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Original Research Article

Effect of pomegranate polyphenols on lipids metabolism in patients with myocardial infarction: a double-blind placebo controlled trial

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ABSTRACT

Background: Myocardial Infarction (MI) is a leading disease globally. Major risk factors for MI are smoking, hypertension, diabetes mellitus, reactive oxygen species (ROS), obesity, coronary artery disease (CAD) and abnormally altered blood lipid levels. It is recommended that for healthy living the risk factors for CAD and ROS should be less. Consumption of natural food supplements rich in antioxidants and polyphenols reduce the risk of MI. One herb is Pomegranate. Pomegranate is polyphenols and antioxidants rich fruit. This prompted us to find out whether the presence of antioxidants in pomegranate offers any prognostic benefits in patients with MI?.
Methods: Pomegranate Extract of Whole Fruit (PEWF) was prepared as tablet of 300mg to investigate its effects in patients with MI. Total 100 participants were included in the trial. Participants were assigned to two groups of 50 each. One group received "Add On" PEWF and other got matching placebo of same colour, shape and size as comparator agent in the dose of 300mg BD for 1 month.
Results: Results were compared by Z test, Chi square test and coefficient of variations. Statistical analysis proves the prognostic effect after active medication ($p<0.05$). Study results indicate the rejection of Null Hypothesis (H_0) and acceptance of Alternative Hypothesis (H1).
Conclusions: Our findings suggest that consumption of antioxidant and polyphenols rich food supplements such as PEWFs for one month reduces the risk factors for CAD.

Keywords: Creatine Phospho kinase MB, Coronary artery disease, High density lipoprotein-c, Oxidized Low density lipoprotein, Reactive oxygen species

INTRODUCTION

Myocardial infarction (MI) is a leading cause of deaths globally. The incidence of MI was 301/100,000 persons/year for men and 48/100,000 person/year for women.[1] The incidence rate of MI in India was 64/1000 person/year for men aged 29-69 years in 2010.[2] The risk factors for MI are smoking, hypertension, diabetes mellitus, reactive oxygen species (ROS), obesity, coronary artery disease (CAD) and abnormally altered blood lipids.[3] Among these CAD, ROS and altered blood lipid level are major risk factors.

The arteries in heart are known as coronaries; which supply blood flow to heart muscles. Due to risk factors, lipids such as cholesterol deposit to form plaque. This plaque with deposition of platelets and cytokines leads to the stoppage of blood supply in heart muscles. This leads to the Atherosclerosis and then ischemia followed by myocardial infarction.[4]

Lipids are essential and bio-regulatory components of human cells. These remain in circulation and transportation in blood by plasma lipoproteins like Chylomicrons, Variable Low Density Lipoproteins Cholesterol (VLDL-C), Low density Lipoproteins Cholesterol (LDL-C) and High Density Lipoproteins Cholesterol (HDL-C), Non High Density Lipoproteins Cholesterol (non HDL-C).

Low density Lipoproteins Cholesterol (LDL-C)

LDL-C is highly atherogenic, which favour cholesterol accumulation in macrophages and lead to the formation of foam cells. In sub-endothelial space LDL particles remain less protected by antioxidants and more frequently exposed to cell derived ROS. The ROS modify LDL particles to oxidized LDL (oxLDL).[5,6] oxLDL are uptaken by macrophages scavenger receptor including Scavenger Receptor-A (SR-A) and B(SR-B), CD36, CD68, lecithin like oxLDL receptor and converted to foam cells. These foam cells triggers the pathogenic mechanism of atherosclerosis.[7]

High Density Lipoproteins Cholesterol (HDL-C)

It is an inverse predictor of future atherogensis. HDL-C shows its anti atherogenic properties by reducing cholesterol accumulation in the artery wall by transporting back it to liver. The HDL-C also shows antioxidative, anti-inflammatory and antithrombotic activity.[8,9] The antioxidative properties of HDL-C are due to the presence of apo-A1, Paroxinase 1(PON 1). By this HDL-C are protecting LDL-C from oxidation and synthesis of oxLDL.[10]

Triglyceride rich lipoproteins

Hypertriglyceridemia leads to the secretion of triglyceride overloaded VLDL apo-B particles. With the use of Cholesterol Ester Transfer Proteins (CETP) and Lecithine Cholesterol Acyl Transferase (LCAT), hypertriglyceridemia leads to reciprocal transfer of lipids and lipoprotein remodeling. Due to this cholesterol rich remnant like lipoprotein particles (RLP) are produced. The RLP exhibit as proatherogenic and proinflamatory features as oxLDL. The RLP play a major role in establishing of endothelial dysfunction and initiation of atherosclerosis.[11,12]

Literature suggests that ROS and RNS stimulate the generation of oxLDL-C and RLP.[13] Generation of oxLDL-C and RLP activates protein kinase C and prevents nitric oxide mediated arterial relaxation and cause atherosclerosis and MI.

For healthy leaving, blood levels of ROS and RNS should be less. There are many ways to reduce body oxidative stress like avoiding toxins, meditation, stress reduction, exercises and prevention of infections. One of the easiest and best ways to reduce ROS and to increase antioxidative level is by consumption of foods items which have high antioxidant potency. Many natural and artificial supplements are available to improve body antioxidant level, among which antioxidant rich food beverages include 100% fruit juice, iced tea and red wine are common. Apart from these, pomegranate fruit has the most potent antioxidant capacity.[14] In present study, we selected pomegranate to evaluate its prognostic effect in patients with MI. Studies showed that levels of polyphenols present in Pomegranate Peels (skin and pericarp) are much higher than that in seeds and pulp.[15] Hence, we got the Pomegranate Extract of Whole Fruit (PEWF) as a tablet of 300 mg; which had the combination of polyphenols from pulp, seeds and peel.

Many interventions are used to keep the coronary profile healthy e.g. by medicines, surgical techniques like angioplasty, stent placement and by coronary artery bypass**.** However, these are not safe and have limitations; but if PEWF administration as tablet of 300 mg twice daily is found to be prognostically effective, this may help the patients in keeping coronary profile healthy without having any side effect.

As PEWF is rich in natural antioxidants and polyphenols, the consumption of this may improve disease condition. Null Hypothesis (H_0) will be implemented during the trial.

METHODS

A randomized, double-blind, placebo controlled, parallel trial was conducted in Base Hospital, Srikot, Pauri-Grahwal, Uttarakhand, India attached to Veer Chandra Singh Garhwali Government Institute of Medical Sciences and Research, Srikot, Pauri Garhwal, Uttarakhand ,India (VCSGGIMSR) and Netaji Subash Chander Bose Subharti Medical College (SMC) and C.S. Subharti Hospital, Meerut (Uttar Pradesh) India, in collaboration with Department of Biochemistry, Pharmacology and Medicine.

A total 100 patients of both men and women with MI and satisfying inclusion and exclusion criteria were enrolled in this trial by applying the formula.

$n = [(z\sigma)/E]^2$

Where, ‘Z’ is constant value 1.96 for the confidence level 95%, ‘σ’ is standard deviation for the sample size, which is ±3, ‘E’ is error which is 0.9, ‘n’ is total number of participants in each group.

Inclusion criteria

- Men/women: Aged 20-60 years,
- MI as per signs and symptoms: Chest pain (radiating to left arm or left side of the neck), shortness of breath, nausea, sweating, anxiety, palpitation,
- Permanent residents of the trail area.

Confirmatory tests

- E.C.G. with changes - Q and ST segment elevation of 1mm or more in two neighboring leads.
- Creatine Phospho Kinase MB (CPK-MB): Levels more than the reference ranges (0-25 U/L at 370c).
- Troponin T' level more than the reference range. (>0.1 ng/ml).

Exclusion criteria

- Patients with acute illness, pregnant, lactating, and postoperative patients.
- Patients with CNS disorders, systemic chronic diseases e.g. renal failure and chronic hepatic disease.
- Postoperative conditions like angiography, angioplasty or any other surgical intervention.

Method of randomization

Selected participants were randomized as per criteria given in Table 1 by generating a list of sequential assignments to the treatment group, using the "random seed" function in the Statistical Package for the Social Sciences (SPSS) software program, version 16.0.

Table 1: Assignment of participants.

Dose	PEWF(Active) 1 BD x 1 month	Placebo 1 BD x 1 month
Myocardial infarction	n=50	n= 50

Assessment of treatment effect

Fasting 4ml venous blood samples were collected in vacutainer. After coagulation, samples were centrifuged at 8000RPM for 15min and serum was collected in separate test tube. Samples were processed on the same day for bio-chemicals markers for MI viz. Total cholesterol, serum triglyceride, high density lipoproteins (HDL), non-HDL cholesterol, low density lipoproteins (LDL), and oxidized LDL (OXLDL) to check pre and post drug effects on fully automated biochemistry analyser-Cobas 6000.

Trial medicines

Trial medicines were given on "add-on basis" in addition to other prescribed medicines.

Description of the medicine

The active medicine had PEWF. Matching placebo of same color, shape, size and weight was used. The PEWF/placebo were given orally, as tablets of 300mg twice daily (BD) for one month.

Trial procedure

Duration of treatment

Participants were treated daily with either active medicine or placebo for one month. Regular follow-up of patients was done by frequents visits and personal communication.

Visit I (Week 0), screening visit (pre-dug analysis)

After obtaining an informed consent, nested cases of MI were included. Venous blood sample was collected for assessment of biochemical parameters related to MI. Baseline titer was obtained and recorded.

Visit II (Week 1)

The participants were under the "add-on" therapy of PEWF/placebo and the treatment doses were issued for 15 days initially and participants were recalled for next visit.

Visit III (Week 3)

Follow-up information was obtained regarding any adverse effects of intervention. The participants were questioned regarding any missed doses of trial medicine and next dose of medicines were issued for next 15 days.

Visit IV (Week 5), final visit (post drug analysis)

The participants were questioned regarding any missed doses of the trial medicine. All biochemical parameters related to risk factors for MI were redone.

Assessment of Compliance

The participants; who had 80% consumption of PEWFs/placebos, were considered to be compliant.

RESULTS

Total 100 participants, of either gender between 40-60 years participated in this trial (Table 2). Amongst these, 20 were from 40-50 years age group, 33 from 50-55 years and 47 from 55-60 years age group. We did not get participants of 20-40 years. The participants were distributed randomly to either of two groups of 50 each. One group of 50 participants (44 men and 6 women) received PEWF (active) and second group of 50 participants (47 men and 3 women) received placebo. All

participants were instructed to continue with prescribed medicines by the clinicians uninterrupted. The PEWF/placebo was given as "add-on" basis for one month.

Table 2: Age and group wise distribution of participants with mi in groups.

Age (Years)	PEWF (Active)	Placebo	Total
40-50	11	09	20
50-55	15	18	33
55-60	24	23	47
Total participants	n=50	n=50	100
	Men:44	Men:47	
	Women:06	Women:03	

n= Total number of participants

Table 3 summarizes the descriptive statistics of PEWF on lipid profile in both pre and post drug analysis. In this table, serial numbers 01 to 07 are showing pre and post drug effects on biochemical parameters for 50 participants with MI. The mean level of total cholesterol and triglyceride (Sr.No.1 and 2) in pre drug analysis was 314.55mg/dl and 287.1mg/dl and in post drug analysis these were 274.8mg/dl and 268.9mg/dl. Serial numbers 3, 4 and 5 highlight the effects on biochemical markers such as HDL-C, non HDL-C and LDL-C in pre and post drug effects. Thus, there is decrease of major risk factors such as lipid profile in post drug. The decrease in mean level of risk factors and improvement in HDL indicates the prognosis. The mean levels of Lp(a) and ox-LDL-C in pre drug analysis are 47.4mg/dl and 1.91mg/dl and in post drug analysis are 44.2mg/dl and 1.54mg/dl. This is a good sign of prognosis.

Table 3: Descriptive statistics for PEWF (active) medication in pre and post drug post intervention.

N=50	Pre dug analysis		Post drug analysis	
Parameters	**Mean (X)**	**Std. Deviation (±SD)**	**Mean (X)**	**Std. Deviation (±SD)**
Total cholesterol (mg/dl)	281.24	81.85	231.10	46.55
Serum triglyceride (mg/dl)	313.15	110.85	271.04	61.77
HDL-C (mg/dl)	24.15	4.82	76.07	8.82
Non-HD cholesterol (mg/dl)	160.86	10.70	140.05	2.82
LDL-C (mg/dl)	109.48	13.93	88.07	8.92
Lp(a) (mg/dl)	45.6	12.4	43.4	11.2
OXLDL-C (mg/dl)	2.31	0.70	0.84	0.21

n= Total number of participants

Table 4: Descriptive statistics for placebo in pre and post drug analysis.

N=50	Pre dug analysis		Post drug analysis	
Parameters	**Mean (X)**	**Std. Deviation (±SD)**	**Mean (X)**	**Std. Deviation (±SD)**
Total cholesterol (mg/dl)	314.55	82.02	275.8	79.74
Serum triglyceride (mg/dl)	287.1	128.93	268.9	115.64
HDL-C (mg/dl)	26.16	4.48	41.4	5.98
Non-HD cholesterol (mg/dl)	163.83	15.33	156.55	11.43
LDL-C (mg/dl)	108.3	11.00	101.02	10.64
Lp(a) (mg/dl)	47.4	11.7	44.2	09.7
OXLDL-C (mg/dl)	1.91	0.89	1.54	0.77

n= Total number of participants

Table 5: Z statistics of PEWF (active) for post drug in comparison to pre drug analysis.

Paired Samples Test				
Pairs	**Parameters**	**Z Test**	**Degree of Freedom**	**Sign (2 Tailed)**
Pair 1	Total cholesterol for pre and post drug analysis	241.37	7254	0.00
Pair 2	Serum triglyceride for pre and post drug analysis	223.94	7254	0.00
Pair 3	HDL-C for pre and post drug analysis	421.75	7254	0.00
Pair 4	LDL-C for pre and post drug analysis	254.07	7254	0.00
Pair 5	Non-HDL cholesterol for pre and post drug analysis	173.50	7254	0.00
Pair 6	OXLDL-C for pre and post drug analysis	202.83	7254	0.00

Table 4 summarizes the descriptive statistics for placebo in both pre and post drug effects. Serial numbers 1 to 7 present post drug effects of placebo on lipid profile in patients with MI. The mean level of total cholesterol,

triglycerides, non HDL-C, LDL, Lp(a) and ox-LDL-C decreased in post drug effects. The reason behind this is that all the patients were taking their prescribed medicine regularly.

Table 6: Z statistics of placebo for post drug in comparison to pre drug analysis.

Paired Samples Test				
Pairs	**Parameters**	**Z Test**	**Degree of Freedom**	**Sign (2 Tailed)**
Pair 1	Total cholesterol for pre and post drug analysis	89.952	4528	0.00
Pair 2	Serum triglyceride for pre and post drug analysis	71.119	4528	0.00
Pair 3	HDL-C for pre and post drug analysis	54.648	4528	0.00
Pair 4	LDL-C for pre and post drug analysis	115.280	4528	0.00
Pair 5	Non-HD cholesterol for pre and post drug analysis	143.667	4528	0.00
Pair 6	OXLDL-C for pre and post drug analysis	141.385	4528	0.00

Table 7: Chi square test of PEWF (active) for post drug in comparison to pre drug analysis.

Paired Samples Test				
Pairs	**Parameters**	**Chi square**	**Degree of Freedom**	**Sign (2 Tailed)**
Pair 1	Total cholesterol for pre and post drug analysis	190.187	20	0.00
Pair 2	Serum triglyceride for pre and post drug analysis	79.700	15	0.00
Pair 3	HDL-C for pre and post drug analysis	48.74	139	0.00
Pair 4	LDL-C for Pre and post drug analysis	2.705	139	0.00
Pair 5	Non-HD cholesterol for pre and post drug analysis	26.817	139	0.00
Pair 6	OXLDL-C for pre and post drug analysis	54.964	2	0.00

Table 8: Chi square test of placebo for post drug in comparison to pre drug analysis.

Paired Samples Test				
Pairs	**Parameters**	**Chi square**	**Degree of Freedom**	**Sign (2 Tailed)**
Pair 1	Total cholesterol for pre and post drug analysis	191.912	20	0.00
Pair 2	Serum triglyceride for pre and post drug analysis	313.259	25	0.00
Pair 3	HDL-C for pre and post drug analysis	0.206	1	0.00
Pair 4	LDL-C for pre and post drug analysis	48.010	4	0.00
Pair 5	Non-HD cholesterol for pre and post drug analysis	33.217	139	0.00
Pair 6	OXLDL-C for pre and post drug analysis	168.469	12	0.00

Table number 5 and 7 summarize the Z and chi square test of PEWF in both pre and post drugs analysis. In table 5; pair number 1 to 6 shows that $p<0.05$, which indicates that Lipid profile such as total cholesterol, triglyceride, HDL-C, non HDL-C and LDL and ox-LDL-C improved in post drug analysis after active and placebo medication. This indicates the prognosis.

Statistical significance ($p<0.05$) indicates the rejection of Null Hypothesis (H_0), which means that alternative hypothesis (H1) will be implemented. Z test showed statistical significance because all the patients were on treatment of MI. Pomegranate extract of whole fruit was given as "ADD-ON" basis.

Table number 6 and 8 show the z and chi square test of placebo in post drug analysis for biochemical parameters related to MI and CAD. In pair 1 to 6 there was significant decrease ($p<0.05$) of cardiac risk factors like Total cholesterol, triglyceride, HDL-C, non HDL-C, LDL-C and ox-LDL-C. The significance was $p<0.05$; which indicates the reduction of risk factor related to MI and CHD; which proves the prognostic effect after medication. These results indicate the rejection of Null Hypothesis (H_0) and acceptance of alternative Hypothesis (H1).

Table 9 shows the difference in coefficient of variations (C.V.) of post drug analysis after PEWF (active) and placebo medication. The C.V. of total cholesterol, serum triglyceride, HDL-C, Non HDL cholesterol, LDL-C and ox-LDL-C as after consumption of PEWF was 0.18, 0.22, 0.21, 0.06, 0.5 and 0.25. The C.V. of total cholesterol, serum triglyceride, HDL-C, Non HDL cholesterol, LDL-C and ox-LDL-C after consumption of placebo was 0.26, 0.29, 0.16, 0.15, 0.7 and 0.5. This highlights that C.V. of

all above parameters except HDL after active medications are lesser than placebo. This shows that active medicine has much higher prognostic effect.

Table 9: Coefficient of variations for PEWF (active) and placebo medicine after post drug analysis.

Descriptive statistics	PEWF (active)			Placebo		
Post Drug Analysis	**Mean (X)**	**Std. Deviation (±SD)**	**CV**	**Mean (X)**	**Std. Deviation (±SD)**	**CV**
Total Cholesterol	231.1	46.55	0.18	296.88	79.74	0.26
Serum Triglyceride	271.0	61.77	0.22	396.93	115.64	0.29
HDL-C	76.07	8.82	0.21	30.45	5.98	0.16
Non HDL-C Cholesterol	160.87	10.97	0.06	139.95	3.14	0.15
LDL-C	88.07	8.92	0.5	101.16	10.64	0.7
OXLDL-C	0.84	0.21	0.25	1.54	0.77	0.5

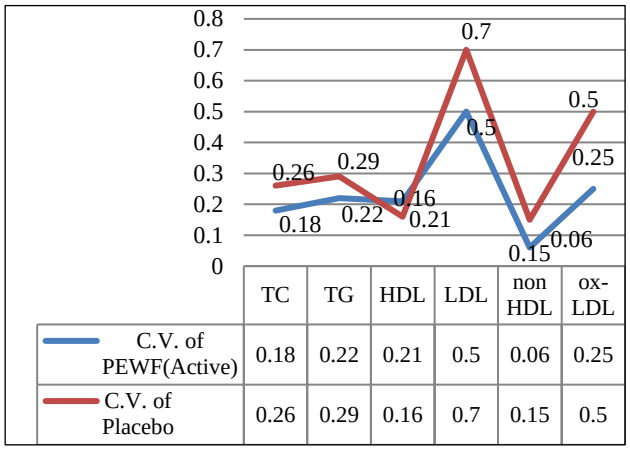

Figure 1: Coefficient of variations for post drug effects after consumption of PEWF (active) and placebo medicine.

In Figure 1, when C.V. for post drug effect of active medicine and placebo are compared with each other by graphical analysis, it is highlighted that the C.V. of active medicine are lesser in all parameters except HDL. This is good sign for prognosis.

This suggests that coronary risk factors have been reduced significantly after active medication. This proves the effectiveness of PEWF as a dietary supplementation for MI and MI risk patients.

DISCUSSION

Oxidative stress leads to generation of ROS and Reactive Nitric Species (RNS). ROS contains one or more unpaired electron in their outer orbit. The generation of ROS and RNS are lethal. These lethal ROS are categorized as superoxide anion radical (dioxide or O_2^-), hydroxyl radical (OH) and peroxynitrite anion ($ONOO^-$). The ROS are generated from oxygen and RNS are derived from NO_2, ONOO, N_2O_3 and HNO_2.

These ROS and RNS may cause alterations in DNA by different mechanisms like nicking, base pair mutations, rearrangement, deletions, sequence amplifications, nitration, nitrosation and deamination etc. The mutated DNAs or nucleic bases are responsible for synthesis of mutated proteins and are cause for pathogenesis of many diseases such as atherosclerosis, cancer, diabetes mellitus, rheumatoid arthritis, post-ischemic perfusion injury and myocardial infarction. The patho-physiology of ROS damages are as follows:

ROS may stimulate the leukocyte adhesions to smooth vascular cell by activation of a cytokine like vascular cell adhesion molecules (VCAM). This action promotes the Monocytes adhesion to vascular endothelial cell and promotes the entry of monocytes to intima. This mechanism is further stimulated by signaling molecules like selectin, integrins and Monocyte chemoattractent protein1 (MCP1). This leads to CAD and atherosclerosis.[16,17]

Literature suggests that ROS and RNS stimulate the generation of ox-LDL-C. Generation of ox-LDL-C activates protein kinase C and prevents nitric oxide mediated arterial relaxation and cause atherosclerosis and MI.[18]

Many natural and artificial supplements are available in market with claims of improving the body antioxidant level, among which are antioxidant rich beverages such as 100% fruit juices, iced tea and red wine. Apart from these pomegranate fruit has the most potent antioxidant capacity. The level of antioxidants and polyphenols in pomegranate is much higher than black grapes and red wine.[19]

Literature suggests that food items rich in polyphenols improve lipid profile by reducing cholesterol and lipids absorption in brush border cells by interacting with cholesterol carrier and its transporters across the brush border cells.[20,21] One of the mechanisms suggests that

food items which are rich in polyphenols can reduce the lymphatic absorption of lipids in intestinal brush border cells.[22] Another mechanism suggests that polyphenols down regulate the HMG CoA Reductase. HMG CoA Reductase is a key regulatory enzyme of cholesterol biosynthesis. Overall, the polyphenols decrease the plasma cholesterol levels.[23] Our trial results recommend that parameter of ox-LDL-C is decreased significantly as compared to placebo after consumption of PEWF. The mechanism is based upon the action of enzyme Paraoxonase-1, which is an aryldialkylphosphatase [EC3.1.8.1]. The PON1is located on HDL which acts as chain breaking antioxidant. This enzyme catalyzes the hydrolysis of toxic insecticides, nerve gases and oxidized phospholipids. The PON-1 enzyme prevents the accumulation of lipid peroxides in LDL. The PON1 hydrolyzes the pro- inflammatory cytokines such as platelet activating factors.[24] Literature reviews suggests that PON1 is much effective than apo A1 and LCAT in preventing the oxidation of LDL.[25]

Our trial indicates that consumption of polyphenols and antioxidants rich food items such as PEWF has the potential to decrease lipid profile and reduce the cardiac risk factor. These are good sign of prognosis, which shows that pomegranate is cardio protective in nature. These food items should be included as an integral part of human diet and may be given as food supplements.

CONCLUSION

Risk factor of MI is CAD. Pathogenesis of CAD is based on increased production of ROS/RNS and inflammatory cytokines such as Interleukin 18(IL-18), Interleukin 6(IL-6), vascular cell adhesion molecule-1(VCAM-1), Selectins, Integrins, Monocytes Chemo-Attractant Protein-1 and tumor necrosis factor (TNF-α). The cytokines activate the natural killer cells such as CD16 and CD56 and lead to the production of oxLDL. The overall effect enhances the atherosclerosis.

Our findings suggest that consumption of antioxidant and polyphenols rich food supplements such as PEWFs for one month reduces the risk factors for CAD. In conclusion, polyphenols and antioxidants rich fruit supplements like pomegranate should be taken in diet for healthy living.

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