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Plasma Phospholipids, including Plasmalogens, after Consumption of Diets Enriched in Long-chain n-3 Fatty Acids

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The level of long-chain n-3 fatty acids in chicken and pork can be increased by changing the diet of the animals. Increased levels of these essential fatty acids improve cardiovascular health in humans. The purpose of this study was to study the effects of the consumption of pork and chicken enriched in docosahexaenoic acid (DHA) on plasma lipids. The consumption of these products decreased the levels of two cardiovascular risk factors, LDL-cholesterol and triacylglycerols, in the plasma of female college students. The effect on LDL-cholesterol differed from that of fish oil, which does not affect the level of LDL-cholesterol. The proportions of DHA in the triacylglycerols and the glycerophospholipids were increased markedly. The greatest changes in the glycerophospholipids were in the ether types of the ethanolamine glycerophospholipids. Dietary DHA appears to be incorporated preferentially into the plasma ethanolamine plasmalogens, which can act as antioxidants. This agrees with our hypothesis that DHA stimulated the transcription of the genes for peroxisomal enzymes that are required for plasmalogen synthesis.

Keywords: Choline glycerophospholipids, Ethanolamine glycerophospholipids, Plasmalogens, Alkylacyl glycerophospholipids, n-3 fatty acids, Omega-3 fatty acids, Linoleic acid, Linolenic acid, Arachidonic acid, Eicosapentaenoic acid, Docosahexaenoic acid, LDL-cholesterol, Triacylglycerols, Plasma, Ether phospholipids, Chicken, Pork

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Introduction

Consumption of foods containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) prevents sudden death from myocardial infarction (Siscovick *et al.*, 1995; Leaf *et al.*, 1999). These fatty acids exert antithrombotic effects through alterations of blood lipids (Harris, 1989; Leaf *et al.*, 1999) and lipoproteins (Fisher *et al.*, 1998). In addition to their antithrombotic effects, n-3 fatty acids also have antiarrhythmic effects in the heart (Leaf *et al.*, 1999) and therefore play an important role in their overall benefit to cardiovascular health.

The n-3 Fatty acids are essential fatty acids, necessary from conception through pregnancy, and infancy to adulthood (Connor, 2000). They cannot be synthesized by humans and must be obtained from the diet. A typical Western diet has 20-fold more linoleic acid, a precursor for arachidonic acid (AA), than a-linolenic acid, a precursor for docosahexaenoic acid (DHA) (Simopoulos, 1991; Galli *et al.*, 1994). In order to achieve a better balance between DHA and AA in the Western diet, strategies have been developed to alter the DHA content of the lipids in animal products through dietary changes (Caston and Leeson, 1990; Jiang and Sim, 1992; Hargis and Van Elswyk, 1993). The fatty acid composition of plasma triglycerides reflects the composition of the most recent meals (Yeo and Holub, 1990). This composition has been used to monitor dietary changes in subjects following special diets. On the other hand, the composition of plasma phospholipids and cholesterol esters reflects the medium-term, weeks to months, dietary intake of fatty acids (Zock *et al.*, 1997). The fatty acid composition of milk, eggs, and chicken meat can be changed by dietary manipulation (Hargis and Van Elswyk, 1993; Farrell, 1998; Sim, 1998; Van Elswyk *et al.*, 1998; Wright *et al.*, 1998; Leskanich and Noble, 1990).

Diets enriched in DHA produce substantial increases in plasma and tissue DHA contents with variable incorporation

into different phospholipid classes in different tissues (Yeo *et al.*, 1989a; Choi *et al.*, 1993; Froyland *et al.*, 1996). There are complex interactions of n-6 and n-3 fatty acids in plasma and cellular lipids after dietary changes. For example, n-3 fatty acids interfere with several steps of the metabolism of arachidonic acid (Weaver and Holub, 1985; Yeo *et al.*, 1999). Studies on the effect of n-3 fatty acids on serum total and low-density-lipoprotein (LDL) cholesterol have been inconsistent; however, a mild effect elevating high-density-lipoprotein (HDL) cholesterol has been reported (Weaver and Holub, 1988; Harris, 1989). The enrichment of DHA in milk, eggs, chicken meat, and pork has been described (Farrell, 1998; Horrocks and Yeo, 1999). Consumption of these products decreased the levels of plasma LDL-cholesterol in young women (Horrocks and Yeo, 1999). Nothing has been reported on the effects of DHA-enriched chicken or pork on plasma phospholipids. In the present study we report the effects of DHA-enriched chicken and pork on plasma lipids, especially the ether types of choline and ethanolamine glycerophospholipids, in normal human subjects.

Materials and Methods

Subjects Twenty women, aged 20 years, were recruited from entering students at Kyungpook National University, Taegu, Korea. Each subject consumed 200 g chicken breast meat daily for 4 weeks in the autumn. After consuming a normal diet, each subject then consumed 200 g pork loin daily for 4 weeks during the following spring. The Edison™ chicken breast meat and pork loin were enriched with n-3 fatty acids. The meats were produced using a feed supplement made by the Edison Company, Ltd., Taegu, Korea. The feed contained fish oil and various antioxidants including vitamin E.

Methods Blood samples were collected before and after the experimental diets. Blood was drawn from the antecubital vein into bags containing acid-citrate-dextrose. The plasma from each donor was removed by centrifugation. Lipoprotein cholesterol was measured with the Cholestech LDX system (Cholestech Corporation, Hayward, USA).

Plasma samples from human subjects were homogenized in chloroform/methanol (2 : 1, v/v) with a Polytron homogenizer. The total lipids were extracted (Folch *et al.*, 1957). Lipid extracts were separated into individual phospholipid fractions by thin-layer chromatography (TLC) on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany) developed with chloroform/methanol/acetic acid/water (50 : 37.5 : 3.5 : 2, by volume) (Yeo and Holub, 1990). The bands corresponding to the individual phospholipids were identified with phospholipid standards (Sigma Chem. Co., St. Louis, USA). The system for separation of triacylglycerols (TAG) employed TLC developed with heptane/isopropyl ether/acetic acid (60 : 40 : 3, by volume). The various lipid bands were visualized under ultraviolet light after spraying with 6-p-toluidine-2-naphthalene sulfonic acid (TNS) (Eastman Kodak Co., Rochester, USA) and exposure to ammonium hydroxide fumes.

For studies on the types of the choline and ethanolamine glycerophospholipids, the phospholipid bands from two TLC plates were pooled and eluted. The diacyl, alkylacyl, and alkenylacyl types were isolated as their acetate derivatives, following phospholipase C hydrolysis and acetylation, as described previously (Yeo *et al.*, 1989b). Following phospholipase C hydrolysis, the resulting diradylglycerols were acetylated with 1 ml of acetic anhydride and five drops of pyridine at 80°C for 1 h. The diradylglycerol acetates were separated by TLC using a solvent system of petroleum ether/diethyl ether/acetic acid (90 : 10 : 1, by volume), followed by a second development in toluene. The three diradylglycerol acetates were visualized under ultraviolet light after exposure to TNS spray. The fractions were scraped from the plates and transmethylated with 6% sulfuric acid in methanol at 90°C for 1 h. Known amounts of monopentadecanoin (Nu Chek Prep, Elysian, USA) were used as an internal standard to give subsequent mass determinations of the fatty acyl chains. The fatty acid methyl esters from the alkenylacyl type were separated from the aldehyde derivatives by TLC using toluene as a solvent. The fatty acid compositions of the three diradylglycerol acetates subclasses were determined with a Hewlett-Packard Model 5890 A gas chromatograph equipped with a DB-225 megabore column 30 m in length and 0.53 mm internal diameter (Chromatographic Specialties, Brockville, Ontario, Canada). The fatty acid methyl esters were identified by comparison of their retention times with those of known standards (Nu Chek Prep). The flow rate of the nitrogen was 36 ml/min and the oven temperature during these isothermal runs was 210°C. Since the acyl group compositions of the two ether lipid types are derived only from the sn-2 position, whereas the acyl groups from the diacyl type represent the sn-1 plus sn-2 positions, the molar amounts of the total methyl esters derived from the two ether lipid types were multiplied by 2 to calculate the amounts of these three types. The composition results, expressed as mol % of the total fatty acids, were analyzed by one-way analysis of variance for comparisons between the two groups.

Results

Lipids in Meats The feeding of both broiler chickens and swine with feed supplemented with fish oil and antioxidants produced meat with a lower content of fat (Table 1). The lipid content of the leg meat and the breast meat was 13% and 26% less than that in the control meats, respectively. Both meats

Table 1. Total lipid content of chicken and pork meats.

	Control	Enriched
Chicken leg	2.05	1.79
Chicken breast	1.05	0.78
Pork loin	3.84	3.30
Pork belly	3.71	3.65

Values are expressed as g per 100 g of tissue. Control meats were from animals fed with commercial feedstuffs in Korea. Enriched meats were Edison™ products enriched with omega-3 fatty acids.

Table 2. Polyunsaturated fatty acids in chicken lipids.

PUFA	Leg		Breast	
	Control	Enriched	Control	Enriched
18:2 n-6	24.7	20.4	24.7	19.3
18:3 n-3	1.6	3.0	1.2	2.2
20:4 n-6	0.9	2.0	1.1	2.4
20:5 n-3	0.1	1.7	0.1	2.0
22:6 n-3	0.2	5.9	0.4	8.7
n-6/n-3	13.6	2.1	15.2	1.7

Values represent means, mol %, for 10 chickens per group fed for 3 weeks with the commercial (control) or Edison™ feed.

from supplemented broilers were very markedly enriched in EPA and DHA, which constituted more than 7% of the total fatty acids (Table 2). The ratio of n-6 to n-3 fatty acids was also very markedly decreased in the supplemented broilers.

The pork loin from supplemented animals contained 14% less lipid than the controls (Table 1). The ham, loin, shoulder, and neck meats from supplemented swine were enriched several-fold with ALA, EPA, and DHA (Table 3). These three n-3 fatty acids comprised less than 5% of the total fatty acids. The low level of AA was decreased in all types of pork from the supplemented swine. The ratio of n-6 to n-3 fatty acids was also very markedly decreased in the supplemented swine. No differences were found in the taste of control and n-3-enriched chicken or pork.

Plasma Neutral Lipids After four weeks of consuming 200 g of enriched meat daily, the plasma from the female university students contained lower levels of LDL-cholesterol (Table 4). The reduction was 20% with chicken and 16% with pork. The level of HDL-cholesterol was the same before and after the experimental period. The levels of plasma triacylglycerols were reduced by 27 and 25%, respectively, but the variance was too large for statistical significance with the pork experiment. After the completion of the chicken study, the subjects consumed a diet of their choice for more than six months before their participation in the pork study. During that time, their plasma lipids returned to their previous values, except for LDL-cholesterol, which rose to a value 29% higher than when they entered the university. The enriched meats were effective in reducing the LDL-cholesterol levels from either starting point.

The polyunsaturated fatty acid composition of the plasma triacylglycerols reflected that of the meats consumed (Table 5). The levels of n-3 fatty acids were increased after the consumption of enriched meats. During the academic year when enriched meats were not being consumed, the levels of EPA and DHA returned to pre-enrichment levels.

Table 3. Polyunsaturated fatty acids in pork lipids.

PUFA	Neck		Shoulder		Loin		Ham	
	Control	Enriched	Control	Enriched	Control	Enriched	Control	Enriched
18:2 n-6	14.7	13.0	15.7	12.1	13.2	13.0	13.4	15.7
18:3 n-3	0.5	2.2	0.9	2.2	0.3	2.6	0.4	2.3
20:4 n-6	2.8	1.1	2.5	1.1	4.4	1.2	3.2	0.8
20:5 n-3	0.2	0.9	Tr	1.0	0.2	1.0	0.1	0.8
22:6 n-3	0.2	1.2	0.1	1.1	0.2	1.5	0.1	1.2
n-6/n-3	13.1	3.3	11.1	3.0	12.7	2.8	18.3	4.0

Values represent means, mol %, for 20 hogs per group fed for 4 weeks with the commercial (control) or Edison™ feed.

Table 4. Levels of various lipid classes and glucose in plasma before and after eating chicken or pork enriched with omega-3 fatty acids.

Class	Chicken		Pork	
	Before	After	Before	After
Total cholesterol	155.2±8.8	132.1±8.6	180.4±11.4	157.1±10.3
Triacylglycerols	100.3±13.9	73.1±7.4[a]	96.0±16.5	72.1±11.4
Glucose	94.0±1.3	88.7±1.9	93.3±1.8	89.0±1.7
HDL-cholesterol	49.9±4.4	49.4±4.7	52.6±4.7	50.9±5.2
LDL-cholesterol	85.2±6.4	68.1±4.4[a]	110.0±8.0	91.8±7.3[a]
TC/HDL-cholesterol	3.2±0.2	2.8±0.2	3.8±0.3	3.3±0.2

Values represent mg/dl, means±S.E. for 20 female subjects, aged 20, per group.
Each subject ate 200 g chicken breast or pork loin daily for 4 weeks. TC/HDL-cholesterol is the ratio of total cholesterol to HDL-cholesterol.
[a]Indicates significant difference from the before eating group, $P<0.05$.

Table 5. Polyunsaturated fatty acids (PUFA) in human plasma triacylglycerols before and after eating chicken or pork enriched with long-chain n-3 fatty acids.

PUFA	Chicken		Pork	
	Before	After	Before	After
18: 2n-6	21.0±1.3	17.6±3.4	17.2±1.2	15.0±1.2
18: 3n-3	0.8±0.2	0.9±0.1	1.2±0.3	2.7±0.3[b]
20: 4n-6	1.8±0.3	1.5±0.7	0.9±0.2	0.7±0.2
20: 5n-3	0.2±0.1	0.5±0.1	0.1±0.0	0.6±0.1[a]
22: 6n-3	0.5±0.2	1.5±0.5[a]	0.5±0.2	2.2±0.8[a]

Values represent means±S.E. for 20 human subjects (female aged 20)/group.
Each subject had 200 g chicken breast or pork loin daily for 4 weeks.
[a]Indicates significant difference from the before eating group, P<0.01.
[b]Indicates significant difference from the before eating group, P<0.05.

Table 6. Composition of the plasma choline and ethanolamine glycerophospholipids before and after eating chicken or pork enriched with omega-3 fatty acids.

Phospholipid type	Chicken		Pork	
	Before	After	Before	After
Choline Gpl				
Alkenylacyl-GPC	1.4±0.4	1.6±0.3	2.2±0.5	1.8±0.3
Alkylacyl-GPC	6.9±0.6	4.1±0.7	9.0±2.2	4.2±0.8[a]
Diacyl-GPC	91.6±3.2	94.3±1.2	88.8±1.8	94.0±1.4
Ethanolamine Gpl				
Alkenylacyl-GPE	17.2±1.7	15.6±1.6	19.2±1.6	21.0±1.6
Alkylacyl-GPE	8.2±0.5	8.2±1.9	15.2±1.5	12.5±0.9
Diacyl-GPE	74.6±3.5	75.8±5.1	65.6±2.0	66.6±1.6

Values represent means±S.E. for 20 female subjects, aged 20, per group.
Each subject ate 200 g chicken breast or pork loin daily for 4 weeks.
Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; Gpl, Glycerophospholipids.
[a]Indicates significant difference from the before eating group, P<0.01.

Table 7. Polyunsaturated fatty acids (PUFA) in human plasma choline glycerophospholipids before and after eating chicken enriched with long-chain n-3 fatty acids.

PUFA	Diacyl		Alkenylacyl		Alkylacyl	
	Before	After	Before	After	Before	After
18:2 n-6	18.8±0.4	15.3±1.3	19.4±1.3	16.0±3.4	15.9±0.7	14.9±0.5
18:3 n-3	0.3±0.1	0.3±0.1	3.6±0.8	4.3±1.1	0.7±0.4	1.9±0.6[a]
20:4 n-6	5.3±0.8	5.5±0.3	13.1±3.6	10.2±2.1	16.0±1.0	14.5±0.5[b]
20:5 n-3	0.3±0.1	0.4±0.0	Tr	4.8±0.1[a]	0.2±0.1	1.4±0.5[a]
22:6 n-3	1.3±0.4	2.3±0.1[b]	1.4±0.4	6.1±0.5[a]	2.5±0.6	6.3±0.2[a]

Values represent means±S.E. for 20 female subjects, aged 20, per group.
Each subject ate 200 g chicken breast daily for 4 weeks.
[a]Indicates significant difference from the before eating group, P<0.01.
[b]Indicates significant difference from the before eating group, P<0.05.

Plasma Glycerophospholipids The types of choline and ethanolamine glycerophospholipids were analyzed (Table 6). The only significant change from consuming DHA-enriched meats was a decrease in the proportion of alkylacyl glycerophosphocholine after consuming DHA-enriched pork. The proportion of alkylacyl glycerophosphoethanolamine was much higher at the beginning of pork consumption than at the end of chicken consumption. This tended to decrease during the consumption of DHA-enriched pork.

In the choline glycerophospholipids, the proportions of

Table 8. Polyunsaturated fatty acids (PUFA) in human plasma choline glycerophospholipids before and after eating pork enriched with long-chain n-3 fatty acids.

PUFA	Diacyl		Alkenylacyl		Alkylacyl	
	Before	After	Before	After	Before	After
18:2 n-6	16.8±4.0	12.7±4.4	12.0±2.4	10.5±1.1	18.6±2.0	16.6±0.9
18:3 n-3	0.3±0.1	1.2±0.3[a]	0.7±0.1	2.7±1.4.	0.8±0.3	1.1±0.4
20:4 n-6	6.0±0.6	3.4±0.7[a]	9.0±2.4	7.4±1.2	15.3±1.0	10.3±1.5[b]
20:5 n-3	0.3±0.1	1.2±0.1[a]	0.3±0.3	2.3±0.1[b]	0.5±0.1	2.3±0.1[a]
22:6 n-3	1.9±0.6	3.9±1.3[b]	1.9±1.0	5.2±4.4[b]	3.8±1.1	6.5±0.5[a]

Values represent means±S.E. for 20 female subjects, aged 20, per group.
Each subject ate 200 g pork loin daily for 4 weeks.
[a]Indicates significant difference from the before eating group, P<0.01.
[b]Indicates significant difference from the before eating group, P<0.05.

Table 9. Polyunsaturated fatty acids (PUFA) in human plasma ethanolamine glycerophospholipids before and after eating chicken enriched with long-chain n-3 fatty acids.

PUFA	Diacyl		Alkenylacyl		Alkylacyl	
	Before	After	Before	After	Before	After
18:2 n-6	15.0±4.4	12.5±3.8	9.3±1.0	7.3±0.2	9.5±2.1	4.9±0.3[b]
18:3 n-3	1.0±0.8	3.8±0.8[b]	2.3±0.3	3.1±1.2	6.5±1.3	16.2±8.2[a]
20:4 n-6	5.6±0.6	4.5±0.5	7.3±2.4	5.2±0.2	12.5±2.8	10.9±1.4
20:5 n-3	0.6±0.4	1.8±0.4[a]	0.2±0.2	1.6±0.4[a]	0.5±0.5	2.5±0.7[a]
22:6 n-3	1.7±0.9	4.5±0.4[a]	3.9±1.2	18.8±2.[a]	1.5±1.0	11.6±2.0[a]

Values represent means±S.E. for 20 female subjects, aged 20, per group.
Each subject ate 200 g chicken breast daily for 4 weeks. [a]Indicates significant difference from the before eating group, P<0.01.
[b]Indicates significant difference from the before eating group, P<0.05.

Table 10. Polyunsaturated fatty acids (PUFA) in human plasma ethanolamine glycerophospholipids before and after eating pork enriched with long-chain n-3 fatty acids.

PUFA	Diacyl		Alkenylacyl		Alkylacyl	
	Before	After	Before	After	Before	After
18:2 n-6	13.9±0.2	8.6±0.0[a]	17.8±0.1	6.6±0.2[a]	6.7±0.2	1.6±0.1[a]
18:3 n-3	0.7±0.1	0.5±0.1	2.2±0.2	5.3±1.2[b]	6.1±0.5	7.5±0.1
20:4 n-6	15.9±1.5	13.6±0.6	8.9±0.4	7.1±0.4	13.9±0.5	6.6±0.2[a]
20:5 n-3	0.7±0.1	2.2±0.1[a]	0.5±0.1	3.2±0.5[a]	Tr	2.0±0.3[a]
22:6 n-3	5.8±0.4	7.6±1.4	2.5±0.2	20.2±1.1[a]	31.8±5.2	51.4±2.7[a]

Values represent means±S.E. for 20 female subjects, aged 20, per group.
Each subject ate 200 g pork loin daily for 4 weeks.
[a]Indicates significant difference from the before eating group, P<0.01.
[b]Indicates significant difference from the before eating group, P<0.05.

DHA and EPA were most markedly increased in the ether types, after consumption of either chicken or pork (Tables 7 and 8). Although the starting EPA levels were low, 0.5% or less, they were increased to levels of 1.4 to 4.8%. The ending levels of DHA were between 5.2 and 6.5%. The proportions of AA were decreased somewhat in the alkylacyl glycerophosphocholine.

The largest changes in the polyunsaturated fatty acids were seen in ethanolamine glycerophospholipids (Tables 9 and 10). After chicken or pork consumption, the DHA levels were very high. The levels in the alkenylacyl type, ethanolamine plasmalogens, were increased from less than 4% to more than 18%. In the alkylacyl glycerophosphoethanolamine, the DHA level was over 11% after eating chicken, and over 51% after eating pork. The increases in the diacyl type were more modest. The proportions of EPA were increased markedly by the consumption of DHA-enriched meats, but did not exceed 3.2% in any type.

Discussion

Previous studies on the effects of n-3 fatty acids have described the effects of purified fish oils on known risk factors of heart disease (Sanders and Hinds, 1992; Conner *et al.*, 1993; Conquer and Holub, 1998). These factors include plasma lipids, blood pressure, platelet aggregability, and lymphocyte function. In our study, we investigated the effects of consuming n-3 fatty acids contained in enriched milk, eggs, chicken breast, and pork loin. Boys (14 years old) who drank n-3 enriched milk and young women (20 years old) who ate n-3 enriched eggs and chicken meat showed significant increases in blood n-3 fatty acid levels (Horrocks and Yeo, 1999). Similar results were obtained with plasma lipids and platelet phospholipid fatty acids in humans who consumed n-3 enriched milk and eggs (Holub, 1994; Ferrier *et al.*, 1995; Wright *et al.*, 1998). Alterations in the alkylacyl and alkenylacyl subclasses of plasma phospholipids from individuals fed n-3 enriched chicken and pork are similar to those reported by other investigators using fish oil supplements (Aukema and Holub, 1989; Croset *et al.*, 1992). Much of the long-chain n-3 fatty acids in the plasma are contained in the alkenylacyl type of ethanolamine glycerophospholipids (ethanolamine plasmalogens). The DHA is also preferentially incorporated into ethanolamine plasmalogens in human platelets producing decreased platelet reactivity. Furthermore, plasmalogens may act as antioxidants and can scavenge peroxy radicals in a lipid environment (Reiss *et al.*, 1997). Plasmalogens are decomposed during oxidation of LDL. The degradation products may be effective modulators of reactive oxygen species generated in platelets, macrophages, and other tissues during phagocytosis. Consumption of n-3 fatty acid enriched eggs, chicken, or pork results in inhibition of collagen, ADP and epinephrine-induced platelet aggregation in 20 year-old human females (Horrocks and Yeo, 1999). A similar inhibition of platelet aggregation was found with healthy male volunteers who consumed a daily fish oil supplement (Sanders and Hinds, 1992).

Diets enriched with n-3 fatty acids may exert some of their beneficial effects by inhibiting platelet aggregation. Thus, consumption of such diets has beneficial effects on cardiovascular function. However, high doses of these fatty acids both increase the degree of unsaturation in membrane phospholipids and susceptibility to lipid peroxidation (Palozza *et al.*, 1996). Without higher levels of vitamin E and other antioxidants, there could be an increased risk of heart disease. The effect of n-3 fatty acids on plasma phospholipids seems to be a transient effect. A diet enriched in n-3 fatty acid should be consumed regularly during adulthood for protection against heart and other diseases. The supplementation of n-3 fatty acids in the human diet may also have long-term positive effects on human health by stimulating transcription of the genes responsible for the synthesis of the plasmalogen type of phospholipids in liver, brain, and heart (Horrocks and Yeo, 1999).

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