge, this is the first observation of such statin effects in an elderly patient sample.

In conclusion, in elderly statistical twins selected from a randomized general population sample, we did not observe significant differences in the serum levels of a set of proinflammatory chemokines (MCP-1, IP-10, IL-8, IL-18) in simvastatin treated and untreated subjects with similar LDL-C level. Although this early observation has to be repeated on larger patient samples, it contributes to putting in perspective the potential relevance of the statins' pleiotropic effects beyond their well-known ability to reduce LDL-cholesterolemia.

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