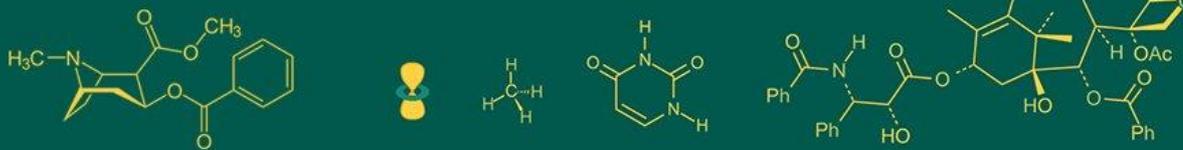


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## Serum lipoproteins response to twelve months changes in antioxidants: Influence of nutraceuticals and functional foods intervention on age and gender

Nweze CC, Solomon M and Haruna GH

### Abstract

The bioactive compounds in encapsulated-nutraceuticals have contributed to reducing oxidative stress. The cost of these encapsulated extracts makes them unaffordable to the low income groups in developing countries such as Nigeria. Thus, the search for alternative affordable antioxidant functional foods is best alternative. This study investigated effect of two antioxidants on blood lipids and lipoproteins. The study was undertaken by healthy volunteers of 96 males and 54 females within the ages of 30-74 years old. Volunteers were randomly divided into three groups and blood samples collected at intervals of baseline and six months. Volunteers were randomized to either 1 capsule per day of antioxidant nutraceutical, antioxidant functional foods or placebo. Blood samples were analysed for Cholesterol, Tryglyceride, LDLc, HDLc, and Uric acid at baseline and twelve months. All were significantly reduced ( $p < 0.05$ ), exception of HDLc that significantly increased ( $p < 0.05$ ). The study indicates that functional foods antioxidants improved the serum lipids and lipoproteins indices.

**Keywords:** Nutraceuticals, functional foods, antioxidants lipoproteins, age, gender

### 1. Introduction

The immune system is a biological structure within an organism that protects against diseases; immune system detects a wide variety of agents from viruses to parasitic worms and distinguishes them from the organism's own healthy tissue. Immune cells are influenced by antioxidant/oxidant balance; the antioxidant levels play a vital role in maintaining immune cells in a reduced environment and in protecting them from oxidative stress. Because the immune system is critically dependent on accurate cell-cell communication in order to mount a response, immune cell integrity is essential. The effects of antioxidants are very beneficial during period of oxidative stress like the periods of infections, and in elderly. The presence of at least three of five criteria (obesity, low HDL-C, hyperglycerilaemia, hypertension and elevated glucose concentration) in man is term a metabolic syndrome [1, 2]. Metabolic syndrome however is one of risk factors for cardiovascular disease [2]. Some of these risk factors and criteria for metabolic syndrome results from poor nutrition of saturated fats and high cholesterol diets which however led to an increase in cholesterol, LDL-C and triglyceride levels in the blood [3] and high level of triglycerides and cholesterol leads to decreased in the level of HDL-C in the blood [4]. Others risk factors are also involved however not controllable, those include age [5], sex [6] race and heredity [5, 6]. Increase in serum level of cholesterol is reported to be associated with early atherosclerosis in adolescent and young adults [7].

Coelho *et al.*, [8] reported that atherosclerosis begins at childhood in a slow progression and characterized for the formation of atheromas that causes the restriction of blood flow. Atherosclerosis is a multi-risk factor and its incidence contributes both genetic and environmental factors leading to changes in plasma lipids and lipoproteins which eventually cause cardiovascular diseases. Hence, oxidation of LDL lipoprotein in vessels increases progression of cardiovascular diseases, but high level or increase in plasma HDL prevent progress of cardiovascular diseases [9]. Many studies have indicated that increased level of serum cholesterol and LDL and a corresponding decreased in HDL levels are the primary factors for predicting atherosclerotic and cardiovascular disease [9, 10].

The protective effects of antioxidants may be mediated through inhibition of the oxidative modification of LDL-C which is reported to be a key step in the pathogenesis of atherosclerosis [11].

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The effects of antioxidants in reducing platelet adhesiveness [12], preserving endothelium vessel function [13] and stabilizing plaques [14] supports its role in the prevention of atherosclerosis and thrombosis and in the regulation of vasomotor tone [15]. Several reports have suggested that higher intake of antioxidants may be an important factor in reducing the incidence of cardiovascular diseases in the general population [16]. Antioxidants are known to prevent LDL oxidation [17] and little evidence of the efficacy of a long term intervention of antioxidant nutraceuticals and functional foods on lipoproteins in healthy individuals. However, limited or no data is available in support of comparative effects of antioxidant nutraceuticals and functional foods on lipoproteins in these subjects according to age and sex distribution. Hence, the present study was undertaken to evaluate the effect of antioxidant nutraceuticals and functional foods on lipoproteins in healthy subjects according to age and sex distribution.

## 2. Materials and Methods

### 2.1 Study design and site

The study was conducted in Nasarawa State University, Keffi, Nigeria.

Cross-sectional study design was employed in this study.

### 2.2 Study Population, inclusion and exclusion criteria

The study subjects included a total of 150 healthy adults of 96 men and 54 women aged between 30 and 74 years. All volunteers are staff of Nasarawa State University, Keffi, Nigeria. A randomized, prospective, parallel group, comparative, open dose and single centre study was undertaken by the 150 healthy subjects. The inclusion and exclusion criteria were adopted.

### 2.3 Ethical Review and Independent Monitoring

The scope, nature, aim and objectives of this study were thoroughly explained to voluntary participants for their consent, and all of them were made to sign an informed consent letter and a questionnaire. Confidentiality was assured and privacy protected for the information collected by using numbers instead of names. The protocol was reviewed and approved by the Chairman Ethical committee, Federal Ministry of Health Abuja, Nigeria.

### 2.3 Baseline sample collection and six months antioxidant nutraceuticals and functional foods intervention

Baseline samples were collected before the six months dietary intervention after a 12-14 hour fast, in a 0.1% EDTA tubes for serum lipoproteins. Volunteers were at baseline randomly assigned to groups of three. Control group volunteers were given antioxidant nutraceuticals ((Forever living product) 1 capsule per day (containing vitamin E 10mg, vitamin C 60mg, and  $\beta$ - carotene 2,000mcg of vitamin A). The treatment group volunteers were given antioxidant functional foods of equivalent vitamin composition (oranges 100g, carrots 100g, and soybean drink 75 cl and 1 heaped table spoon of soybean powder 35g/day). The placebo group volunteers were giving clean drinking water (ordinary Swan table water 75 cl with no antioxidants) for twelve months.

### 2.4 Lipids and Lipoprotein Analysis

Total cholesterol was determined with lithium hyperinized

plasma on Reflotron system [18]. Triglycerides concentrations were determined with reflatron in Lithium hyperinized plasma (based on GPO-PAD method, Tietz). Low density lipoprotein cholesterol (LDL-C) was calculated with Chawla [19] equation.  $LDLc = \text{Total cholesterol} - (\text{Triglyceride}/2.2 - \text{HDL})$  mmol/L or  $\text{Total cholesterol} - (\text{triglyceride}/5 - \text{HDL})$  mg/dL. Reference values were taken from Third Report of the National cholesterol Education, Evaluation Programme (NCEP) [18]. High density lipoprotein cholesterol were determined with reflatron in Lithium hyperinized plasma based on precipitation method [18] expert panel on Detection, Evaluation and Treatment of High blood cholesterol in Adult in 2001 [18].

## 2.5 Statistical Analysis

Pair-wise comparisons of the means were validated using Analyze-It for Microsoft Excel Version 10, where a p-value < 0.05 was considered statistically significant. Further Post hoc test: the Fischer's least significant difference (LSD) was used together in the analysis of variance (ANOVA).

## 3. Result

Results of the plasma lipoproteins of the samples analyzed are presented in Table 1. There is a positive decrease in the cholesterol levels after antioxidant dietary intervention. There was a significant difference in the Cholesterol (CHOL) of different ages of male/female after treatment with LSD-Male (p-value) 0.59 (< 0.0001), and LSD-Female (p-value) 0.66 (0.0004). No significant difference was observed in the Cholesterol (CHOL) of different ages of male/female after treatment with LSD-Gender (p-value) 0.67 (0.1669).

The result showed a positive decrease in triglyceride after antioxidants intervention. No significant difference was noted in the triglyceride (TRIG) level on ages of male/female after treatment with LSD-Gender (p-value) 0.42 (0.3604), LSD-Male (p-value) 0.32 (0.090), and LSD-Female (p-value) 0.33 (0.2862).

Results of low density lipoproteins showed a significant decrease after antioxidant intervention. No significant difference was noted in the plasma Low density lipoprotein cholesterol (LDL-C) in all the ages of male/female after dietary intervention with LSD-Gender (p-value) 0.74 (0.6788) and on females with LSD-Female (p-value) 0.54 (0.1139) but a significant difference on the LDL-C of the male with LSD-Males (p-value) 0.49 (0.0291).

High density lipoproteins showed positive increase after dietary intervention. There was no significant difference in high density lipoprotein (HDL-C) on ages of male/female with LSD-Gender (p-value) 0.77 (0.6432). However, a significant difference in HDL-C levels among the males with LSD-Male (p-value) 0.47 (0.0073), and a significant difference in HDL-C among the females with LSD-Female (p-value) 0.30 (0.0007).

The results of CHOL after treatment showed that their concentrations in the blood are lower at age range of 50-59 in males (Male =  $6.90 \pm 0.07$ ) than in females (Female =  $6.60 \pm 0.04$ ) and higher in males at age range of 30-39 in males (Males =  $5.72 \pm 0.02$ ), 40-49 (Males =  $6.90 \pm 0.07$ ), 60-69 (Males =  $7.85 \pm 0.02$ ) and 70-79 (Males =  $7.90 \pm 0.02$ ) than in females (Female =  $6.84 \pm 0.05$ ), (Female =  $6.60 \pm 0.04$ ), (Female =  $8.17 \pm 0.01$ ) and (Female =  $8.20 \pm 0.00$ ) of the same age ranges respectively.

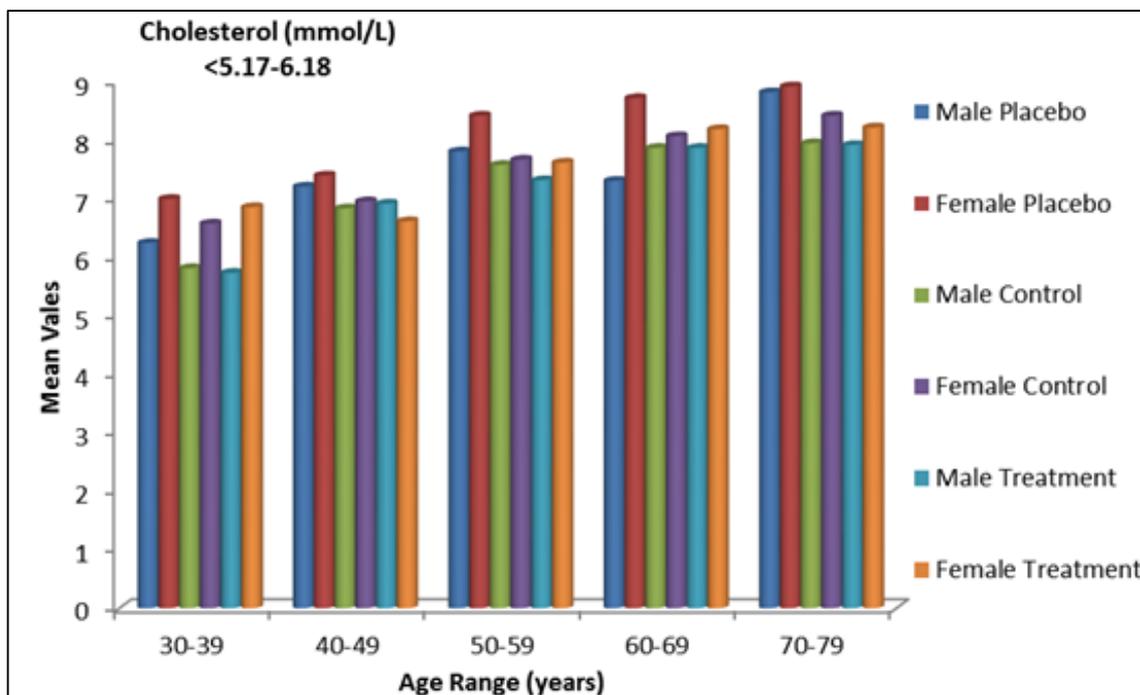


Fig 1: Effect of antioxidants Cholesterol (CHOL) after twelve months treatment

The triglyceride level after treatment showed higher concentrations males at all the age ranges 30-39 in males (Males =  $2.51 \pm 0.07$ ), 40-49 (Males =  $2.73 \pm 0.08$ ), 50-59 (Males =  $2.80 \pm 0.06$ ), 60-69 (Males =  $2.82 \pm 0.03$ ) and 70-

79 (Males =  $2.83 \pm 0.01$ ) than in females of all age ranges (Female =  $2.35 \pm 0.05$ ), (Female =  $2.45 \pm 0.05$ ), (Female =  $2.50 \pm 0.03$ ), (Female =  $2.55 \pm 0.01$ ) and (Female =  $2.58 \pm 0.00$ ) respectively.

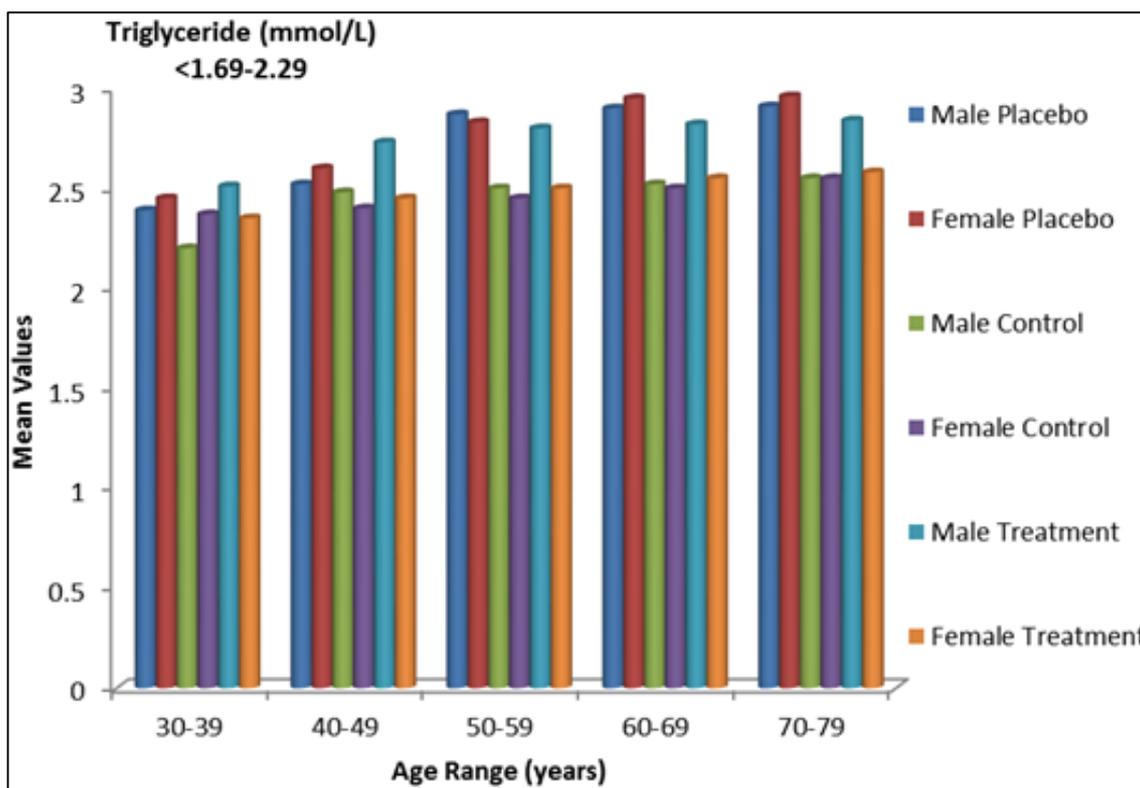


Fig 2: Effect of antioxidants on Triglyceride after twelve months treatment

The LDL-C level after treatment showed higher concentrations males at all the age ranges 30-39 in males (Males =  $3.36 \pm 0.10$ ), 40-49 (Males =  $3.71 \pm 0.08$ ), 50-59 (Males =  $3.95 \pm 0.05$ ), 60-69 (Males =  $4.00 \pm 0.03$ ) and 70-

79 (Males =  $4.20 \pm 0.02$ ) than in females of all age ranges (Female =  $3.25 \pm 0.07$ ), (Female =  $3.60 \pm 0.06$ ), (Female =  $3.80 \pm 0.05$ ), (Female =  $3.90 \pm 0.01$ ) and (Female =  $4.05 \pm 0.00$ ) respectively.

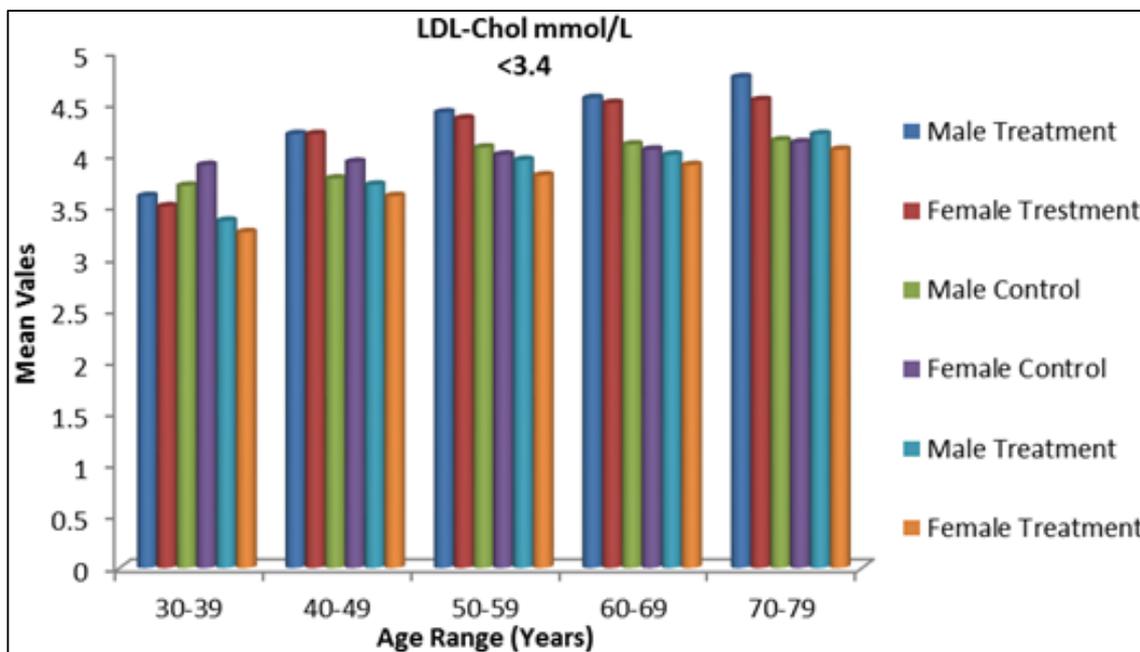


Fig 3: Effect of antioxidants on LDL-C after twelve months treatment

The HDL-C level after treatment showed higher concentrations males at all the age ranges 30-39 in males (Males =  $1.93 \pm 0.03$ ), 40-49 (Males =  $1.24 \pm 0.04$ ), 50-59 (Males =  $1.02 \pm 0.03$ ), 60-69 (Males =  $1.00 \pm 0.02$ ) and 70-79 (Males =  $0.93 \pm 0.01$ ) than in females of all age ranges

(Female =  $1.65 \pm 0.04$ ), (Female =  $1.05 \pm 0.03$ ), (Female =  $0.95 \pm 0.03$ ), (Female =  $0.90 \pm 0.02$ ) and (Female =  $0.90 \pm 0.00$ ) respectively. HDL-C of all the sexes reduced with increase in age.

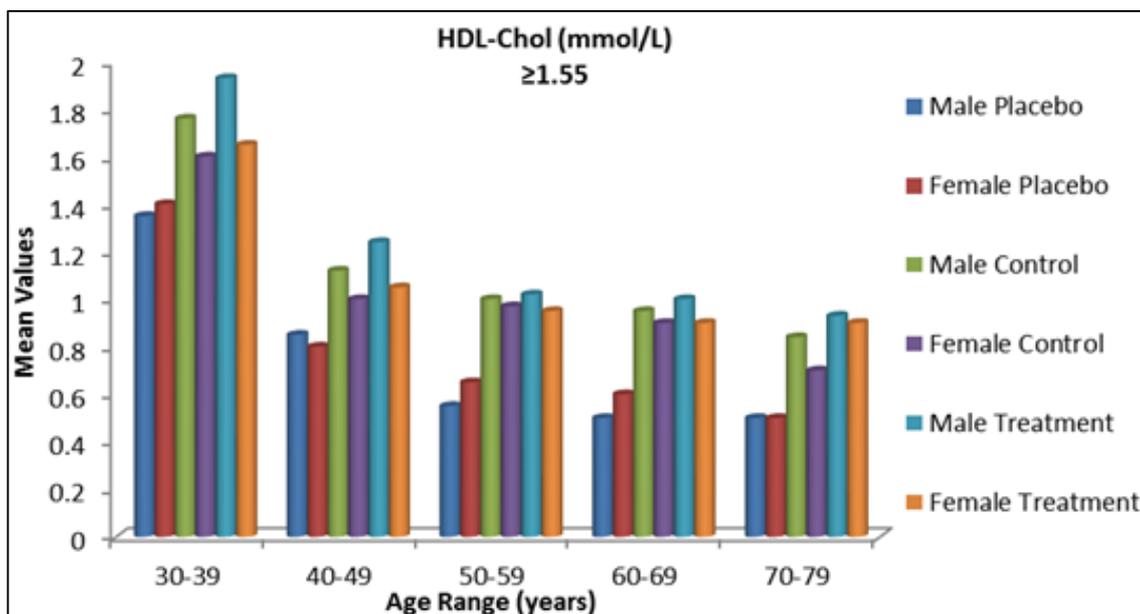


Fig 4: Effect of antioxidants on HDL-C after twelve months treatment

#### 4. Discussion

The process of assimilation of lipids from the diet, their synthesis in the liver, adipose and peripheral tissues are inter-related and the levels of various lipoproteins in blood are well regulated. However, age, genetic and environmental factors (diet, smoking etc) can disturb the balance and lead to alterations in blood levels of one or more of the lipoproteins. Alteration in lipoprotein in blood is associated with arterogenesis and thrombogenesis which in turn may affect the immune level [20]. The blood levels of certain lipoproteins can serve as a diagnostic tool. High serum levels of cholesterol can cause diseases and death by

contributing to the formation of atherosclerosis plaques in arteries throughout the body [21]. This excess cholesterol is present in the form of LDLc so called ‘‘bad cholesterol’’. The ratio of cholesterol in the form of HDLc sometimes refer as ‘‘good cholesterol’’, to that of LDLc can be used to evaluate the susceptibility to the development of heart disease [22].

The current results for lipoproteins and effect of antioxidants revealed three main findings; Firstly, The antioxidant intervention positively influenced the lipoproteins compare to the placebo group with the antioxidant functional foods showing a better influence than

the antioxidant nutraceuticals. Secondly, the females tend to have significantly higher HDL-cholesterol, triglycerides, and total cholesterol compared to the males, while males have higher LDLc compare to females. Lastly, the age group of the subjects who achieved higher serum lipid levels increased with increase in age with age 70-79 having the highest value of total chol, tig, LDLc, and uric acid while the HDL reduces with increase in age. Cholesterol is carried through the blood; Packaged in LDL particles, LDL is responsible for depositing cholesterol in walls of the arteries. These deposits from the fatty plaques that eventually narrow the arteries and could possibly lead to a heart attack. Scientists now know that before LDLc can have this effect; it first has to be modified by a free radical to form an oxidized LDL. Free radicals are unstable because they are missing electrons, which must be replaced. So they seek out other compounds in the body and steal electrons to restore stability. If the compound giving up its electrons is the fat or protein in an LDLc molecule, the result is the formation of fatty lesions in the walls of the blood vessels – the hallmark of arteriosclerotic diseases.

Diet, weight, physical activity, age and gender have been known to affect the lipoproteins especially the cholesterol level. As men and women get older their cholesterol levels rise mainly in the female after menopause, the cholesterol of the female is higher than that of the male. This may be due to estrogen level. Estrogen is associated with higher levels of HDLc and withdrawal of this natural estrogen by menopause leads to lower level of HDLc thus increasing the LDLc which is a risk factor of heart disease. Estrogen is present in significant amounts in both men and women. They are present in significantly higher amount in women after menarche until menopause. The primary function of estrogen is development of female secondary sexual characteristic (breast, menstrual cycle, endometrium etc. In males estrogen helps in maturation of the sperm and maintenance of a healthy libido. Oxidation of LDL particles is believed to be a significant contributor to atherogenesis. Susceptibility to oxidation varies from LDL particles to LDL particles and is determined by, among other factors, particle size (small, dense LDL particles are more easily oxidized than are large particles), particle composition (e.g. high content of polyunsaturated fatty acids increases susceptibility to oxidation), and level of antioxidants such as vit E within the particle. HDL protects LDL from oxidation, but is itself susceptible to oxidation. Such oxidation has been shown to increase HDL clearance and could be pro-atherogenic. Estrogen share structural similarities with vit E and other lipophilic antioxidants and are thus able to function as scavengers for lipid peroxy radicals and interrupt the chain reaction of lipid peroxidation. Estrogen protects HDL from oxidation, an effect that should preserve the beneficial function of HDL, including the protection of LDL from oxidation.

A plausible explanation suggest that the physiological and pathological roles of plasma lipoproteins likely change with age, data obtained support the hypothesis that “continuous reshaping in lipid physiology occurs with age and is a critical factor for survival and successful aging<sup>[6]</sup>. A major consequence of this reshaping in lipid is that changes in the serum/plasma level of proteins, lipids, and lipoproteins are considered risk factors for atherosclerotic vascular diseases with advancement in age. Thus, this study is in line with general hypothesis that a continuous remodelling develops

with time as a result of the continuous adaptation to changes occurring in the body with age in response to internal and external damaging agents<sup>[21]</sup>.

High levels of LDLc, and Trig are complicated structures their oxidative modification has been reported to be involved in cardiovascular diseases. The oxidative modifications of LDLc are associated with many degenerative diseases which are associated with ageing<sup>[23]</sup>, The study confirmed that Serum level varies in different ages with cholesterol, LDLc and triglyceride levels of the young being significantly lower than that of healthy elderly, whereas the HDLc was higher in the young subject. Physiologically low estrogen levels associated with menopause was proved to minimize LDL clearance by the liver and hence increase LDL-cholesterol in postmenopausal women. Ageing in females was proved to increase high density lipoprotein subfraction 3 (HDL3) cholesterol and decrease concentrations of high density lipoprotein subfraction 2 (HDL2) cholesterol. The high HDL-cholesterol in the females group of the current study is possibly secondary to increased HDL3 fraction.

Figures (1, 2, 3, 4) showed that the antioxidant functional foods positively affected the lipoproteins more than the antioxidant nutraceuticals. The antioxidant functional foods dietary intervention may have affected the lipoproteins positively because of their components: The soybean taken among the functional foods contains concentration of isoflavonoids phytoestrogen which is a group of biphenolic compounds with weak estrogen activity. Isoflavonoids in LDL particles have protective effect by reducing or eliminating concentrations of preformed lipid hydroperoxides which enhances initiation rate of oxidation. Thus, the soybean isoflavonoids may have lowered initiation rates because of reduced hydroperoxide formation *in vivo*; The fact that the binding of  $cu^{2+}$  to apolipoprotein B in LDL initiates lipid peroxidation. But Isoflavonoid phytoestrogens known to bind tightly to soybean protein and could be attached to binding sites of apolipoprotein B normally occupied by  $cu^{2+}$ , thus inhibiting formation of lipid hydroperoxide.

The soybean also contains vitamin E which is a good antioxidant it is an oil soluble vitamin is absorbed in fat globules (chylomicrons) that travel through lymphatic system of the small intestine and into the general blood circulation within the body. Vit E is highly lipophilic found in membranes of lipoproteins where it reacts at considerable rates with variety of free radical species with emphasis on lipid peroxy radicals formed during lipid peroxidation. Vitamin E generally consists of phenols or aromatic amines. The initial step involves a very rapid transfer of phenolic hydrogen to the recipient free radical with the formation of a phenoxyl radical ( $\alpha$ -tocopheroxy radical) from vitamin E. The phenoxyl radical is resonance stabilized and is relatively underactive towards lipid or oxygen. It does not therefore continue the chain. However, the phenoxyl radical ( $\alpha$ -Toc $\bullet$ ) is no longer an antioxidant and to maintain the antioxidant properties of membranes, it must be recycled or repaired (i.e. reconverted to vitamin E) because the amount of vitamin E present in membranes can be several thousand-fold less than the amount of potentially oxidizable substrate. Water-soluble vitamin C is the popular candidate for this role, but thiols and particularly GSH can also function in this role in vitro. The vitamin C in the oranges helps to reactivate the phenoxyl radical from vitamin E. Vitamin C

or ascorbic acid (AH<sup>-</sup>) is a water-soluble vitamin that reacts with several radical species producing semidehydroascorbic acid or ascorbyl radical (A<sup>•-</sup>). Vitamin C is a powerful antioxidant because it can donate a hydrogen atom and form a relatively stable ascorbyl free radical (i.e. L-ascorbate anion). As a scavenger of ROS, ascorbate has been shown to be effective against the superoxide radical anion, hydrogen peroxide, the hydroxyl radical, and singlet oxygen. It is a powerful antioxidant because it can donate a hydrogen atom and form a relatively stable ascorbyl free radical (i.e. L-ascorbate anion). The ascorbyl free radical can be converted back to reduced ascorbate by accepting another hydrogen atom or it can undergo further oxidation to dehydroascorbate. Dehydroascorbate is unstable but is more fat soluble than ascorbate and is taken up 10–20 times more rapidly by erythrocytes, where it will be reduced back to ascorbate by GSH or NADPH from the hexose monophosphate shunt. Vit C may have exerted a uricosuric effect, increasing glomerular filtration and/or competition for renal absorption by vit C and uric acid being reabsorbed via anoin-exchange transport at proximal tubules. This increase in glomerular filtration include the fact that vit C has an antioxidant effect that may reduce micro vascular ischemia in glomeruli and leads to increase blood flow at the site, dilation of afferent arterioles, and competition for reabsorption with ions such as sodium and potassium that exert osmotic effects.

The dietary intervention with carrot as part of the functional food is rich in  $\beta$ -carotene.  $\beta$ -Carotene does not have the structural features commonly associated with chain-breaking antioxidants. In fact, the extensive system of conjugated double bonds in the molecule imparts pro-oxidant character, making it very susceptible to attack by the addition of peroxy radicals. The resulting carbon-centered radical,  $\beta$ -car, reacts rapidly and reversibly with oxygen to form a new, chain-carrying peroxy radical,  $\beta$ -car-OO<sup>•</sup>. Indeed,  $\beta$ -carotene readily undergoes autoxidation. The reactivity of  $\beta$ -carotene towards peroxy radicals and the stability of the resulting carbon-centered radical,  $\beta$ -car, are two important features that give the molecule antioxidant capability. Firstly, the reactivity of  $\beta$ -carotene means that it has the potential to compete, even when present in low concentration, for peroxy radicals derived from other lipid molecules (e.g., polyunsaturated phospholipid). Secondly, the stability of the carbon-centered radical,  $\beta$ -car That is formed means that at sufficiently low O<sub>2</sub> partial pressures

this form can predominate over the chain carrying peroxy radical form. In addition,  $\beta$ -car The radical can be removed from the system in a reaction with another peroxy radical. The reactivity of  $\beta$ -carotene towards peroxy radicals and the stability of the resulting carbon-centered radical,  $\beta$ -car, are two important features that give the molecule antioxidant capability.

The combination of functional foods in group 3 (soybean, oranges and carrots) and nutraceuticals in group 2 (vitamin C, vitamin E, and B-carotene of forever living product) as dietary intervention may have affected the positive effect on the lipoproteins by synergistic effect of the antioxidants vitamin E, vitamin C and  $\beta$ -carotene. Probably, due to nutrient-nutrient interaction and presence of other antioxidants that are present in the functional foods group it has more positive influence than the nutraceuticals group on the lipoproteins. Previous studies support the result that 4-tocopherol, tomatoes, vitamin C, B-carotene, and phenol compounds inhibit LDLc oxidation and protect lymphoid cells against cytotoxic effects of oxidized LDLc [22, 27].

A plausible explanation suggest that the physiological and pathological roles of plasma lipoproteins likely change with age [8, 28], study support the hypothesis that “continuous reshaping in lipid physiology occurs with age and is a critical factor for survival and successful aging” [25]. A major consequence of this reshaping in lipid is that alterations in the serum/plasma level of proteins, lipids, and lipoproteins are considered risk factors for atherosclerotic vascular diseases with advancement in age [3, 26, 29]. The study is in line with general hypothesis that a continuous remodelling develops with time as a result of the continuous adaptation to changes occurring in the body with age in response to internal and external damaging factors [30].

## 5. Conclusion

This study showed a significant improvement on the lipoproteins with antioxidant dietary intervention. A plausible explanation suggests that the physiological and pathological roles of plasma lipoproteins likely change with age [22]. Thus, the study is in line with general hypothesis that a continuous remodelling develops with time as a result of the continuous adaptation to changes occurring in the body with age in response to internal and external damaging agents [19]. Data of the present study suggest that the dietary intervention of antioxidant nutraceuticals and functional foods intervention is likely to affect lipoprotein levels.

**Table 1:** Effect of antioxidant nutraceuticals and functional foods on Serum Lipoprotein after twelve months treatment

Group	Age(years)	CHOL (mmol/L)	TRIG (mmol/L)	LDLc (mmol/L)	HDLc (mmol/L)
		Male	Male	Male	Male
Placebo (n=31)	30-39(n=08)	6.23±0.05	2.39±0.04	3.60±0.07	1.35±0.03
	40-49(n=09)	7.19±0.07	2.52±0.05	4.20±0.05	0.85±0.03
	50-59(n=06)	7.79±0.06	2.87±0.03	4.41±0.04	0.55±0.02
	60-69(n=05)	7.29±0.05	2.90±0.02	4.55±0.03	0.50±0.02
	70-79(n=03)	8.80±0.01	2.91±0.01	4.75±0.02	0.50±0.01
Control (n=32)	30-39(n=10)	5.80±0.07	2.20±0.06	3.70±0.10	1.76±0.05
	40-49(n=10)	6.82±0.08	2.48±0.05	3.77±0.07	1.12±0.05
	50-59(n=06)	7.56±0.05	2.50±0.03	4.07±0.05	1.00±0.04
	60-69(n=05)	7.85±0.03	2.52±0.05	4.10±0.03	0.95±0.04
	70-79(n=01)	7.93±0.00	2.55±0.00	4.14±0.00	0.84±0.02
Treatment (n=31)	30-39(n=09)	5.72±0.02	2.51±0.07	3.36±0.10	1.93±0.03
	40-49(n=10)	6.90±0.07	2.73±0.08	3.71±0.08	1.24±0.04
	50-59(n=07)	7.30±0.05	2.80±0.06	3.95±0.05	1.02±0.03
	60-69(n=03)	7.85±0.02	2.82±0.03	4.00±0.03	1.00±0.02
	70-79(n=02)	7.90±0.02	2.83±0.01	4.20±0.02	0.93±0.01

		Female	Female	Female	Female
Placebo (n=19)	30-39(n=07)	6.98±0.07	2.45±0.06	3.50±0.08	1.40±0.03
	40-49(n=06)	7.38±0.06	2.60±0.06	4.20±0.07	0.80±0.04
	50-59(n=03)	8.40±0.04	2.83±0.05	4.35±0.05	0.65±0.02
	60-69(n=02)	8.70±0.02	2.95±0.03	4.50±0.01	0.60±0.01
	70-79(n=01)	8.90±0.00	2.96±0.00	4.53±0.00	0.50±0.00
Control (n=18)	30-39(n=05)	6.56±0.05	2.37±0.06	3.90±0.07	1.60±0.03
	40-49(n=05)	6.94±0.04	2.40±0.05	3.93±0.06	1.00±0.03
	50-59(n=04)	7.65±0.03	2.45±0.04	4.00±0.05	0.97±0.02
	60-69(n=02)	8.05±0.02	2.50±0.02	4.05±0.01	0.90±0.01
	70-79(n=02)	8.40±0.01	2.55±0.01	4.12±0.01	0.70±0.01
Treatment (n=19)	30-39(n=06)	6.84±0.05	2.35±0.05	3.25±0.07	1.65±0.04
	40-49(n=06)	6.60±0.04	2.45±0.05	3.60±0.06	1.05±0.03
	50-59(n=04)	7.60±0.03	2.50±0.03	3.80±0.05	0.95±0.03
	60-69(n=02)	8.17±0.01	2.55±0.01	3.90±0.01	0.90±0.02
	70-79(n=01)	8.20±0.00	2.58±0.00	4.05±0.00	0.90±0.00
LSD- Gender (p-value)		0.67 (0.1669*)	0.42 (0.03604*)	0.74 (0.6788*)	0.77 (0.6432*)
LSD-Male (p-value)		0.59 (< 0.0001)	0.32 (0.090*)	0.49 (0.0291)	0.47 (0.0073)
LSD-Female (p-value)		0.66 (0.0004)	0.33 (0.2862*)	0.54 (0.1139*)	0.30 (0.0007)

\* Not Significant

## 6. Acknowledgment

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## 7. Conflict of Interest

The authors declare no conflict of interest.

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