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Research Article

Evaluation of s.CD40 Ligand and Monocyte Chemo-attractant Protein-1 Levels In Patients With Acute Myocardial Infarction And Acute Ischemic Stroke

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Abstract

Introduction: Atherosclerosis is a complex disease process in which inflammatory pathways play a critical role in virtually all phases, from development of the fatty streak to plaque rupture and thrombus formation, and its clinical manifestations, i.e., CAD, peripheral artery disease, and stroke. Levels of acute reactant substances and platelet-driven inflammatory cytokines have been reported to be elevated in a variety of acute ischemic vascular events. Newly emerging markers such as sCD40L and Monocyte Chemo-attractant Protein (MCP)-1 may not only aid in discriminating those patients at increased risk for atherosclerosis and cardiovascular events, but may also serve as therapeutic targets in the future. sCD40L is gaining much attention for its role in the initiation and progression of atherosclerosis. CD40 ligand (CD40L, CD154) is a transmembrane glycoprotein, expressed on stimulated CD4-positive T cells, mast cells, basophils and platelets. MCP-1 is a member of the C-C chemokine family that is produced by monocytes/ macrophages, smooth muscle cells, and endothelial cells within atherosclerotic plaques. MCP-1 stimulates tissue factor and superoxide production by monocytes contributing to the transition from stable atherosclerosis to the acute ischemic syndrome phenotype. **Aim of the work**: we aim at investigating whether patients with acute myocardial infarction or cerebral ischemic stroke possessed high levels of sCD40L and MCP-1 as a step towards validating these biomarkers for risk stratification and therapeutic interventions. **Patients and Methods:** This study was carried out in Ain-Shams University hospitals over about 2 years and it enrolled 34 patients with a current diagnosis of acute myocardial infarction (AMI) both NSTEMI and STEMI; 32 with acute ischemic cerebro-vascular stroke (IS) and 24 healthy sex- and age- matched subjects with a comparable atherosclerotic risk profile serving as controls. Detection of serum sCD40L and MCP-1 was performed using quantitative ELISA kits. Normal values range from 0.03-3.98 ng/ml for CD40L and from 74-760 pg/ml for MCP-1 respectively. **Results:** Both AMI and IS patients showed a high level of sCD40L compared to the control group with the difference being of a high statistical significance. The same pattern was shown with MCP-1 measurement where both AMI and IS patients showed a high level of MCP-1 compared to the control group with the difference being of a high statistical significance. sCDL40 and MCP-1 levels did not differ significantly in relation to any of the risk or ischemic profiles (i.e. gender, presence of diabetes or hypertension, etc did not have any significant influence on the level of sCD40L). There was no significant correlation between the levels of MCP-1 and sCD40L neither in the AMI nor the IS group. **Conclusion:** Inflammatory biomarkers have become integral in helping the diagnosis, risk stratification, and treatment of patients with acute coronary syndrome and acute stroke. Among them, MCP-1 and sCD40L are strongly implicated in the patho-physiology of acute atherosclerotic events. Our study found that these inflammation cytokines were significantly increased by at least 2-folds in patients who had AMI or IS compared with controls. We also found that serum levels of both sCD40L and MCP-1 did not differ between IS and MI patients. Finally, the levels of these biomarkers were not influenced by known risk factors for these diseases such as hypertension, diabetes, smoking, or alcohol consumption.

Keywords: Biomarkers, sCD40L, MCP-1, coronary, cerebral, infarction.

1.Introduction

Obesity represents one of the most important public health problems due to its prevalence and associated Atherosclerosis is a complex disease process in which inflammatory pathways play a critical role in virtually all phases, from development of the fatty streak to plaque rupture and thrombus formation, and its clinical manifestations, i.e., coronary artery disease, peripheral artery disease, and stroke.[1] Interestingly, the complicated interface between inflammation and thrombosis is currently becoming apparent.[2] Levels of acute reactant substances and platelet-driven inflammatory cytokines have been reported to be elevated in a variety of acute ischemic vascular events.[3,4]

So far, the underlying mechanisms for the pro-inflammatory and pro-thrombotic milieu found in acute coronary or cerebral ischemia are only partially understood. Established biomarkers such as troponins and BNP have become cornerstones for diagnosis and risk stratification in coronary artery disease (CAD) and acute coronary syndromes (ACS). While CRP appears to have value on a population basis, it does not specifically reflect the inflammatory processes in the developing atheroma. Newly emerging markers such as sCD40L and Monocyte Chemo-attractant Protein (MCP)-1 may not only aid in discriminating those patients at increased risk for atherosclerosis and cardiovascular events, but may also serve as therapeutic targets in the future, given their respective patho-physiologic roles in inflammation and platelet activation and aggregation.

sCD40L is gaining much attention for its role in the initiation and progression of atherosclerosis. CD40 ligand (CD40L, CD154) is a transmembrane glycoprotein, originally thought to be restricted to stimulated CD4-positive T cells mast cells and basophils.[5,6] Subsequent studies demonstrated its expression on platelets, carrying preformed CD40L, which is rapidly translocated onto the cell surface within seconds of platelet activation and then cleaved to generate a soluble, trimeric fragment, sCD40L. Furthermore, it was demonstrated that platelets could release large amounts of sCD40L when stimulated ex vivo by a thrombin receptor-agonist peptide, which in turn induced a dose-dependent increase in the release of Monocyte chemo-attractant protein (MCP-1) from monocytes.[7]

MCP-1 is a member of the C-C chemokine family that is produced by monocytes/ macrophages, smooth muscle cells, and endothelial cells within atherosclerotic plaques.[7] It is recognized by CCR-2 receptors on monocytes and serves as a chemotactic agent to recruit monocytes into the vascular wall.[8] Monocyte chemo-attractant protein-1 stimulates tissue factor and superoxide [9,10] production by monocytes, which may contribute to the transition from stable atherosclerosis to the acute ischemic syndrome phenotype.

2-Aim of the work:

In this study, we aim at investigating whether patients with acute myocardial infarction or cerebral ischemic stroke possessed high levels of sCD40L and MCP-1 as a step towards validating these biomarkers for risk stratification and therapeutic interventions.

3-Patients:

This study was carried out in Ain-Shams University hospitals in the period from August 2011 to April 2013. The study enrolled 34 patients with a current diagnosis of acute myocardial infarction (AMI patients) and involved both non-ST-elevation MI (NSTEMI; based on typical electrocardiographic changes and/or high level of troponin I) and ST elevation myocardial infarction (STEMI); 32 with acute ischemic cerebro-vascular stroke (IS patients; defined according to World Health Organization criteria as rapidly developing clinical signs of focal disturbance of cerebral function with no apparent cause other than that of vascular origin and based on a positive MRI diffusion study) and 24 healthy sex- and age- matched subjects with a comparable atherosclerotic risk profile serving as controls.

Exclusion criteria:

Stroke patients with symptoms lasting for >24 hours were excluded from the study. Further exclusion criteria were infections, malignancies,

autoimmune diseases, surgery within the previous 12 months, and intra-cerebral hemorrhage.

4-Methods:

Blood Sampling Protocol:

Peripheral venous blood (8 ml) was drawn into blood collection tubes containing sodium citrate within 6 hours after admission and before the administration of any medications, including thrombolytic agents. Blinded blood samples were either centrifuged to obtain platelet-rich plasma. Non-citrated blood was immersed in melting ice and allowed to clot for 1 hour before centrifugation. The supernatant was stored at -30°C until analysis. Grossly hemolyzed and lipemic samples were avoided.

sCD40L and MCP-1 detection:

Detection of sCD40L and MCP-1 was performed on the stored serum using quantitative ELISA kits (human sCD40LBMS239TEN / MCP-1, BMS281, Bender MedSystems GmbH; Campus Vienna Biocenter 2; A-1030 Vienna, Austria, Europe). The tests were performed according to the manufactures instructions.

The principle of the test in brief is that CD40L/MCP-1 presents in the sample binds to anti CD40L/MCP-1 monoclonal coating antibody adsorbed onto microwells. A HRP-conjugated monoclonal anti-CD40L/MCP-1 antibody is added and binds to CD40L/MCP-1 captured by the first antibody. Following incubation, unbound enzyme conjugated anti CD40L/MCP-1 is removed during a wash step and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of CD40L/MCP-1 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven CD40L/MCP-1 standard dilutions and CD40L/MCP-1 serum concentrations determined.

Normal values range from 0.03-3.98 ng/ml for CD40L and from 74-760 pg/ml for MCP-1 respectively. The intra-assay variation among the triplicates for all samples was less than 10%. For the purpose of statistical analysis, non-detectable concentrations were imputed as zero.

5-Statistical Analysis

Statistical analysis was performed using Statistica® software to calculate basic statistical parameters and to perform the qui-square statistics, Pearson correlation coefficient, and by t test for independent samples. Fisher's exact test was used to compare differences in qualitative parameters between IS and MI groups. A multiple linear regression analysis was performed to further quantify the relationship between the assessed biomarkers and the variables in study. All values are reported as mean ± SD. Statistical significance was considered to be indicated by a value of $P<0.05$ while a p value <0.001 was considered as highly statistically significant.

Results

The clinical characteristics of the studied patients are listed in ***(table 1)***. Both AMI and IS showed homogenous distribution of gender, age, and risk factors profile with no significant difference in-between these groups or between each of them and the control.

All patients included in the AMI group underwent coronary angiography procedure, while the IS group were documented both clinically and by MRA.

Measurement of circulating biomarkers:

Both AMI and IS patients showed a high level of sCD40L compared to the control group with the difference being of a high statistical significance ($p<0.0001$). On the other hand IS patients showed a higher level of sCD40L than AMI patients but the difference failed to reach a statistically significant level ***(table 2)***. However, a greater degree of inter-individual variability of sCD40L

level was observed in AMI patients than among patients with IS ***(figure 1)***.

The same pattern was shown with MCP-1 measurement where both AMI and IS patients showed a high level of MCP-1 compared to the control group with the difference being of a high statistical significance ($p<0.0001$). While IS patients showed a higher level of MCP-1 than AMI patients but the difference also failed to reach a statistically significant level ***(table 2).*** In contrast to sCD40L, a greater degree of inter-individual variability of MCP-1 level was observed in IS patients than among patients with MI ***(figure 2).***

Results in the AMI group:

sCDL40 level did not differ significantly in relation to any of the risk or ischemic profiles. i.e. gender, presence of diabetes or hypertension, smoking, troponin positivity, enzymatic elevation, AMI recurrence, lesion location, or type of infarction had any significant influence on the level of sCD40L.

The same pattern applies to MCP-1 level except that patients with anterior MI showed higher levels of MCP-1 compared to those with inferior MI, however that fell short of achieving statistical significance ($p=0.07$) ***(table 3).***

Results in the stroke group:

sCDL40 level and MCP-1 showed the same insignificant difference all of the risk and ischemic profiles. i.e. gender, presence of diabetes or hypertension, smoking, size of cerebral vessel affected or location of cerebral ischemic insult ***(table 4).***

Etiology of stroke according to the TOAST criteria was large-artery atherosclerosis in 71.88% (23 of 32 patients) of all patients with stroke, small-artery occlusion 28.13% (9 of 32 patients).

Correlation between sCD40L and MCP-1:

There was no significant correlation between the levels of MCP-1 and sCD40L neither in the AMI ***(figure 3)*** nor the IS ***(figure 4)*** group ($P>0.05$).

Table (1): Baseline clinical characteristics of the studied patients

		AMI Group (n=34)	IS Group (n=32)	P-value	Control Group (n=24)	P-value vs AMI	P-value vs IS
Sex	**Male**	29 (85.29%)	22 (68.75%)	>0.05	18 (75%)	>0.05	>0.05
	Female	5 (14.07%)	10 (31.25%)		6 (25%)		
Age (years)		54 ± 9	57.5± 12.7	0.14	54.1 ± 11.8	0.84	0.31
BMI		28 ± 3	28.4 ± 3.2	0.88	28.6 ± 3.0	0.75	0.86
Hypertension		18 (52.94%)	24 (75%)	>0.05	13	>0.05	>0.05
DM		16 (47.06%)	16 (50%)	>0.05	11	>0.05	>0.05
Smoking		18 (56.25%)	17	>0.05	13	>0.05	>0.05
Dyslipidemia		29 (85.29%)	28	>0.05	22	>0.05	> 0.05
Total Cholesterol		186 ± 54	192.15 ±	0.64	179.9 ± 52	0.67	0.36
LDL		105 ± 44	101.9 ±	0.75	96.8 ± 38.6	0.46	0.62
HDL		43 ± 11	43.8 ± 12.1	0.75	43.4 ± 11.7	0.86	0.90
Triglycerides		189 ± 76	188.3 ± 77.8	0.99	183.8 ± 82.4	0.82	0.83

Table (2): Circulating levels of the studied biomarkers in different groups

	AMI Group (n=34)	IS Group (n=32)	P-value	Control Group (n=24)	P-value vs AMI	P-value vs IS
sCD40L	7 ± 4	9.06 ± 3.47	0.09	1.29 ± 0.32	<0.0001	<0.0001
MCP-1	582 ± 314	652.5 ± 370	0.4	212.9 ± 54.2	<0.0001	<0.0001

Table (3): Per-risk factor analysis of the studied biomarkers in the AMI group

	sCD40L (n=34)	P value	MCP-1 (n=34)	P value
Males **Females**	7.5 ± 4.7 6.7 ± 3.1	0.71	582.95 ± 333.3 580 ± 183.2	0.99
Diabetics **Non-diabetics**	6.72 ± 4.13 7.92 ± 4.83	0.44	592.5 ± 404.3 573.1 ± 215.54	0.86
Hypertensives **Normotensives**	6.89± 4.88 7.88 ± 4.11	0.53	675 ± 365.5 477.8 ± 207.3	0.06
Smokers **Non-smokers**	6.09± 4.75 9.04 ± 3.98	0.06	517.5 ± 210.79 646.79 ± 421.24	0.26
Troponin +ve **Troponin –ve**	7.64 ± 4.91 5.88 ± 2.15	0.40	555.7 ± 263.67 731.7 ± 500	0.22
Elevated Enzymes **Normal Enzymes**	7.68 ± 4.98 5.88 ± 2.15	0.39	559.8 ± 268.02 731.7 ± 500.3	0.20
First **Recurrent**	7.58 ± 4.46 7.19 ± 4.79	0.81	547.94 ± 282.9 600 ± 351.5	0.64
Anterior infarction **Inferior infarction**	8.4 ±6.1 6.8 ± 3.1	0.59	784.4 ± 282.5 515.0 ± 132.9	0.07
NSTEMI **STEMI**	7.03 ± 4.5 7.73 ± 4.57	0.65	513.1 ± 351.15 660.1 ± 253.77	0.17

Table (4): Per-risk factor analysis of the studied biomarkers in the ischemic stroke group

	sCD40L (n=32)	P value	MCP-1 (n=32)	P value
Males **Females**	9.3 ± 3.8 8.6 ± 2.7	0.6	648 ± 313.8 662.0 ±491.2	0.90
Diabetics **Non-diabetics**	8.59 ± 2.96 9.5 ± 3.96	0.45	608.1 ± 224.6 696.9 ± 477.6	0.50
Hypertensives **Normotensives**	8.73 ±2.9 10.1 ± 4.9	0.35	698.8 ± 372.7 513.8 ± 346.7	0.20
Smokers **Non-smokers**	8.59 ± 2.8 9.3 ± 4.4	0.60	757.1 ± 435.02 530.8 ± 240.8	0.10
Small vessels **Large vessels**	9.5 ± 2.5 8.9 ± 3.8	0.66	626.7 ± 271.2 662.6 ± 406.99	0.80
Anterior circulation **Posterior circulation**	9.4 ± 3.7 8.4 ± 3.03	0.47	645.2 ± 413.5 668.5 ± 268.1	0.87

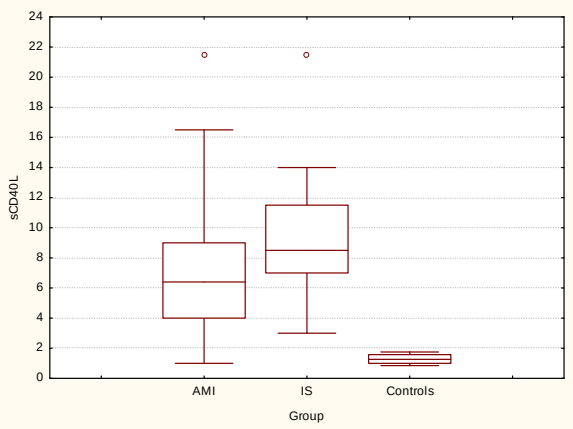

Figure 1: *sCD40L levels (ng/mL) in serum of patients with AMI (n=34), ischemic stroke (IS, n = 32), and control subjects (n = 20). The data are expressed as whisker box plots; the box represents the 25–75th percentiles, the median is indicated by a bar across the box, the whiskers on each box represent the 10–90th percentiles.*

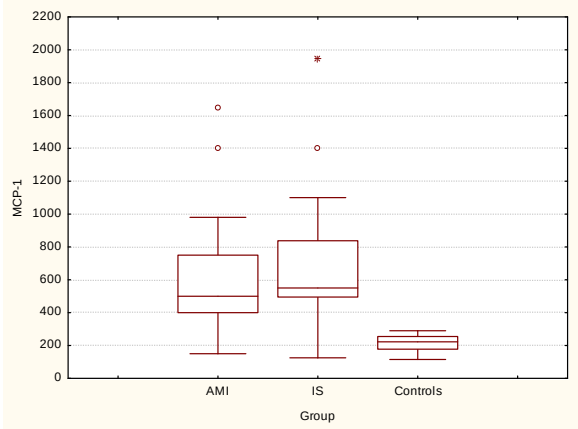

Figure 2: *MCP-1 levels (pg/mL) in serum of patients with AMI (n=34), ischemic stroke (IS, n = 32), and control subjects (n = 20). The data are expressed as whisker box plots; the box represents the 25–75th percentiles, the median is indicated by a bar across the box, the whiskers on each box represent the 10–90th percentiles.*

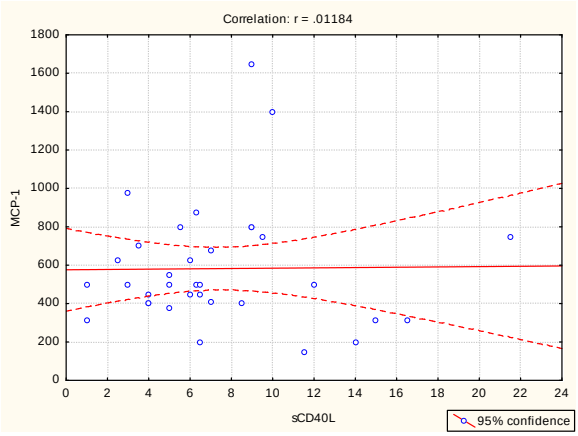

Figure 3: *Correlation between sCD40L and MCP-1 in AMI patients.*

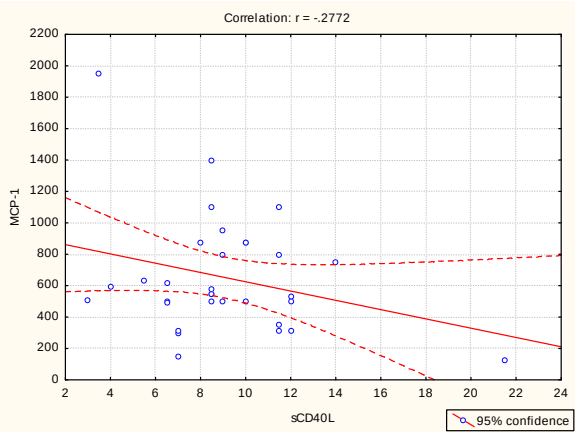

Figure 4: *Correlation between sCD40L and MCP-1 in IS patients.*

Discussion

Activation and infiltration of platelets and monocytes/macrophages are crucial for the onset of unstable ischemic syndromes.[11] Activated platelets aggregates with monocytes, which subsequently interact with injured vascular endothelial and smooth muscle cells, inducing the up-regulation of matrix degrading enzymes, a key factor in atheromatous plaque rupture through breakdown of the fibrous cap.[12]

Inflammatory biomarkers have become integral in helping the diagnosis, risk stratification, and treatment of patients with acute coronary syndrome and acute stroke. Among them, MCP-1 and sCD40L are strongly implicated in the patho-physiology of acute atherosclerotic events.

In the present study, we evaluated these two important members of the panel of circulating inflammatory markers in AMI and ischemic stroke patients. These two atherosclerosis associated complications share many similarities, but at the same time they have at least quantitative if not qualitative differences in their risk factors.[13] This trend is preserved in our patients' characteristics: our IS patients were older and the different incidence in males and females is less pronounced in IS compared to MI. Also, IS patients were more prone to hypertension than MI patients although this trend did not reach statistical significance.

Our study found that these inflammation cytokines were significantly increased by at least 2-folds in patients who had AMI or IS compared with controls. We also found that serum levels of both sCD40L and MCP-1 did not differ between IS and MI patients. Finally, the levels of these biomarkers were not influenced by known risk factors for these diseases such as hypertension, diabetes, smoking, alcohol consumption, etc.

This comes in concordance with ***MSV Elkind etal*** who showed that sCD40L is a powerful biochemical marker of inflammatory thrombotic activity in patients with ACS. Platelet activation correlated closely with sCD40L, and that inhibition of glycoprotein 2b/3a receptors by abciximab abolished the increased risk in patients with ACS and elevated levels of sCD40L. But the effect of abciximab on lowering the risk of a recurrent event was seen only in those with elevated levels of CD40L, indicating that CD40L could be used as a marker of response to therapy. [14]

But in addition to anti-platelets, ***Charalambos etal*** found that statin treatment and thiazolidinediones (peroxisome proliferator-activated receptor-alpha agonists) reduce plasma sCD40L levels in patients with CAD and stroke patients. In addition he found that circulating sCD40L levels in stable or unstable coronary syndromes are also affected by genetic determinants. Genetic polymorphism A3459G on sCD40L has been reported as a determinant of sCD40L levels, with carriers of 3459G allele having elevated sCD40L levels during ACS.[15]

Charalambos etal stated also a gradual increase in sCD40L levels with ACS progression. Trans-coronary sCD40L levels exhibit an early peak just 9 hrs after onset of AMI or unstable angina. However, there is no significant difference between AMI and unstable angina patients in circulating sCD40L levels. Interestingly, intracoronary CD40L levels are higher in the culprit coronary artery than in the peripheral circulation, reflecting the activation of a potent local inflammatory process [15]. ***MSV Elkind etal*** also showed the upregulation and the gradual increase of the CD40 on monocytes, and CD40L expression on platelets and T cells in patients with acute cerebral ischemia in vivo compared with sex and age matched controls and this up regulation continues for at least 3 months after the infarction. So sCD40L may be an appropriate and useful marker of future risk of recurrent cardiovascular events in stroke patients [14].

sCD40 was significantly high in patients diagnosed with ACS (4.54 ±1.73 ng/ml) when compared to controls (1.57±0.83 ng/ml). The optimum cutoff value above which sCD40L was considered positive is 2.99 ng/ml. The sCD40L levels were higher non-significantly in patients diagnosed as unstable angina when compared to Non-ST Segment Elevation Myocardial Infarction. So as a matter of fact, sCD40L can identify the subgroup of patients at higher risk of cardiac events even among those with negative troponin as well as identification of patients with acute coronary syndrome at heightened risk of death and recurrent MI independent of other predictive variables, including cTnI and CRP.[16]

Recent studies suggest that sCD40L contributes to the progression of atherosclerotic and consequently to the destabilization of atherosclerotic plaques by inducing the expression of cytokines, chemokines, growth factors, matrix metallo-proteinases and pro-coagulant factors in a variety of atheroma-associated cell types. So sCD40 is implicated in restenosis in CAD patients.[17]

sCD 40 is also involved in post-stroke epilepsy , being significantly higher in such patients compared to healthy control . Patients with acute ischemic stroke in comparison to control subjects without acute ischemic stroke had significantly higher CD40 L levels and Fetuin-A serum levels.[18, 19]

In myocardial infarction, elevation of circulating MCP-1 has been already reported [20,21] and, therefore, our findings of increased MCP-1 in our patients with MI are of a confirmatory nature. Moreover, ***Wang etal*** [4] found that MCP-1 was significantly increased in patients who had stable CAD compared with controls and this may indicate low-grade of vascular inflammation, with cellular and endothelial activation. Moreover the elevated MCP-1 can regulate migration and infiltration of monocytes and macrophages to the sites of inflammation and so it may reflect the presence of a clinically silent unstable plaque. However, in the same study,[4] the biomarkers such as sCD40L, IL-8, and P-selectin which represent the platelet activation status (rather than inflammation) showed no significant difference between stable angina patients and the control group suggesting that platelet activation may be an important phenomenon in the pathogenesis and clinical outcome of AMI. ***Losy and Zaremba*** [22] reported that blood MCP-1 levels were not increased in his group of 23 patients with IS. Also ***Boyle etal*** found that MCP-1 release from the human heart is suppressed following MI [23]. By contrast and in line with our results, ***Reynolds etal*** [24] demonstrated elevation of the MCP-1 in the blood from a big group of more than 200 stroke patients. This discrepancy may occur due to low number of subjects included in the study by ***Losy and Zaremba*** [22] and possibly also by marked variability of circulating MCP-1 levels.

MCP-1 is correlated strongly with carotid intimal medial thickness, a subclinical marker of atherosclerosis ($r=0.59$, $p<0.0001$). MCP-1 levels are also increased in patients with unstable angina and acute MI but not in those with stable angina, indicating that it may be a marker of instability or plaque activity.[14]

De Lemos *etal* [25] confirmed this finding within the coronary domain and prospectively confirmed that an MCP-1 threshold of 238 pg/ml, derived from a previous large study of patients with ACS, identifies patients at increased risk for adverse events in both the acute and the chronic setting

after AMI. Patients with levels persistently above this threshold seemed to be at particularly high risk for death compared with those with persistently lower levels, or levels that were only transiently above this threshold. This is concordant with our results where all our control subjects had an MCP-1 level that fall well below this threshold while AMI patients had a mean value of 582 ± 314 pg/ml.

There are at least two mechanisms by which the observed upregulation of MCP-1 protein levels in circulation of patients with IS and MI can be achieved. Firstly, it may be the direct consequence of atherosclerosis development. The main sources of MCP-1 are endothelial and macrophage like cells,[26] which are known to play a significant role in atherosclerosis development and plaque formation. In vitro studies have revealed that endothelial cells are able to produce MCP-1 in response to LDL (an important atherosclerosis triggering factor) [27,28].A study by **Hughes *etal*** [29] demonstrated that MCP-1 deficiency is protective in a murine stroke model. The same processes are taking place in heart during MI.[30,31]

Unexpectedly, in our study none of the four investigated common risk factors for AMI and IS influenced serum sCD40L or MCP-1 levels. In number of previous studies in patients with myocardial infarction, the association of MCP-1 levels with several risk factors such as hypertension and diabetes has been reported. [32] However, these were large-population-based studies where even slight differences may be identified. It is, therefore, possible that in our relatively small patient population of 32 AMI patients, differences of similar magnitude may not be visible.

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