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γ-Linolenic acid does not augment long-chain polyunsaturated fatty acid ω-3 status

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Long-chain-polyunsaturated fatty acids (LCPUFA) of the ω3 and ω6 series are used for energy generation and storage, as building blocks of cellular membrane phospholipids, and as precurors of eicosanoids. They derive either from the diet or synthesis from the parent essential fatty acids α-linolenic (18:3ω3; ALA) and linoleic (18:2ω6; LA) acids. Rich dietary LCPUFA sources are meat [arachidonic acid (20:4ω6; AA)] and fish [eicosapentaenoic (20:5ω3; EPA) and docosahexaenoic (22:6ω3; DHA) acids]. Synthesis occurs by alternating desaturation and chain elongation reactions, in which Δ6-desaturation is rate limiting. The synthesis outcome is dependent on competition between ALA, LA and some of their higher products for Δ-6 desaturation, the influence of various hormones on desaturation activity, and negative feed-back of the formed LCPUFA at both the Δ-6 and Δ-5 desaturases (1). Conversion of ALA to LCPUFAω3 seems to occur with difficulty, possibly due to a combination of the high dietary intake of its competitor LA via the Western diet and the preference of ALA for β-oxidation (1). Various studies with both humans and rats indicated that administration of γ-linolenic acid (18:3ω6; GLA) augments LCPUFAω3 status (2) probably by its ability to stimulate the conversion of ALA to LCPUFAω3 through an increase of Δ6-desaturation activity (3,4).

Epidemiological studies showed an inverse relation between the intake of LCPUFAω3 from fish and coronary artery disease (CAD) risk (5,6). In addition, many fish oil supplementation studies suggested beneficial effects on important factors in CAD development such as coagulation, vascular response and production of cellular growth factors (7). These effects are largely ascribed to EPA, which competes with AA for incorporation into cellular phospholipids, and thereby alters the eicosanoid balance to a state of less platelet aggregation and less vasoconstriction (8). It is possible that beneficial effects on CAD development by improving LCPUFAω3 status can also be reached by an increase of the dietary ALA/LA ratio. The 7 countries study showed that the Cretan Mediterranean diet is associated with a 2-3 fold lower CAD mortality rate, despite comparable serum cholesterol levels (9). The Cretan diet is characterised by abundance of plant foods (e.g. bread, vegetables, fruits, nuts), low to moderate amounts of fish, poultry, dairy products, eggs and wine, and little amounts of red meat. It provides a high intake of oleic acid from olive oil at the expense of LA from sunflower oil, which together with a comparable ALA intake gives rise to a higher ALA/LA ratio. Beneficial effects of the Mediterranean diet were demonstrated in the 'Lyon diet heart study'. The intervention caused 22% higher plasma EPA contents in conjunction with a 70% reduction of all cardiac events in patients who recovered from myocardial infarction (10,11). The favourable outcome may derive from many factors, including augmented LCPUFAω3 status.

We investigated whether a short term intake of GLA was able to augment LCPUFAω3 status in 15 apparently healthy adults. Seven of them took 2.1 g ALA daily and the remaining 8 took 1.4 g GLA daily during 4 weeks. In the consecutive 4 weeks all 15 took 2.1 g ALA plus 1.4 g GLA. The ALA dose was chosen to reach the same dietary ALA/LA ratio as used in the Lyon diet heart study (0.21). The LCPUFAω3 status was monitored by measurement of the fatty acid compositions of erythrocytes (RBC), platelets (PLT), plasma cholesterol esters (CE) and plasma triglycerides (TG).

SUBJECTS and METHODS

Study group and study design

Healthy subjects, aged 20-50 years were eligible to participate in this study. Participants were recruited by advertisement from hospital employees and students of the Pharmacy Department of the Groningen University. Exclusion criterion was premature atherosclerosis. Written and informed consent was obtained from all participants. The study protocol was approved by the medical ethical committee of the Groningen University Hospital and was in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

Fifteen apparently healthy subjects [9 males, 6 females; ages 32±3 years (mean±SEM); BMI 22.2±0.7 kg/m^2] participated in this study. They were divided into two groups. Group A consisted of 7 adults (male/female 4/3) who took 4 flaxseed oil capsules (1 g oil/capsule; 52.7% ALA; Banner, Tilburg, The Netherlands) daily for 4 weeks during breakfast (1 capsule), lunch (n=1) and the hot meal (n=2). Group

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B consisted of 8 adults (male/female 5/3) who took 12 starflower oil capsules (500 mg oil/capsule; 23.9% GLA; Scherer, Blagrove, Swindon, Wilts, UK) daily for 4 weeks during breakfast (4 capsules), lunch (n=4) and the hot meal (n=4). In the consecutive 4 weeks, all 15 subjects took the combination of 4 flaxseed oil capsules and 12 starflower oil capsules daily. The dosage regimen was the same as in the first 4 weeks. Their usual intakes of total fat, saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were estimated at baseline, using a food frequency questionnaire (Fat express, Agriculture University Wageningen, The Netherlands).

Samples and sample processing

EDTA anti-coagulated blood was collected in the fasting state, at baseline (week 0), after 4 weeks supplementation with either ALA or GLA (week 4) and after 4 weeks supplementation with ALA+GLA (week 8). The sample was separated into PLT rich plasma and a RBC pellet by centrifuging for 20 min at 120xg and 4°C. An aliquot of 2.0 ml PLT rich plasma was transferred to a Teflon sealable glass tube and the remaining volume was put into a glass tube. Both tubes were centrifuged (10 min 1500xg, 4°C) and their supernatants (PLT poor plasma) were pooled. The PLT poor plasma was stored at -20°C for the analyses of the CE and TG fatty acid compositions. The PLT pellet isolated from 2.0 ml PLT rich plasma was washed three times with 1 ml aliquots of 0.9% NaCl solution. Each wash was followed by centrifugation for 10 min at 800xg and 4°C. Antioxidant (1 mg butylated hydroxytoluene in 100 µl methanol), internal standard (50 µg C17:0 in 100 µl methanol) and transmethylation mixture (2 ml methanol/HCl 6 mol/l=5:1 v/v) were added after the last wash. The RBC pellet was washed three times with equal volumes of 0.9 % NaCl solution. Each wash was followed by centrifugation for 10 min at 800xg and 4°C. The washed RBC were finally resuspended in 0.9% NaCl solution to a haematocrite of approximately 50%. A 200 µl aliquot of the RBC suspension was put into a teflon sealable tube and antioxidant (1 mg butylated hydroxytoluene in 100 µl methanol), internal standard (25 µg C17:0 in 50 µl methanol) and transmethylation mixture (2 ml methanol/HCl 6 mol/l=5:1 v/v) were added.

Analytical methods

Plasma CE and TG were isolated by solid phase extraction (12). Fatty acid compositions of RBC, PLT, plasma CE and plasma TG were determined as their methyl esters by capillary gas chromatography with flame ionisation detection (12).

Data evaluation and statistics

Fatty acid compositions were expressed in mol%. Time-dependent changes in RBC, PLT and plasma CE and TG fatty acid were analysed with the paired Student's t-test at $p<0.05$ (13). Differences between week 0 and 4, and between week 4 and 8 were tested separately for each of the subgroups. Bonferroni correction was employed to correct for type 1 errors. Data of both subgroups were pooled to investigate changes between weeks 0 and 8 with the paired Student's t-test at $p<0.05$.

RESULTS

The mean±SD dietary fatty acid intakes (in energy %) of the 15 participants at baseline amounted to: 39.4±4.1 (total fat); 14.9±1.8 (SAFA); 15.2±2.4 (MUFA) and 7.6±1.4 (PUFA). The PUFA intakes amounted to 28.7±5.7 and 14.8±3.3 g/day for men (n=9) and women (n=6), respectively. Taking into account a mean dietary ALA/LA ratio of 0.17 and 0.23 for Dutch men and women aged 19-50 years (14), respectively, these intakes correspond roughly with daily ingestions of 24.9±4.9 g LA and 4.2±0.8 g ALA for the men, and 12.1±2.7 g LA and 2.8±0.6 g ALA for the women. Using these background intakes from the usual diets it was calculated that daily supplementation with 4 g flaxseed oil (containing 2.2 g ALA and 0.6 g LA), 6 g starflower oil (4.5 g LA) and the combination of flaxseed and starflower oils gave rise to a change of the dietary ALA/LA ratio from about 0.20 to 0.31±0.07 (n=7), 0.15±0.01 (n=8) and 0.25±0.04 (n=15), respectively.

Figure 1 shows the ALA, EPA and DHA contents of RBC, PLT plasma CE and plasma TG for groups A (4 weeks ALA, 4 weeks ALA+GLA) and B (4 weeks GLA, 4 weeks ALA+GLA). ALA increased from 0-4 weeks in RBC, CE and TG of group A ($p\leq0.04$), from 4-8 weeks in all compartments of group B ($p\leq0.05$) and from 0-8 weeks in all compartments of group A+B ($p\leq0.001$). EPA increased from 0-4 weeks in TG of group A ($p=0.03$) and from 0-8 weeks in PLT and TG of group A+B ($p<0.05$). It decreased from 0-4 weeks in RBC of group B ($p=0.02$). DHA decreased in RBC of groups A and B from 0-4 weeks ($p=0.004$), RBC of groups A+B from 0-8 weeks ($p<0.001$), CE of group A from 0-4 weeks ($p=0.03$) and PLT of group B from 4-8 weeks ($p=0.02$). DHA increased in TG of group A from 0-4 weeks ($p=0.01$) and RBC of group A from 4-8 weeks ($p=0.03$).

Figure 2 shows the GLA, 20:3ω6 (DGLA) and AA contents of RBC, PLT, plasma CE and plasma TG for groups A and B. GLA increased in RBC, CE and TG ($p<0.001$), and DGLA increased in all compartments ($p\leq0.005$) of group A from 4-8 weeks. GLA and DGLA increased in all compartments of group B from 0-4 weeks ($p\leq0.05$) and in all compartments of groups A+B from 0-8 weeks ($p\leq0.02$). AA decreased in RBC of group A from 0-4 weeks ($p=0.002$) and increased in RBC of groups A and B from 4-8 weeks ($p\leq0.004$). AA also increased in CE of group A from 4-8 weeks ($p=0.01$), CE of group B from 0-4 weeks ($p=0.01$), and PLT of groups A+B from 0-8 weeks ($p=0.02$).

DISCUSSION

We investigated whether a short term supplementation of GLA (1.4 g daily during 4 weeks) augmented the LCPUFAω3 status of 15 apparently healthy

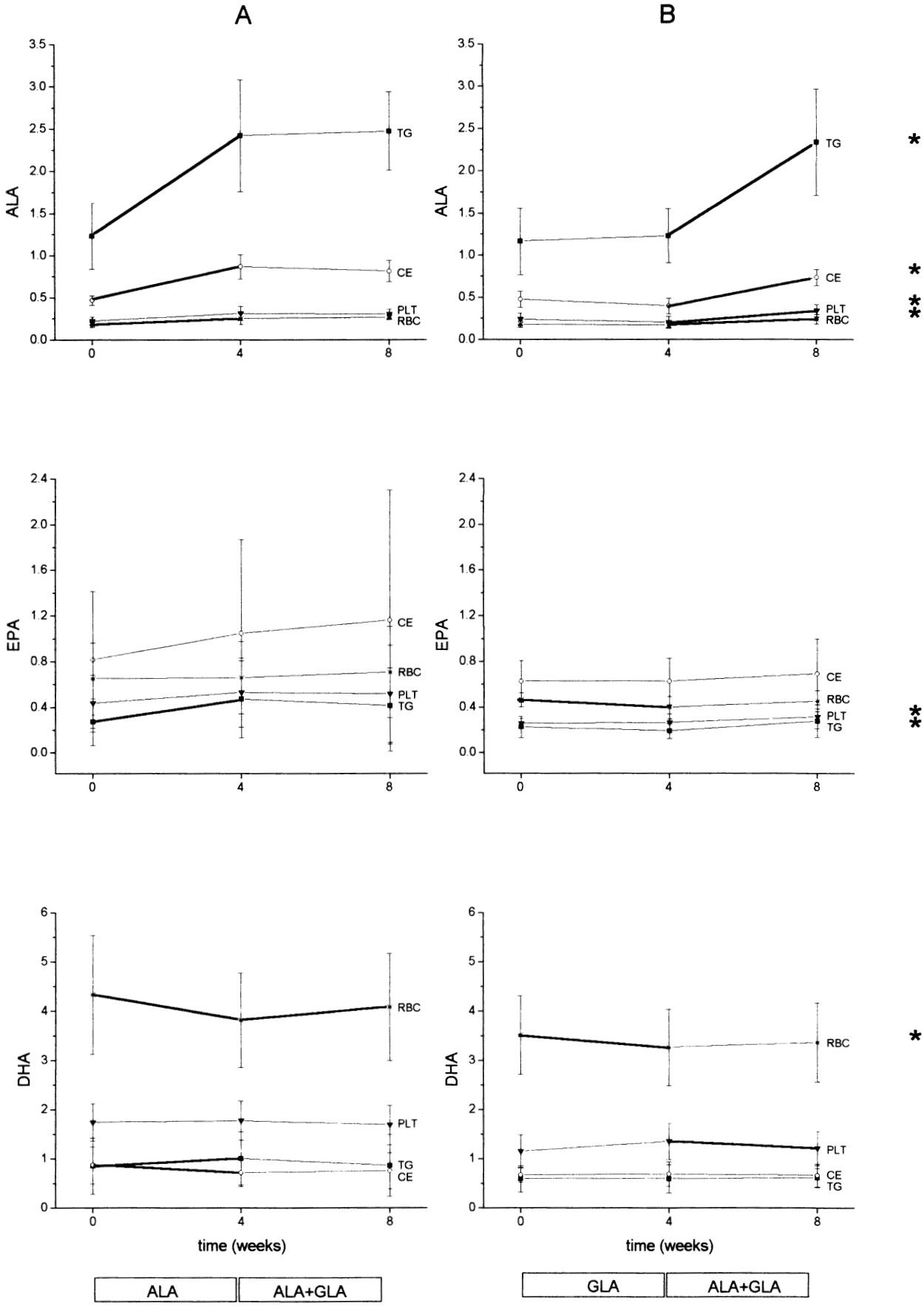

Figure 1. Contents of α-linolenic, eicosapentaenoic and docosahexaenoic acids in circulating cells and plasma lipids. Group A received ALA from 0-4 weeks and ALA+GLA from 4-8 weeks, group B received GLA from 0-4 weeks and ALA+GLA from 4-8 weeks. Significant changes for groups A and B (paired Student's t-test, $p<0.05$) are indicated by bold lines; asterisks at the right hand side indicate significant changes for group A+B from 0-8 weeks. ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosa-

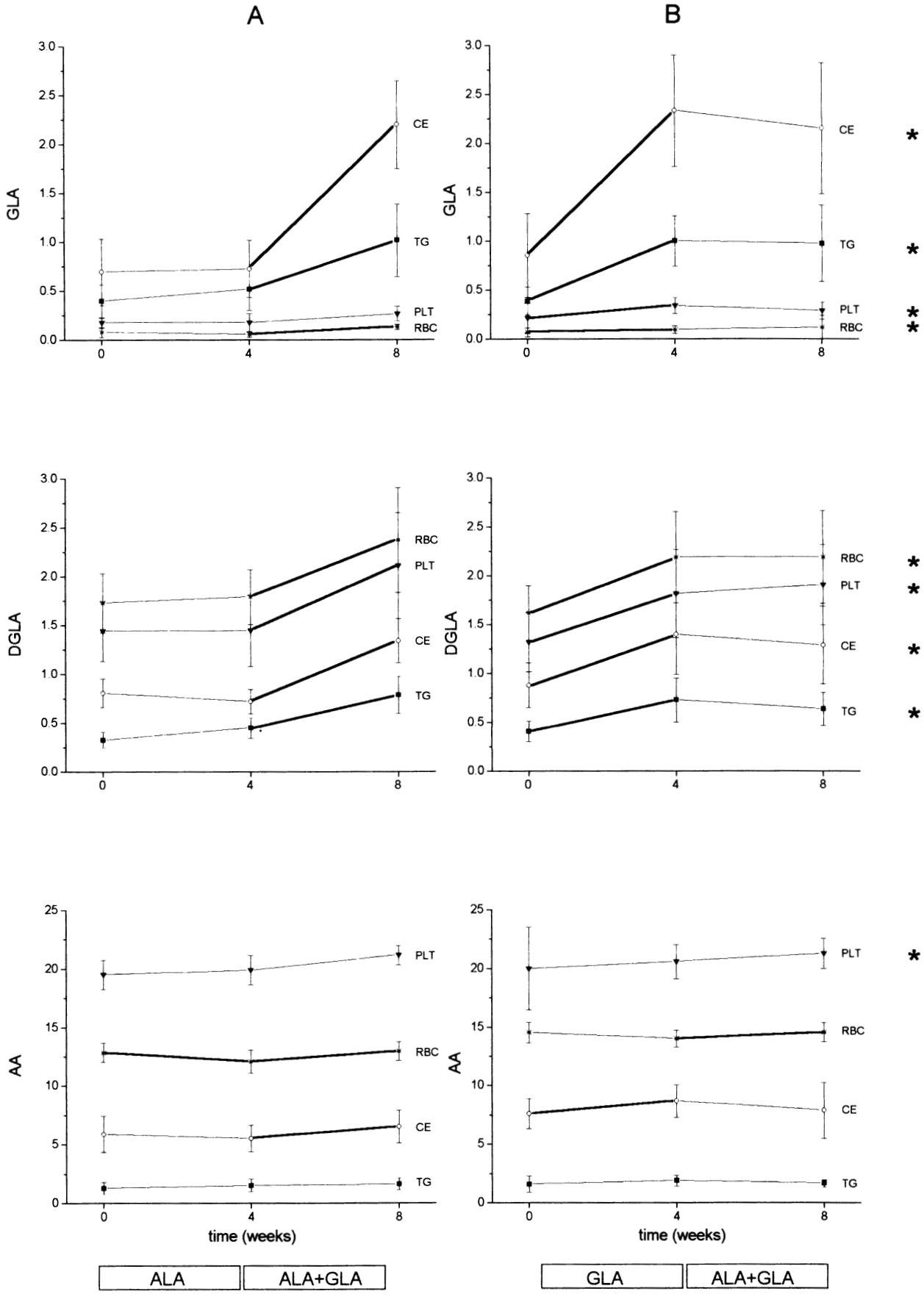

Figure 2. Contents of γ-linolenic, dihomo-γ-linolenic and arachidonic acids in circulating cells and plasma lipids. GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; AA: arachidonic acid. For other abbreviations, study design and indication of statistical significance see legend of Figure 1.

adults, who also received ALA (2.1 g daily during 4 weeks). Prior to the ALA+GLA supplementation 7 participants (group A) received ALA for 4 weeks and 8 participants (group B) received GLA only, to investigate the influence of each of these fatty acids separately. The supplements gave rise to a change of the dietary ALA/LA ratio from about 0.20 to 0.31 (during ALA supplementation), 0.15 (during GLA) and 0.25 (during ALA+GLA), respectively. Our results indicate that ALA and GLA supplementation caused statistically significant increases of ALA and GLA in nearly all studied compartments, that GLA caused an increase of DGLA in all studied compartments, but that the supplements had negligible effects on the levels of AA, EPA and DHA (Figures 1 and 2).

An increase of DGLA and no effects on AA in RBC (15), serum lipids (16,17) and PLT phospholipids (18) following GLA supplementation of humans is well documented. Studies in both humans and rats indicated that administration of GLA augments LCPUFAω3 status in RBC-phospholipids (2). The underlying mechanism of action was conceivably augmentation of the conversion of ALA to LCPUFAω3 through an increase of Δ6-desaturation activity (3,4). We did not find that GLA augments LCPUFAω3 status, neither when administered to subjects on their usual diet (group B from week 0-4), nor in a group who received both GLA and ALA (group A+B from week 4-8). Our results are in line with those of other investigators who studied the effect of GLA supplementation in healthy adults (15,18).

Increased dietary ALA intake is associated with increases of EPA and decreases of DHA in plasma lipids and in phospholipids of various circulating cell types (19). However, results of several studies indicate that augmentation of the LCPUFAω3 status by an increase of the dietary ALA intake and thereby the ALA/LA ratio remains difficult, even at high ALA/LA ratios of 1.6 (20), 2 (21) and 4 (22,23). For instance, Mantzioris et al. (20) showed that a daily dietary ALA intake of 13.7 g (ALA/LA=1.6) causes modest increases of EPA in plasma phospholipids (from 0.8 to1.9%), CE (0.8-2.0%) and TG (0.2-0.6%), whereas replacement of ALA for a 8.5 times lower dose of fish oil (1.62 g EPA, 1.08 g DHA) gives rise to a further increase of EPA to 5.4% in phospholipids, 6.0% in CE and 2.1% in TG. Similar results were obtained by Freese and Mutanen (23) in an ALA and fish oil parallel study design, indicating that augmentation of the LCPUFAω3 status by ALA supplementation is poor compared to fish oil. The discrepancy is explained by preferential oxidation of ALA, as has been demonstrated in rats that exhibited increased activities of the perixosomal and mitochondrial β-oxidation enzymes in the liver upon ALA administration (24). It is also possible that we are dealing with an inhibitory effect of dietary AA from the omnivorous diet, since AA exerts negative feed-back inhibition on the desaturating enzymes (1,25). The dietary AA intake from the omnivorous diet is estimated at 96-130 mg/day (26) and 222±107 mg/day (27) and it is not known how these figures relate to the daily AA need. Reduction of the LA intake and an increase of ALA consumption, causing an increase of the ALA/LA ratio from 0.04 to 0.21, together with decreased intake of meat, the principle source of dietary AA, may explain the increase of plasma EPA (from 0.75 to 0.92) noted in the Lyon diet heart study (11). Studies on the regulation of the conversion of ALA to LCPUFAω3 in vegans may shed more light on the influence of the dietary AA background.

We conclude that GLA, given at a daily dose of 1.4 g with or without 2.1 g ALA for 4 weeks, does not augment the LCPUFAω3 contents in RBC, PLT, plasma CE and plasma TG of apparently healthy adults. The underlying reason for the poor conversion of ALA to LCPUFAω3 remains to be elucidated.

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Summary

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Augmentation of long chain polyunsaturated ω3 fatty acid (LCPUFAω3) status decreases the risk for coronary artery disease. It can be reached by consumption of LCPUFAω3-rich fish oils or improvement of the conversion of α-linolenic acid (ALA) to LCPUFAω3 by desaturation and chain elongation. Since it has been suggested that γ-linolenic acid (18:3ω6, GLA) activates the rate limiting Δ-6 desaturation, we investigated whether GLA augments LCPUFAω3 status. Seven apparently healthy adults (23-47 years; female/male=3/4) received a daily oral dose of 4 g linseed oil (2.2 g ALA) for 4 weeks, and subsequently a combination of 4 g linseed oil and 6 g starflower oil (2.2 g ALA+1.4 g GLA) daily for another 4 weeks. A second group of eight adults (22-49 years; female/male=3/5) received 6 g starflower oil for 4 weeks, and subsequently the same combination during the second 4 weeks. EDTA-blood was collected in the fasting state at 0, 4 and 8 weeks. Erythrocytes, platelets, plasma cholesterol esters and plasma triglycerides were isolated and their fatty acid compositions were determined by capillary gas chromatography with flame ionization detection. ALA and GLA administration augmented their contents in each of the investigated compartments. GLA, either alone or as GLA+ALA combination, increased 20:3ω6, but did not change arachidonic acid, 22:4ω6, or 22:5ω6. ALA, either alone or as ALA+GLA combination, did not significantly augment EPA and DHA contents. We conclude that the LCPUFAω3 status can not be improved by supplementation with 4 g linseed oil, and that cosupplementation of 6 g starflower oil does not augment LCPUFAω3 status either. Poor conversion of ALA to LCPUFAω3 may be caused by preferential β-oxidation of ALA, negative feedback of the background intake of arachidonic acid from the omnivorous diet, or by the remaining low dietary ALA/LA ratio.

Key-words: coronary artery diseas; α-linolenic acid; γ-linolenic acid; supplementation; desaturation/chain elongation; β-oxidation; arachidonic acid

De prognostische waarde van p53 mutaties voor