

# Short-term Effects of Whey, Creatine, and L-carnitine Supplementation on Muscle Hypertrophy Marker Candidates in Young Males: A Randomized Placebo-controlled Pilot Study

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**Abstract** Previous research showed that resistance exercise could induce muscle development, noticeably from the increased level of several markers from blood samples. However, no study had been performed to explore the effect of combination of resistance exercise and proper nutrition supply on those markers. The aim of this study to investigate the effect of whey protein, creatine, and L-carnitine; on potential molecular markers of muscle hypertrophy (*arg1* and *mmp9*) from blood samples. Twelve healthy male participants were randomly categorized into supplement (SUPP) or placebo (PLAC) treatment, and performed resistance training three times in a one-week period. Blood sampling was carried out before (day one) and 2 hours after the exercise (day one, day three and day five). The level of *mmp9* gene expression was increased along with the progress of the resistance training program. Moreover, participants who received supplementation (SUPP) showed a higher level of *mmp9* gene expression compared to resistance training only (PLAC). A significant difference was observed between two treatments in the first day, 2 hours after the resistance training session ( $p = .04$ ); and between SUPP group on the fifth day, 2 hours after the resistance training; compared to the first day, before the resistance training session ( $p = .02$ ). The effect was not observed on *arg1* gene. A combination of resistance training with supplementation; was considered to enhance the muscle hypertrophy process, compared to resistance training only. The results also suggested that *mmp9* could act as a blood-derived molecular marker of muscle hypertrophy.

**Keywords:** muscle hypertrophy, resistance training, whey protein, L-carnitine, *mmp9*, *arg1*

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## 1. Introduction

Resistance training is a kind of sport which can improve general fitness status and induce muscular hypertrophy, by performing movements against force in the opposite direction. Studies showed positive effects of resistance exercise to muscle cross-sectional area, cellular markers, protein markers, and gene expression markers; yet, muscle biopsy was still the method of preference for sampling [1,2,3]. Muscle biopsy is known as an invasive method and potential to cause a scar in the participant's body. Therefore, molecular markers from blood sample are preferable because they give less invasive effect to the participants.

Research showed that there were two genes which upregulated in 2 hours after acute resistance exercise: *arg1* and *mmp9* [4]. Those genes were analyzed from blood

samples. Arginase-1 (Arg1) is an enzyme which metabolizes arginine to ornithine, which furtherly modified to proline, a constituent of collagen which important in hypertrophy process [5,6,7]. Meanwhile, matrix metalloproteinase 9 (MMP9, gelatinase B) is an enzyme which is capable of degrading extracellular matrices, particularly type IV and V collagen [8,9]. This enzyme helps the movement of leukocytes toward the damaged area of muscle tissue, improve the ability of myoblast migration, and help the synthesis of new myofiber [10,11]; thus, the hypertrophy process can occur.

The combination of resistance training and proper nutrition supply will help the body-builders to achieve enhanced muscular development. Administration of nutrients, particularly whey protein, creatine, and L-carnitine would further enhance the process of muscle hypertrophy. Researches showed that whey protein and creatine could improve myoblast proliferation and differentiation [12,13]. Meanwhile, L-carnitine was involved in muscle recovery

[14] and reduced muscle tissue damage [15]. However, there had been no study which observes the effect of combination between nutrition and resistance training on muscle hypertrophy, at the level of gene expression. This study was the first to observe the changes of gene expression in blood samples after resistance training, combined with nutrition supplementation. Therefore, the aim of this research was to investigate the effect of nutrient administration (whey protein, creatine, and L-carnitine) towards potential molecular markers of muscle hypertrophy (*arg1* and *mmp9*) from blood samples.

## 2. Materials and Methods

### 2.1. Participants

A total of 12 healthy male participants were recruited, in which each had to fulfill the following criteria: 1) aged between 18 to 35 years old; 2) did not smoke and drink alcoholic beverages; 3) did not consume prescribed medicines; 4) was not overweight; 5) was not diagnosed to have diabetes; 6) had no records of heart disease; 7) less active, which performed less than 2 hours of structured physical activity at free time [16,17]; and 8) had never done resistance training program for the last 2 years prior to this study. The design was double-blind randomized, in which all participants were randomly categorized into supplement (SUPP) or placebo (PLAC) treatment; in which, neither the participants nor the researcher knew about the participant grouping.

During the study period, participants were asked not to drink high protein and ergogenic supplements, apart from the drinks given as treatment. The use of anabolic steroids was not allowed during the study. They were also asked not to perform any resistance exercise in three weeks prior to this study. Before participating in this study; all the participants were provided with written informed consent, which complied with the Declaration of Helsinki and was approved by the ethics committee of Atma Jaya Catholic University of Indonesia.

### 2.2. Body Composition Measurement

Body composition measurement was performed with the InBody720 instrument (Biospace, Gangnam-gu, Seoul, Korea), which utilized bioelectrical impedance method. The measurement was performed at the beginning of the study to screen the compatibility of the prospective

participants with the criteria used in this study (not overweight, which  $BMI \leq 23 \text{ kg/m}^2$ ).

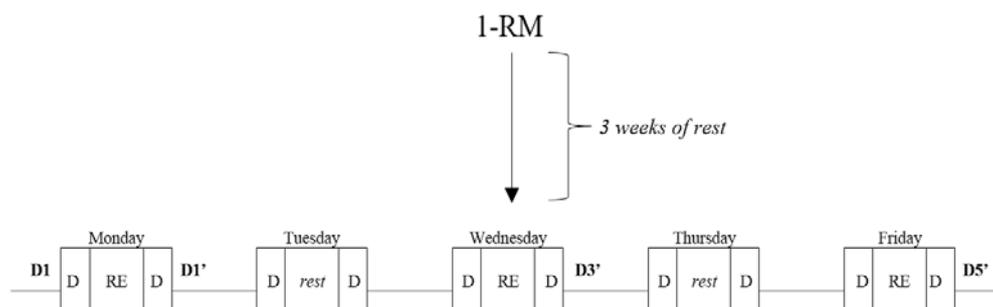
### 2.3. 1-RM Measurement, Resistance Training and Supplementation

All participants performed 1-RM measurement three weeks prior to this study (Figure 1). 1-RM measurement involved the following exercise: smith machine bench press, smith machine close grip bench press, smith machine bent over row, dumbbell standing reverse curl, and dumbbell one arm standing curl. Every successfully lifted weight was followed by three minutes of rest and additional 2.5 kg weight for the next repetition gradually.

The resistance training was performed in five days period: three days of exercise and two days of resting. Participants were asked to consume supplement (SUPP) or placebo (PLAC) before and after each resistance exercise session during the three days of exercise; as well as twice a day during the resting day. In total, every participant consumed two servings of drink each day and repeated for five days' period.

The supplement drink consisted of the mixture of whey protein, creatine, and L-carnitine (total calories: 130 cal, total fat: 2.5 g, protein: 23 g, total carbohydrate: 3 g). Meanwhile, placebo drink consisted of the isocaloric amount of maltodextrin (total calories: 130 cal, total fat: 2.5 g, protein: 1 g, total carbohydrate: 26 g). Both of the drinks were made as identical as possible in color, taste, and texture. Participants were asked not to discuss the administered drinks during the study. Blood samples (6 mL) were collected from antecubital vein before the first resistance exercise session (day 1) and 2 hours after each resistance exercise session (day 1, day 3 and day 5). Blood samples were placed in EDTA vacutainer tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). The overall experimental design was described in Figure 1.

The resistance exercise of each session consisted of following motions: smith machine bench press, smith machine close grip bench press, smith machine bent over row, flutter kick, plank, leg raise, dumbbell standing reverse curl, and dumbbell one arm standing curl. Each exercise consisted of three sets, with exercise volume of 60% 1-RM with 12 repetitions for the first set; 65% 1-RM with ten repetitions for the second set; 70% 1-RM with eight repetitions for the third set (exception for flutter kick, plank, and leg raise exercises because their 1-RM values were not measured).



**Figure 1.** Experimental design. 1-RM: 1-RM measurement; RE: resistance exercise session; D: drink administration according to their group; D1, D1', D3', and D5': time points of the study, according to blood sampling periods. Time point with no apostrophe sign indicated blood sampling before exercise. Time points with apostrophe sign (') indicated blood sampling two hours after exercise

## 2.4. RNA Isolation from Blood Sample and cDNA Synthesis

RNA from each blood sample was isolated with QIAamp® RNA Blood Mini Kit (Qiagen, Venlo, Limburg, Netherlands) according to the instructions provided by the manufacturer. RNA concentration and purity were assessed with NanoDrop 2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was done with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions provided by the manufacturer in GS 482 PCR instrument (G-Storm, Somerton, Somerset, Great Britain).

## 2.5. qPCR of *arg1* and *mmp9* Genes

Primer sequences used were described in Table 1. Gene quantification was done with StepOnePlus Real-Time PCR System instrument (Thermo Fisher Scientific, Waltham, MA, USA). The reaction was done with the following condition: pre-denaturation at 95°C for 2 minutes; denaturation at 95°C for 20 seconds; primer annealing at 55°C (*arg1* dan *mmp9*) or 53°C (*β-actin*) for 20 seconds; and fluorescence acquisition at 72°C for 30 seconds. The whole reaction was done in 40 cycles. Levels of gene expression measurement were done with  $2^{-\Delta\Delta CT}$  relative quantification method [18].

Table 1. Primer sequences used in this study

Name	Sequence	Reference
<i>arg1</i>	5'-ATTGAGAAAGGCTGGTCTGC-3' (forward)	[19]
	5'-CATTAGGGATGTCAGCAAAGG-3' (reverse)	
<i>mmp9</i>	5'-GCTTCTGCCCGGACCAAGGATACAGTT-3' (forward)	[20]
	5'-GCCTCCGCACTGAGGAATGATCTAA-3' (reverse)	
<i>β-actin</i>	5'-CAGCCATGTACGTTGCTATCCAGG-3' (forward)	[21]
	5'-AGGTCCAGACGCAGGATGGCATG-3' (reverse)	

## 2.6. Statistical Analysis

Data obtained were statistically analyzed with IBM SPSS Statistics (Version 22 for Windows; IBM, Armonk, NY, USA). Independent t-test was used to analyze the homogeneity of participants (age, BMI, PBF, and SMM) and gene expression difference between different groups. Repeated measures ANOVA with Bonferroni adjustment for multiple comparisons was used to analyze the difference of gene expression between different time points. Significance was considered at p-value < .05.

Table 2. Demographic data from participants

Variable	PLAC (n = 6)	SUPP (n = 6)	p-value
Age (years)	20.83 ± 2.79	20.50 ± 1.38	.80
Body mass index (kg/m <sup>2</sup> )	20.55 ± 1.38	19.57 ± 2.21	.30
Percent body fat (%)	20.85 ± 6.72	18.53 ± 5.55	.53
Skeletal muscle mass (kg)	24.25 ± 2.89	25.42 ± 1.70	.41

## 3. Results

The level of *mmp9* gene expression was increased along with the progress of resistance training program; in which the highest level was found on the fifth day (D5') (Figure 2). Moreover, resistance training with nutrition supplementation (SUPP -whey protein, creatine, and L-carnitine) further induced the level of *mmp9* gene expression in all three time-points measurement (D1', D3', and D5'); compared to resistance training only (PLAC). A significant difference was observed between two treatments in the first day, 2 hours after the resistance training session (\*p = .04); and between SUPP group on the fifth day, 2 hours after the resistance training; compared to the first day, before the resistance training session (\*\*p = .02).

The different result was observed in *arg1* gene expression. The *arg1* gene expression was increased after 2 hours of resistance training on day 1 (D1'); yet, the level was decreased along with the study duration (D3' and D5'). Supplementation of whey protein, creatine, and L-carnitine gave no significant effect on the expression of *arg1* gene (Figure 3).

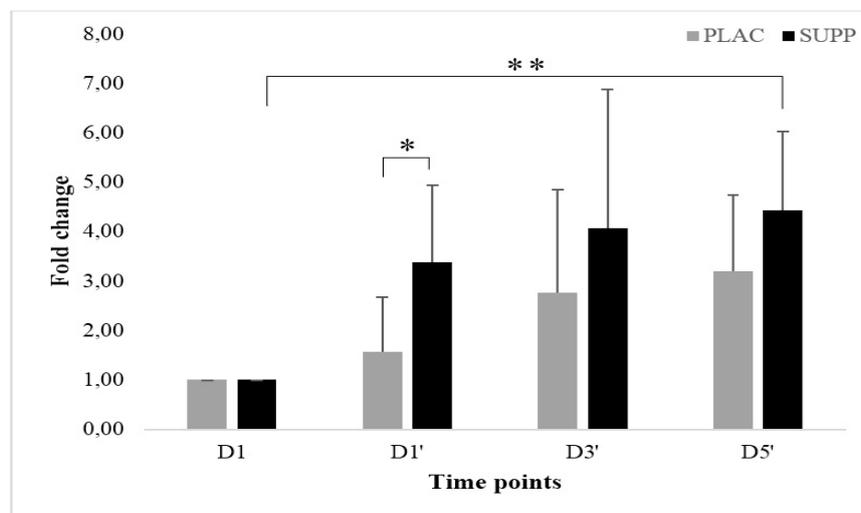
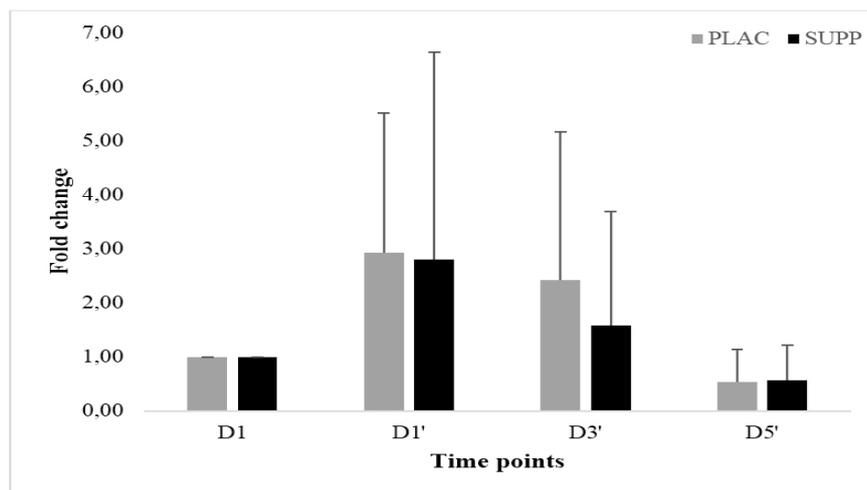


Figure 2. Fold change values of *mmp9* in different time points of the study: baseline at the first day before training (D1), the first day (D1'), the third day (D3'), and the fifth day (D5'). Apostrophe sign (') indicated blood sampling two hours after exercise. Gray and black graph indicated PLAC and SUPP group, respectively. Asterisk sign (\* and \*\*) indicated a significant difference between two values (p < .05)



**Figure 3.** Fold change values of *arg1* in different time points of the study: baseline at the first day before training (D1), the first day (D1'), the third day (D3'), and the fifth day (D5'). Apostrophe sign (') indicated blood sampling two hours after exercise. Gray and black graph indicated PLAC and SUPP group, respectively

#### 4. Discussion

The demographic characteristics of participants in this study were homogeneous; in which they were similar in age, BMI, PBF, and SMM (Table 1). Three weeks prior to the clinical study, all the participants were asked to perform the 1-RM measurement. The purpose of 1-RM measurement was to quantify the maximum amount of weight that could be lifted by each person in each exercise; so, the resistance training weights could be adjusted personally. Later, in the clinical study period; we observed the effect of resistance training combined with nutrition (a mixture of whey protein, creatine, and L-carnitine); and resistance training per se, on the expression of *mmp9* and *arg1* gene.

The study showed that *mmp9* gene expression was upregulated in 2 hours after resistance exercise, which in line with a previous study [4]. This study also showed that a combination of resistance training with a mixture of whey protein, creatine, and L-carnitine induced higher expression of *mmp9* gene expression, compared to resistance training only. The MMP9 protein played a role in the recruitment of leukocytes to the sites of injury [10] and helped the migration of satellite cells by the degradation of basal lamina [11,22]. Satellite cells were those which responsible for muscle hypertrophy process, by furtherly differentiated or fused to myofibers [6,7,23].

Neutrophils, which were able to synthesize MMP9 [24], were the first immune cells recruited to the area of muscle injury; followed with M1 macrophages [25]. Resistance exercise increased the blood neutrophil count up to 3 hours [26]. The increase of viable neutrophils after whey protein application [27] could lead to more recruitment of neutrophils to the damaged muscle area and led to increased *mmp9* gene expression.

Whey protein increased neutrophil responses (superoxide anion formation, chemotaxis, and phagocytic activity) [27] and stimulated the proliferation of resting splenocytes [28]. Moreover, whey protein also increased the levels IL-1Ra and number of cytokines, such as IL-1 $\beta$ , IL-6, MIP-1 $\alpha$ , MIP-1 $\beta$ , and TNF- $\alpha$  [29]. TNF- $\alpha$  was known as the activator of MMP9 protein and its mRNA synthesis in leukocyte [30] and in muscle cell [31].

L-carnitine was also able to modulate the expression of *mmp9* gene by restoring the chemotaxis [32] and phagocytic ability [33] of neutrophils. Several human studies also showed that L-carnitine increased the ATP production of lymphocytes [34] and TNF- $\alpha$  expression in resting human peripheral blood mononuclear cells [35]. Together, these phenomena could lead to an increased level of *mmp9* gene expression.

To date, there was no convincing evidence about the effect of creatine supplementation towards neutrophil. Nevertheless, some researches noted that creatine supplementation played a role in reducing the expression of TNF- $\alpha$  [36,37]. A study showed that the in vitro supplementation of L-carnitine (400  $\mu$ g/mL) induced high increments of TNF- $\alpha$  expression up to 234  $\mu$ g/mL from zero levels after 24 hours of incubation [35]; whereas in vivo supplementation of creatine (20 g/day) only reduced 42% of TNF- $\alpha$  increments in 24 hours after heavy exercise [38]. Furthermore, in vitro supplementation of whey protein extract increased TNF- $\alpha$  expression from zero to the level of  $0.04 \pm 0.01$   $\mu$ g/L [29]. While these studies were not directly comparable, the data suggested that combination of whey protein and L-carnitine elicited a higher impact towards TNF- $\alpha$  expression than creatine. Therefore, we hypothesized that the decreased TNF- $\alpha$  expression effect from creatine was overruled by whey protein and L-carnitine content in the supplement; thus, the level of *mmp9* expression could be increased. Taken together, the gene expression increment after resistance training with supplementation suggested that *mmp9* could act as a molecular marker of muscular hypertrophy taken from blood samples.

On the other hand, the effect of whey, creatine, and L-carnitine was not substantial in modulating the expression of *arg1* gene. This phenomenon might be correlated with the increased expression of nitric oxide synthase (NOS) after resistance exercise. Exercise was known to increase the levels of nitric oxide, which was the result of NOS expression increment [39]. For example, eNOS (endothelial nitric oxide synthase) activity was known to be upregulated after endurance exercise [40] and treadmill running stimuli [41]. On the other hand; iNOS (inducible nitric oxide synthase), which could be produced

by neutrophils and macrophages [42], was increased significantly after resistance exercise with blood flow resistance [43] and running exercise [44]. The existence of iNOS was essential for muscle regeneration [42]; which was also a part of the hypertrophy process.

## 5. Conclusion

A combination of resistance training with supplementation of whey protein, creatine, and L-carnitine; was considered to enhance the muscle hypertrophy process, compared to resistance training only. This was perceived from the increased level of *mmp9* gene expression during the clinical trial duration; which could be due to the effect of whey protein and L-carnitine in improving neutrophil proliferation and chemotaxis, and also upregulating the TNF- $\alpha$  cytokine. The effect was not observed in the *arg1* gene expression. The data suggested that *mmp9* could act as a blood-derived molecular marker of muscle hypertrophy.

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