THE IMPACT OF STATINS ON CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH CORONARY ARTERY DISEASES

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ABSTRACT

Background: Multiple measures of endothelial progenitor cells (EPCs) have been determined. We sought to assess the percentage and function of EPCs in acute and chronic angina patients with and without statin medications and correlation between EPC assay methodologies.
Methods: We assessed EPCs percentage and functions in 81 patients (40 with acute angina and 41 with chronic stable angina) and 24 control subjects. EPCs were identified on the basis of KDR cell surface marker expression (VEGFR-2). EPCs function was assessed by quantitative vWF and VEGFR-2 genes expression and eNOS protein level measurement. Flow mediated dilatation (FMD) was applied to assess the effect of statin on endothelial function.
Results: EPCs percentage based on flow cytometry (FACS) was significant and precise in assessment role of statin medications in angina patients compared to control. As regard EPCs function higher significant increase in quantitative levels of VEGFR-2 and vWF genes expression and eNOS protein level were detected in patients on statins treatment versus patients not on statins treatment (P< 0.001).There was positive correlation (r=0.723) between FMD percentage and plasma eNOS biomarker in patients on statins medication. There was also positive correlation (r=0.475) between KDR flow cytometry percentages.
Conclusions: Statins medication increases the circulating EPCs in coronary artery disease patients. EPCs number was the strongest predictor of FMD. FMD is an indicative test for eNOS bioavailability production. These observations need combined assay (statins & exercise) to precise definition of EPCs in cell therapy research in coronary artery disease patients.

1. INTRODUCTION

Heart failure due to coronary artery disease (CAD) is a major health problem, being the most prevalent cause of premature death in humans and accounting for a significant proportion of all hospital admissions throughout the world, including most low-income and mid-income countries(1)**.** Novel approaches to increase the function and the number of circulating progenitor cells by pharmacological modulation (cytokines, statins) or gene therapy (VEGF) will be highlighted. Advanced stages of heart failure were shown to be associated with reduced levels of EPCs (2)**.**

Recent studies indicate that endothelial dysfunction and injury in the vascular wall are repaired by bone-marrow-(BM-) derived endothelial progenitor cells (EPCs). Evidence suggests that CD133$^+$CD34$^+$KDR (vascular endothelial growth factor receptor 2, VEGFR2) are EPCs antigens. EPCs are mobilized from the BM into the peripheral blood in response to tissue ischemia or injury; these cells migrate to sites of damaged endothelium and differentiate into endothelial cells (ECs), thereby improving blood flow and tissue repair. EPCs contribute to reendothelialisation and neovascularisation (3)**.**

Of the many agents that have been examined to increase EPCs and enhance their function, HMG-COA reductase or statins are one of the most intriguing. Accumulated evidence has demonstrated that statins promote EPC mobilization, proliferation, migration, adhesion, differentiation and reduce senescence and apoptosis independent of their serum lipid-lowering effect (4).

Several studies showed that statins may affect the cardiovascular system beyond their effect on the lipid profile, and it was suggested that they affect the immunological system and vascular inflammation. Many of the beneficial pleiotropic effects of statins occur as a result of modulated endothelial function and reduced inflammatory processes (5).

Numerous clinical trials have demonstrated early reductions in cardiovascular events occurring independently of statins' lipid-lowering effects. These pleiotropic effects have been attributed to anti-inflammatory properties, to atherosclerotic plaque stabilization, and more recently to mobilization of EPCs (6)**.**

Based on this background, we hypothesize that statins improve endothelial function and EPC differentiation in patients with coronary artery disease.

The aim of the present work is tostudy the impact of statin therapy on number and function of EPCs in patients with coronary artery disease. Also, the assessment of the effect of statin on endothelial function estimated by brachial artery flow mediated dilatation*.*

2. ETHICS

The study was approved according to the ethical standards of the kasralainy hospital, faculty of medicine Cairo University responsible committee on human clinical studies. Blood samples were collected after approval written consent from all enrolled participants.

3. SUBJECTS AND METHODS

This study included patients' selection, clinical evaluation and laboratoryendothelial progenitor cells isolation, characterisation and molecular functional assessment investigations.

Subjects grouping

Eighty one patients and twenty four healthy subjects were enrolled in our present study. Allsubjects were divided into 3 groups according to the following chart:
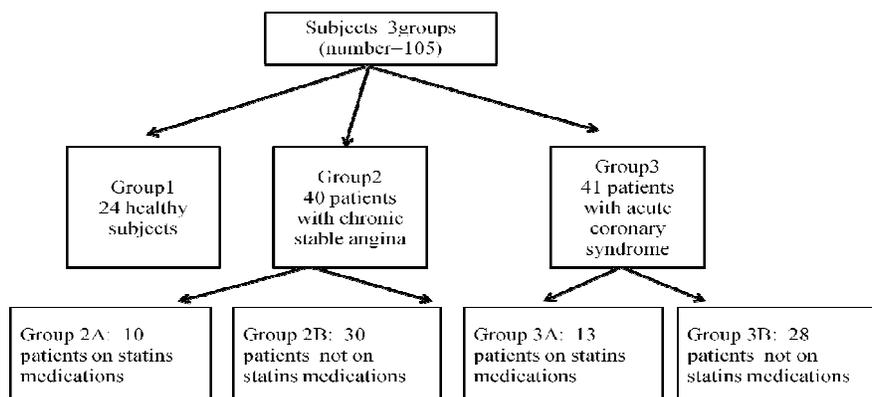
The inclusion criteria:
  1) Patients with stable coronary artery disease on regular statin therapy or not.
  2) Patients with acute coronary syndrome (unstable angina, NSTEMI, STEMI) on statin therapy or not.

The Exclusion criteria:
1) Subjects who had chronic renal failure (serum creatinine>3 mg/dl).
2) Subjects who had concomitant inflammatory or malignant disease.

1.Baseline patients' evaluation:

All patients were subjected to detailed full history and clinical examination with special emphasis on the assessment of (NYHA) functional class, age, gender, presence of risk factors including diabetes mellitus, hypertension, dyslipidemia in case of ischemic heart failure, duration of illness, and the presence of any other co-morbidity that exclude the patient from the study.Comparison between patients on regular treatment with statin and those who were not on statin therapy regarding number and function of EPCs and endothelial function was done.

2.EPCs identification and counting by Flow Cytometry analysis(FACS):

Peripheral blood was collected from all enrolled subjects. The peripheral blood mononuclear cell fraction (PB-MNCs) was isolated from the buffy coats through density-gradient centrifugation with Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY). 20 ml anticoagulated blood sample was carefully layered on 20 ml Ficoll (volume ratio 1:1), and then they were centrifuged for 35 min. at 400xg rpm. The upper layer was aspirated leaving the PB-MNC layer undisturbed at the interphase. The interphase layer PB-MNC layer was carefully aspirated and washed twice in PBS containing 2 mM EDTA and centrifuged for 10 minutes at 200xg rpm at 2 ºC. The cell pellet was resuspended in a final volume of 300 µl of buffer.Then $1x10^5$ cells were incubated with 10µl of KDR PE monoclonal antibody (Beckman coulter, USA) at 4 ◦C in the dark, same specie isotype served as a negative control. After 20 min incubation, 2 ml of PBS containing 2% FCS solution were added to each tube of monoclonal treated cells. The mixtures were then centrifuged for 5 min at 2500 rpm followed by discarding the supernatant and resuspending cells in 500µl PBS containing 2% FCS. Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman coulter, FL, USA) and analyzed using CXP Software version 2.2.

3. EPCs functional assessment:
  I. **QRT- PCR genes expression of VEGFR-2& von Willbrand factor:**
a. RNAextraction:

Total RNA was extracted from identified human EPCs using Qiagene cells/tissue extraction kit (Qiagene, USA) according to instructions of manufacture.

b. cDNA synthesis:

The cDNA 25µl master mix was prepared; first strand buffer (10x) 5µl, 10 mM dNTP's, RNase inhibitor (40 U/µl), MMLV-RT enzyme (50U/µl), and DEPC-treated water. The last mixture was incubated in the programmed thermal cycler (Biometra, Germany) one hour at 37°C followed by inactivation of enzymes at 95°C for 10 minutes, and finally cooled at 4°C. Then cDNA was stored at –20 °C.

c. Real-time qPCR using SYBR Green I:

Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets (Table 1) were optimized at the annealing temperature. All cDNA including previously prepared samples (for VEGFR-2 and vWF genes expression), internal control (for GAPDH gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate.Each 25 µL of reaction mixture contained 12.5 µL of SYBR Green (Fermentas), 1 µL of each primers (10 µmol/L), and cDNA (1 µg/mL) for sample determination. The reaction was initiated by activation of *Taq* polymerase at 95 °C for 5 min, followed by 40 two-step amplification cycles: 10 s denaturizing at 95 °C, 50 s annealing at 55 °C (VEGFR2) or 53 °C (vWF). After the RT-PCR run the quantitative data were expressed in Cycle threshold (Ct) of assessed genes (VEGFR-2 and vWF) and the house keeping gene (GAPDH). Therefore, Relative quantitation (RQ) of target genes expression was assessed and related to housekeeping gene by previously published method RQ = $2^{-(\Delta \Delta Ct)}$ (7, 8).

eNOS assessment by ELISA:
eNOS was quantitatively (pg/ml) measured by ELISA whichwas supplied by (R&D system Minneapolis. USA).
  II. Flow Mediated Dilatation (FMD):
FMD was assessed in the subject's right arm in the recumbent position after a 15-min equilibration period in a temperature-controlled room (22°C to 25°C). Each subject was fasted the previous night for at least 12 h. The artery was longitudinally imaged approximately 5 cm proximal to the antecubital crease, and brachial artery diameter (BAD) were measured at end-diastole. After the baseline resting scan, a pneumatic cuff was placed at the level of the mid-forearm (proximal to the target artery) and was be inflated until no blood flow was detected through the brachial artery with the Doppler probe, and this pressure were held for 5 min. Increased flow were then induced with sudden cuff deflation and a continuous scan was be performed for 1 min. For the reactive hyperemia scan, BAD measurements were taken 45 to 60 s after cuff deflation. Flow-mediated dilation were calculated from the diameters as (reactive hyperemia – baseline)/baseline percent.

4. STATISTICAL ANALYSIS

The data were coded, entered and processed on a PC compatible computer using Graph pad prism (version 6).Results are presented as mean ± standard deviation (SD) for continuous, normally distributed variables and as percentages for categorical data.Student's t-test was used to assess the statistical significance of the difference between two population means in a study involving independent samples.ANOVA (Analysis of Variance) evaluates the equality of several group means; was used to test the difference about mean values of some parameters among multiple groups.Bartlett's test was used to determine which groups were significantly different.Correlation analysis: assessing the strength of association between two variables. Pearson correlation symbolically r, defines the strength and direction of the linear relationship. p value <0.05 was considered statistically significant.

5. RESULTS

Demographic characterization of patients showed no statistically significant differences existed between the groups in terms of age and sex. No significant differences existed statistically between the groups in terms ofrisk factors like smoking, hypertension and diabetes mellitus. AS regards plasma cholesterol, LDL, HDL and triglycerides levelswere significantly higher in all patients groups (2, 3) than the control (P < 0.001) (table 2).
EPCs were selectively identified by specific KDR surface antigen (vascular endothelial growth factor receptor-2). KDR antigen was highly significant expressed on EPCs surfacein patients on statins therapy (group 2B, 3B) compared to patients not on statins therapy (group 2A, 3A) (P< 0.001) (figure 1). EPCs counting were assessed by FACS analysis. Flow Cytometry percentage shows high significant difference between patients on statins therapy(2B, 3B) and patients not on statins therapy (2A, 3A) (P<0.001).The highest significant difference was found at patients on statins treatment (2B, 3B) compared to control and patients not on statins treatment (2A, 3A) (figure 2).
As regard EPCs functionhighersignificantquantitative levels of VEGFR-2 andvWFgenes expression were found in patients on statins treatment (group 2B, 3B) versus patients not on statins treatment (group 2A, 3A) (P< 0.001)The highest significant difference was found at patients on statins treatment (group2B, 3B) compared to other 3 group.(figures 3,4 respectively).
Plasma eNOS protein concentration as easy non invasive biomarker was quantitatively measured in all studied subjects.Significant higher levels were found in eNOS protein level in patients on statins medication (group 2B, 3B) versus patients not on statins medication (group 2A,3A) (P< 0.001) (figure5).
FMD is a diagnostic technique to assess endothelial dysfunction and has prognostic value in PAD.Significant higher levels were found in FMD assessment in patients on statins treatment (group 2B, 3B) versus patients not on statins treatment (group 2A,3A) (P< 0.001) (figure 6).
There was high positive(r=0.723) correlation between FMD percentage and plasma eNOS biomarker in patients on statins medication (group 2B, 3B) and so FMD is an indicative test for eNOS bioavailability production.There was also high positive (r=0.475) correlation between KDR flow cytometry percentages and FMD and this indicates that EPCs number was the strongest predictor of FMD.
There were non significant(p>0.05) negative correlations between risk factors like smoking, hypertension and diabetes mellitus and flow cytometry percentage, quantitative levels of VEGFR-2 and vWF expressed genes and eNOS protein concentrationin all patients enrolled in study either on statins medication or not. But there were significant (p<0.05)negative correlations between dyslipedemia risk factor (e.g:LDL)and flow cytometry percentage, quantitative levels of VEGFR-2 and vWF expressed genes and eNOS protein concentration(figure 7).
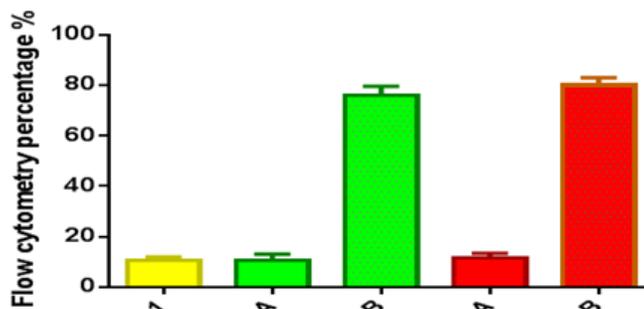

Figure 1

Fig.1. Flow cytometry analysis represents percentage quantification in all different studied groups
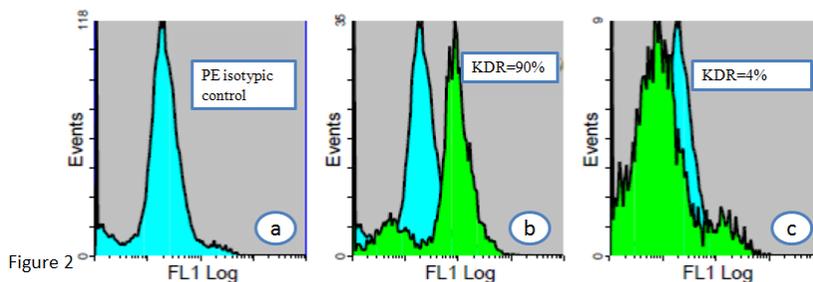
Figure 2

Fig. 2. *Flow Cytometry analysis represents EPCs count (KDR%) in both types of patients*
(either chronic stable angina or acute coronary syndrome) who on statin and not on statin therapy.
a- blue color peak represents PE isotopic control. b- green color peak shifted to right represents KDR =90% in patients
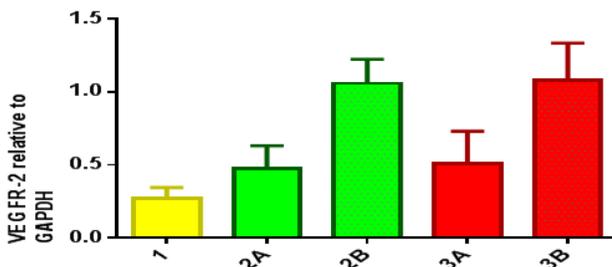*on statin therapy. c- green color peak shifted to right represents KDR =4% in patients not on statin therapy*

Figure 3

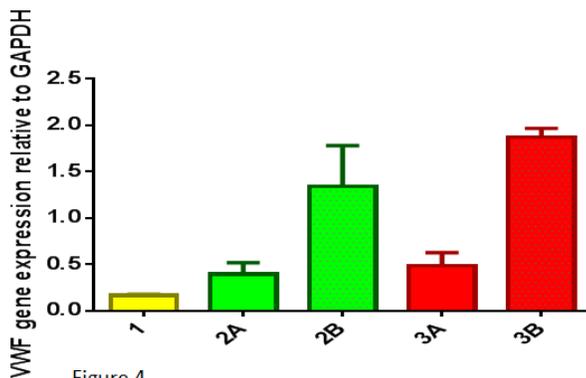***Fig. 3.*** *Represents relative quantitation expression of VEGFR2 gene in all different studied groups*

Figure 4

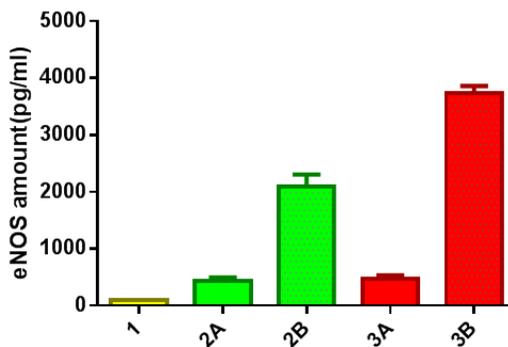***Fig. 4.*** *Represents relative quantitation expression of vWF gene in all different studied groups*

Figure 5

Fig. 5. *Represents quantitation of eNOS (pg/ml) in all different studied groups*
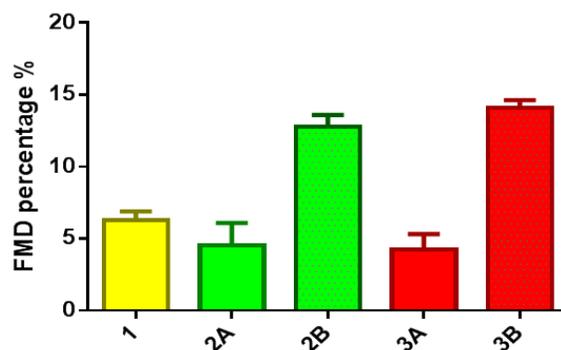
Figure 6

Fig. 6. Represents FMD precentage in all different studied groups
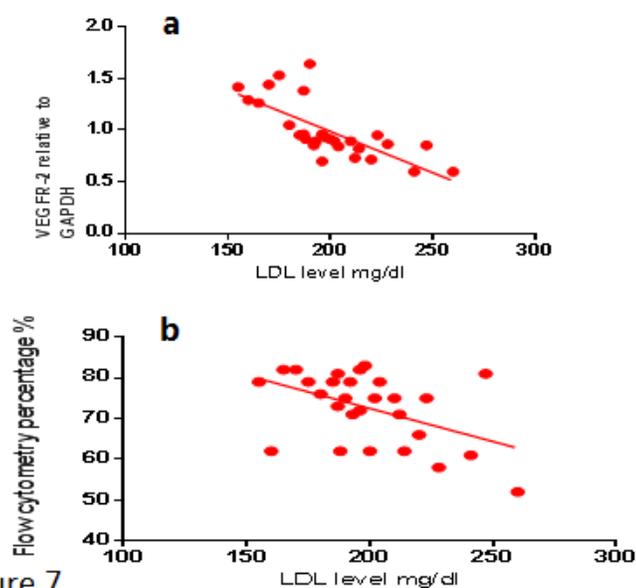

Figure 7

Fig. 7. Represents significant negative correlations between a- quantitative levels of VEGFR-2 and vWF expressed genes and eNOS protein concentration and b- LDL and flow cytometry percentag

6. DISCUSSION

Several lines of evidence indicate that EPCs constitute an important endogenous system that maintains endothelial integrity and vascular homeostasis. Their beneficial effects may be mediated through paracrine secretion of angiogenic factors and cytokines. Patients with cardiovascular diseases, such as CAD, hypertension, heart failure, and diabetes, exhibit reduced EPC number and function. Therefore, reduced EPC levels may reflect a mechanistic link that confers increased risk of adverse cardiovascular outcome. Reversal of EPC dysfunction could therefore potentially prevent the progression of cardiovascular and vascular disease (3).

Statins have been demonstrated to significantly affect the prognosis and outcome of patients with risk factors to atherosclerosis (in primary and secondary prevention trials). Several clinical and recently basic studies have suggested an extra-beneficial effect of the statins in the prevention of atherosclerosis and coronary artery disease (5).Studies in humans suggest that statin therapy mobilizes EPCs into the circulation.Rosuvastatin potently mobilizes EPCs, promotes EPCs de novo differentiation, and significantly enhances neovascularization and blood flow recovery after 'ischemic limb injury.(6)(7).

With interpretation of our results it should be noted that counting of circulating EPCs by flow cytometry wasassessed by KDR[+] surface antigen quantification but there is no standardized approach.During our practical procedure we avoid and exclude EPCs isolation and propagation in vitro (fibronectin cultured EPCs) to exclude any additional external factor that may increase EPCs counting. This allows real statistical comparison between patients on statin and those not on statin medication as regard EPCs counting. This result was in agreement with (8) who found that circulating EPCs highly expressed CD34^{+}/KDR^{+}in blood of patients with systolic heart failure that received rosuvastatin and remained unchanged in placebo group. In addition there is some ambiguity regarding precisely which antigens expressed on stem cells identify those capable of differentiation into endothelial cells an area of ongoing research

Statins induce EPC differentiation via the PI 3-kinase/Akt (PI3K/Akt) pathway as demonstrated by the inhibitory effect of pharmacological PI3K blockers or overexpression of a dominant negative Akt construct. Similarly, the potent angiogenic

growth factor VEGF requires Akt to augment EPC numbers, suggesting an essential role for Akt in regulating hematopoietic progenitor cell differentiation. Given that statins are at least as potent as VEGF in increasing EPCs differentiation, augmentation of circulating EPC might importantly contribute to the well-established beneficial effects of statins in patients with coronary artery disease(9)**.**

In a recent study, use of 40 mg of atorvastatin might increase the number of circulating EPCs in patients with ICM in comparison with 10 mg of atorvastatin, and the effect might be independent of the decrease of lipids, oxLDL(10). Interestingly, considerable benefits have been demonstrated in statins' clinical trials in patients with ischemic heart and peripheral disease, irrespective of the cholesterol concentration (11). In fact, statins have been shown to stimulate angiogenesis by upregulation of the expression and activity of endothelial nitric oxide synthase (eNOS)(12)**.**

The results of our present study revealed that there was a high significant increasing level ofeNOS protein concentration in patients with statin medications compared to patients without statin medications in both groups of ACS and chronic stable angina with higher levels in ACS than chronic stable angina. Such finding is considered a new and recent event; the effect of statins on eNOS in both ACS and chronic stable angina CAD. (13). found that statins increase the stability of eNOS mRNA and induce activation of eNOS through PI3-K/AKt/eNOS pathway restoring endothelial function.

The results of this study revealed that there was an effect of statins on EPCs in the form of increasing levels of angiogenic growth factors like VEGFR2 (KDR), vWF genes expression. This is an indicative of improved endothelial dysfunctions on statins therapy and may be possibility of neovascularisationcompared to control and to patients without statin medications.

These findings agreed with several studies as(14)demonstrated that myocardial ischemia induces EPC mobilization and VEGF release. Both Pravastatin and G-CSF can enhance EPC mobilization from the bone marrow and VEGF release, but G-CSF produces a stronger effect on EPC mobilization in association of VEGF release.(15)stated that a low dose of atorvastatin, but not a highdose, significantly increased regional blood flow. Atorvastatin significantly increased the expressions of VEGF, IL-8, Ang-1, Ang-2, eNOS, and HO-1 proteins in ischemic hindlimbs. Atorvastatin significantly increased the number and colony formation of EPCs and decreased oxidation in mononuclear cells fromspontaneously hypertensive rats. (16) found that CD31 immunostaining revealed an increased capillary density in ischemic gastrocnemius muscle tissue of diabetic rats treated with either simvastatin wor its combination with vitamin C. This effect was accompanied by up-regulated plasma levels of HO-1, NO, VEGF and its intra-muscular receptor type-2 (FIK-1). (17) assessed higher significant effect of atrovastatin on venous thrombotic embolism patients following discontinuation of anticoagulant therapy on IL-1b, IL-6, IL-8,IL-10 andvWF compared to control

FMD provides a technique to assess the integrity of the shear stress–mediated pathway of NO production. FMD of the brachial artery is closely correlated with coronary vasomotor responses. A reduction in FMD is thought to represent an early functional disturbance in the development of CAD(18).

The results of our present study revealed that there was high significant effect of statins on EPCs in the form ofhigh FMD percentage in patients on statin medication compared to those without statin medications.

(19) demonstrated that FMD could not distinguish between the patients who were treated with statin and those not treated with statins with the same demographic, clinical, and angiographic characteristics. The only exception was in the group of patients with a minor coronary disease. Statin treatment had a more pronounced effect in the earlier stages of coronary atherosclerosis.

(20) demonstrated that high dose Atorvastatin therapy pre-PCI improves EPCs number and CAC number and function in humans which may in part explain the benefit in clinical outcomes seen in patients undergoing coronary interventions.

(21)demonstrated thatamong patients who have recently had an acute coronary syndrome, an intensive lipid-lowering statin regimen provides greater protection against death or major cardiovascular events than does a standard regimen. These findings indicate that such patients benefit from early and continued lowering of LDL cholesterol to levels substantially below current target levels. This finding coincides with our results concerning high significant negative correlation between statin therapy in ACS and chronic stable angina and LDL levels compared to control. This is an important conclusive event of our study which demonstrates the high impressive effect of statin on lipid profile in CAD patients.

In conclusion, the present study demonstrates at number of important findings. CAD treated with statin had lower endothelial dysfunction and higher circulating numbers of EPCs, compared with those not treated with statin. These findings may together contribute to the increased demand and importance of regular statin medications in CAD. Future interventional studies will be important in defining a possible mechanistic link between the findings and may improve our understanding of the pathophysiological processes leading to lower rates of CAD in populations have long regular statin medictions. We recommend ongoing research to assess combined effects of statin medication and exercise (recommended therapeutic regimen agents for CAD) on the number and function of circulating EPCs.

Table 1. Primer sequences for all studied genes (from 5' to 3' direction)

Gene	Forward	Reverse	Accession
VWF	TAAGTCTGAAGTAGAGGTGG	AGAGCAGCAGGAGCACTGGT	HF571250.1
VEGFR-2	GATGTGGTTCTGAGTCCGTCT	CAT GGCTCTGCTCTCCTTTG	FJ899739.1
GAPDH	CCTCTACTGGCGCTGCCAAGGCT	GTCCACCACTGACACGTTGG	NT_009759.16

Table 2. Demographic characterization of all studied subject groups

Group / Parameter	Control group (group1) (n= 24)	Patients with chronic stable angina(group2)		Patients with ACS (group3)		P value
		Subgroup2A: not on statins medication (n=10)	Subgroup2B:on statins medication (n=30)	Subgroup3A: not on statins medication (n=13)	Subgroup3A:on statins medication (n=28)	
Age	40.63 ±7.16	44.8±6.23	45.77±10.69	49.08±7.17	46.61±5.08	NS

Sex Males Females	16 8	8(80%) 2(20%)	21(70%) 9(30%)	10(76.9%) 3(23.1%)	24(85.7%) 4(14.3%)	NS
Smoking, no. (%)	Zero	7 (70%)	6 (20%)	4 (30.7 %)	15 (53.57 %)	NS
Hypertension , no. (%)	Zero	6 (60%)	15 (50%)	10 (76.9%)	17 (60.7 %)	NS
Diabetes, no. (%)	Zero	5(50 %)	12(40 %)	10 (76.9%)	12(42.8 %)	NS
Plasma cholesterol mg/dl	137.9±26.9	272.6±80.1	180.1±29.49	268.8±78.6	175±28.8	<0.001
Plasma TAG mg/dl	87.9±18	154.8±29.1	130.7±23.8	141.7±30	121.8±36.7	<0.001
HDL mg/dl	50.6±7.4	28.2±2.9	28.2± 2.3	28.1±2.1	29.4±2.8	<0.001
LDL mg/dl	110.5±26.9	230.7±74.7	155.7± 28.01	251.3±44.3	199.2±25.37	<0.001

Data were expressed as Mean ± SD.P value <0.05 was significant and NS= non significant

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