

## Association of adipose tissue arachidonic acid content with BMI and overweight status in children from Cyprus and Crete

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The relationships between *n*-3 and *n*-6 fatty acids in subcutaneous fat, BMI and overweight status were investigated in eighty-eight children from Crete and Cyprus. Overweight status, BMI and serum lipid levels were similar in children at both locations, but Cretan children had higher levels of total MUFA than Cypriot children (62.2 (SD 2.8) v. 52.2 (SD 2.8) % area, respectively,  $P < 0.001$ ) and consequently Cypriot children had higher levels of total saturated, polyunsaturated, *trans*, *n*-3 and *n*-6 fatty acids. Cypriot children had also higher levels of individual *n*-3 and *n*-6 fatty acids, specifically linoleic,  $\alpha$ -linolenic and dihomo- $\gamma$ -linolenic acids. The variance of BMI was better explained (38.2 %) by adipose tissue arachidonic acid content than any other *n*-3 and *n*-6 fatty acids. Mean levels of arachidonic acid, dihomo- $\gamma$ -linolenic acid and docosahexaenoic acid were higher in overweight and obese subjects. All obese subjects fell in the 4th quartile of arachidonic acid levels, whereas 88.9 % of overweight subjects fell in the 3rd and 4th quartile of arachidonic acid. These results indicate positive associations between adipose tissue arachidonic acid and BMI and overweight status. Further research could clarify whether this association is causal.

### Adipose tissue: Arachidonic acid: Fatty acids: Overweight: Children

The relationship of dietary fat to the increasing prevalence of obesity in adults (Mokhad *et al.* 2003) and children (Nicklas *et al.* 2001) remains controversial and poorly understood. The prevalence of obesity is increasing despite a decrease in dietary fat intake and especially in saturated fatty acids. MUFA intake has also decreased (Bray & Popkin, 1998; Seidel, 1998; Hooper *et al.* 2001), whereas intake of PUFA and refined carbohydrates has increased, in the past 20 years (Nicklas *et al.* 2001; Prentice, 2001). The increased intake of PUFA has been associated with a significant increase in the *n*-6 : *n*-3 fatty acid (FA) balance, mainly as a result of the increased consumption of refined vegetable oils in western Europe (Sanders, 2000) and the USA (Kris-Etherton *et al.* 2000). This imbalance, in particular of *n*-6 FA intake, has been linked to chronic diseases (Simopoulos, 1999; Holub, 2002) and cancer (Bartsch *et al.* 1999; Nkondjock *et al.* 2003).

There are substantial differences in dietary fat intake between different areas. The Cretan population, for instance, has been shown to consume high-fat diets (45 % energy as fat) that are rich in oleic acid (27 % energy; Aravanis *et al.* 1988), whereas Cypriot children have lower total fat consumption (about 35 % energy) and MUFA consumption (about 15 % energy, based on 24 h dietary recall)

than Cretan children (Child Health Foundation, Cyprus database 1997–2002, unpublished results).

Habitual intake of PUFA correlates well with the adipose tissue concentration of these FA (Cantwell, 2000). Linoleic acid (LA), an essential *n*-6 FA, is particularly highly correlated with dietary intake of LA, both in the short term (Plakke *et al.* 1983), but also more longitudinally (Katan *et al.* 1986; Van Staveren *et al.* 1986). Population differences in adipose tissue composition in different European communities exist (Seidel *et al.* 1991) as well as age-related changes, presumably due to a decline in  $\Delta^6$ -desaturase activity (Bolton-Smith *et al.* 1997).

The role of PUFA in adipogenesis is of particular interest, as this has been evaluated in experimental models. In particular, prostaglandins, derivatives of arachidonic acid (AA), play a critical role in adipocyte differentiation. More specifically, prostaglandins have opposing effects on adipogenesis through the use of different mechanisms, one through a nuclear receptor transcription factor and the other through a cell-surface prostanoid receptor; they also have a significant effect on glucose metabolism (Darimont *et al.* 1994; Long & Pekala, 1996; Reginato *et al.* 1998; Ahren *et al.* 2000). Furthermore, it has recently been shown that AA promotes the differentiation of clonal

**Abbreviations:** AA, arachidonic acid; FA, fatty acid; LA, linoleic acid.

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preadipocytes *in vitro*, whereas mice fed diets rich in LA had higher fat mass and adipocyte size compared with mice fed a diet supplemented with a combination of LA and  $\alpha$ -linolenic acid (Massiera *et al.* 2003).

In studies of human subjects it has been shown that obese children have higher serum levels of individual *n*-6 PUFA, and in particular of AA, di-homo- $\gamma$ -linoleic acid and  $\gamma$ -LA in comparison with normal-weight children (Decsi *et al.* 1996). Conversely, malnourished children have lower plasma phospholipids levels of AA in comparison with control subjects (Decsi *et al.* 1998), but no data exist on the relationship of adipose tissue PUFA to obesity.

The aim of the present study, therefore, was to evaluate the relationship of subcutaneous adipose tissue *n*-3 and *n*-6 FA content to the BMI and overweight status in children aged 10–12 years old from Crete and Cyprus.

## Subjects and methods

### Cypriot sample

The Cypriot children (Greek-Cypriot) that participated in the present study were recruited after they were evaluated at their school for the presence of CVD risk factors in 1999. The age range of these subjects was 10–12 years. Written informed consent was obtained from parents or legal guardians of all participants. All children were residents of rural and semi-rural areas in the Nicosia district. A preliminary analysis revealed that there were no differences in subjects' characteristics in rural and semi-rural areas, and therefore subjects were pooled. The evaluation was performed by the Research and Education Foundation of Child Health, Cyprus, under the auspices of the Ministry of Health and the Ministry of Education and Culture of Cyprus. The subcutaneous fat aspiration was performed by a trained physician (C. H.) from the University of Crete.

### Cretan sample

All Cretan children were recruited from the municipality of Rouvas, a rural area in central Crete. All children attending public schools were invited to participate in the baseline survey for CVD risk factors in January and February 2001; written informed consent was obtained from parents or legal guardians of all participants. The participation rate was 95% (190 children). In the final analysis we included only children who were age-matched to the Cypriot sample, that is, children aged 10–12 years.

### Anthropometric measurements

On both islands, measurements were performed by trained personnel. Weight was measured with a portable Seca 762 scale (Vogel & Halke GmbH & Co., Hamburg, Germany) to the nearest 0.5 kg. Height was measured with a portable Seca stadiometer 208 (Vogel & Halke GmbH & Co.) to the nearest 1 mm. Both measurements were taken after breakfast, with the child dressed in light clothing and without shoes. BMI was calculated as: weight (kg)/height (m)<sup>2</sup>. The scale and stadiometer were calibrated every morning with a standard weight and height respectively.

### Overweight definition

Each subject's BMI was classified as normal weight, overweight or obese according to gender- and age-specific cut-offs (6-month intervals) suggested by the International Obesity Task Force (Cole *et al.* 2000).

### Adipose tissue aspiration

Buttock subcutaneous tissue samples were collected by aspiration using the method described by Beynen & Katan (1985). This method has been reported to be rapid and safe, and to cause no more discomfort than a routine venepuncture. Buttock adipose tissue samples can be safely stored for up to 1.5 years without changes in the component FA (Beynen & Katan, 1985). Samples were taken from the left upper outer quadrant of the gluteal area, through the use of a 10 ml vacutainer. Before the procedure, aspiration sites were sprayed with local anaesthetic (ethyl chloride).

### Adipose tissue analysis

Adipose tissue samples were stored in  $-80^{\circ}\text{C}$ . Before analysis, each sample was thawed and the fat transferred with a Pasteur pipette into a 10 ml screw-capped tube containing several drops (about 0.5 ml) of chloroform:methanol (2:1, v/v). When necessary, samples were washed with saline (9 g NaCl/l) in order to remove blood. Methyl esters of the fat-component FA were prepared in the screw-capped vials according to the method described by Metcalfe *et al.* (1966). Briefly, 20–30 mg of the fat sample were saponified with 1.0 ml NaOH (1 mol/l methanol) and the FA methyl esters were prepared with boron trifluoride (140 g/l methanol) following extraction with hexane after washing with 3.0 ml saturated NaCl. The hexane (upper layer) containing the FA methyl esters was transferred to GC vials and stored at  $-20^{\circ}\text{C}$  until analysis. The FA methyl esters were separated on a 100 m  $\times$  0.25 mm SP-2560 fused silica column, coated with 0.2  $\mu\text{m}$  biscyanopropyl polysiloxane provided by SUPELCO (Bellefonte, PA, USA), using a Shimadzu GC (GC-17A; Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-20 auto sampler and a flame ionization detector. The class-VP chemstation software (Shimadzu Corporation) was used for quantitation and identification of peaks. The identification of more than fifty FA methyl esters peaks was accomplished by means of mixed FA methyl esters standards (Sigma, Sigma Chemicals Co. USA). The analytical conditions employed were as follows: volume injected 1  $\mu\text{l}$ , carrier gas He (20 cm/s), injector temperature  $250^{\circ}\text{C}$ , flame ionization temperature  $250^{\circ}\text{C}$ , split ratio 1:25 to 1:30 (depending on the sample quantity: a different quantity was obtained from each subject because it was obtained by aspiration and not biopsy), and oven temperature from 140 to  $240^{\circ}\text{C}$  with stepped temperature programme within total run time of 60 min.

### Serum lipids and lipoproteins

Blood samples were obtained in vacutainers after 12 h fasting, before breakfast in the morning. All samples were put

in ice and transported immediately and analysed at either the Clinical Chemistry Laboratory of the General Hospital of Nicosia for Cypriot subjects, or the Clinical Chemistry Laboratory of the University Hospital of Heraklion for the Cretan subjects. All measurements were reproducible and validated in both laboratories by internal (Boehringer, Mannheim, Germany) and external (Murex Dartford, UK; External Quality Control System, WHO) quality control.

Total cholesterol and triacylglycerol were assayed enzymatically (Hitachi 717 analyser; Hitachi Ltd, Tokyo, Japan) and HDL-cholesterol with a non-immunological enzymatic reaction. LDL-cholesterol was calculated from the Friedewald formula ( $\text{LDL-cholesterol} = \text{total cholesterol} - (\text{HDL-cholesterol} + \text{triacylglycerol}/5)$ ).

### Statistical analyses

Descriptive statistics and FA content in adipose tissue in relation to country of origin and gender are presented as mean values and standard deviations. Continuous variables were examined for skewness and kurtosis; a logarithmic transformation was performed in case they did not follow the normal distribution. The non-parametric Mann-Whitney U test was used to test differences among groups. ANOVA was used to test the trend of mean of certain FA in relation to BMI status.

Adipose tissue FA levels were categorized in quartiles in order to have group comparisons; cross-tabulation was performed with weight status.

Stepwise multiple linear regression models were used to estimate the influence of certain adipose tissue FA on the variance of BMI. BMI was therefore the dependent variable; age, gender and country of origin as well as certain combinations of adipose tissue FA were used as independent variables. A  $P$  value  $< 0.05$  was considered as statistically significant.

### Results

Descriptive statistics are presented in Table 1. The sample consisted of thirty-six Cretan children (thirteen male, twenty-three female, age 11.0 (SD 0.7) years) and fifty-two Cypriot children (twenty-three male, twenty-nine

female, age 11.2 (SD 0.3) years). BMI and incidence of overweight were similar in both islands: BMI, Crete 19.5 (SD 3.7) v. Cyprus 19.5 (SD 4.1)  $\text{kg}/\text{m}^2$ ,  $P=0.806$ ; incidence of overweight, Crete 27.8 v. Cyprus 30.8%,  $P=0.762$ . Serum lipid levels were similar both between country and gender.

The composition of subcutaneous adipose tissue in certain groups and individual  $n-3$  and  $n-6$  FA in relation to country and gender are presented in Table 2. There were no significant differences in groups of FA in relation to gender, whereas there were significant differences in relation to country in all six groups of FA; total MUFA were significantly greater in Cretan children (Crete 62.2 (SD 2.8) v. Cyprus 52.2 (SD 2.8) % area,  $P<0.001$ ), whereas all other groups were greater in Cypriot children. The  $n-3$  and  $n-6$  essential FA,  $\alpha$ -linolenic (18:3 $n-3$ ) and LA (18:2 $n-6$ ), were both higher in Cypriot children, whereas there was no significant difference in AA (20:4 $n-6$ ) content.

The distribution of significant associations of these adipose tissue FA against overweight status is presented in Fig. 1. There was a significant increasing trend of AA ( $P<0.001$ ), di-homo- $\gamma$ -linolenic acid (20:3 $n-6$ ;  $P<0.001$ ) and docosahexaenoic acid (22:6 $n-3$ ,  $P=0.022$ ).

Similarly, a cross-tabulation of AA quartiles against weight status (Fig. 2) revealed that all obese children were in the 4th AA quartile, whereas 88.9% of overweight children were in the 3rd and 4th quartile. There were less consistent results when di-homo- $\gamma$ -linolenic acid and docosahexaenoic acid quartiles were cross-tabulated against weight status (results not shown).

Finally, stepwise multiple linear regression analyses were run with BMI as the dependent variable and certain combinations of FA as well as age, gender and country of origin as the independent variables. The results of these analyses are presented in Table 3. Adipose tissue AA explained better the variance of BMI (38.2%) either alone or in combination with certain other  $n-3$  and  $n-6$  FA included in the models. Adipose tissue LA had no effect either alone or in conjunction with other FA on the variance of BMI. BMI variance was also well explained by adipose tissue di-homo- $\gamma$ -linolenic acid, but when combined with AA in the same model, AA remained as

**Table 1.** Descriptive statistics characteristics of the Cretan and Cypriot samples of certain variables in the two populations (Mean values and standard deviations)

Variable	Country				Gender			
	Crete (n 36)		Cyprus (n 52)		Male (n 36)		Female (n 52)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)	11.0	0.7	11.3	0.3	11.2	0.5	11.1	0.5
Weight (kg)	41.3	10.5	43.5	11.4	42.7	12.8	42.5	9.8
Height (m)	1.448	0.080	1.487*	0.073	1.456	0.075	1.481	0.079
BMI ( $\text{kg}/\text{m}^2$ )	19.5	3.7	19.5	4.1	19.9	4.5	19.2	3.4
Serum total cholesterol (mmol/l)	4.5	0.9	4.4	0.7	4.5	0.9	4.4	0.7
Serum HDL-cholesterol (mmol/l)	1.6	0.4	1.5	0.4	1.5	0.4	1.5	0.4
Serum LDL-cholesterol (mmol/l)	2.6	0.7	2.5	0.6	2.5	0.7	2.5	0.6
Serum triacylglycerol (mmol/l)	0.8	0.3	1.0	0.6	0.9	0.6	0.9	0.4

Mean value was significantly different from that of the Cretan group: \* $P=0.037$ .

**Table 2.** Fatty acid composition of subcutaneous adipose tissue in relation to country and gender† (% area)

Fatty acid in subcutaneous adipose tissue (g/100 g total fatty acids)	Country				Gender			
	Crete (n 36)		Cyprus (n 52)		Male (n 36)		Female (n 52)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total MUFA	62.20	2.80	52.20***	2.80	56.60	5.50	55.90	6.10
Total PUFA	13.60	1.00	19.90***	2.80	17.20	3.70	17.50	4.00
Total saturated fatty acids	21.80	2.50	25.10***	3.00	23.60	3.10	24.00	3.50
Total <i>trans</i> fatty acids	1.45	0.29	1.99***	0.34	1.75	0.37	1.81	0.48
Total <i>n</i> -3 fatty acids	0.80	0.15	1.01***	0.15	0.90	0.19	0.95	0.16
Total <i>n</i> -6 fatty acids	12.30	1.00	18.30***	2.81	16.00	3.80	15.70	3.60
Linoleic acid (18:2 <i>n</i> -6)	11.70	1.00	17.50***	2.80	15.10	3.60	15.30	3.80
$\alpha$ -Linolenic acid (18:3 <i>n</i> -3)	0.37	0.04	1.96***	0.14	0.48	0.15	0.48	0.14
Di-homo- $\gamma$ -linolenic acid (20:3 <i>n</i> -6)	0.20	0.05	0.26***	0.09	0.24	0.08	0.23	0.08
Arachidonic acid (20:4 <i>n</i> -6)	0.31	0.09	0.35	0.13	0.33	0.09	0.34	0.15
EPA (20:5 <i>n</i> -3)	0.04	0.01	0.04	0.03	0.04	0.01	0.05‡	0.03
Docosahexaenoic acid (22:6 <i>n</i> -3)	0.11	0.03	0.11	0.04	0.10	0.03	0.12†	0.03

Mean values were significantly different from those of the Cretan group: \*\*\* $P < 0.001$ .

Mean values were significantly different from those of male subjects: † $P < 0.05$ , ‡ $P < 0.01$ .

‡ For details of subjects and procedures, see Table 1 and p. 644.

the most significant predictor. The addition of any other combinations of FA in the regression analysis models did not increase further the overall significance of the models.

## Discussion

The present study has identified a positive relationship between adipose tissue AA and BMI in children aged 10–12 years old from two different Mediterranean islands. Adipose tissue AA, di-homo- $\gamma$ -linolenic acid and docosahexaenoic acid were higher in overweight and obese subjects compared with normal-weight subjects, but AA explained BMI variance better than any other *n*-6 or *n*-3 FA; all obese children were in the 4th AA quartile and 88.9% of overweight children were in the 3rd and 4th AA quartile.

There are some shortcomings in the present study. These include the relatively small number of subjects included in the analysis and the cross-sectional nature of the study. Therefore, a causal relationship between AA and overweight risk cannot be established.

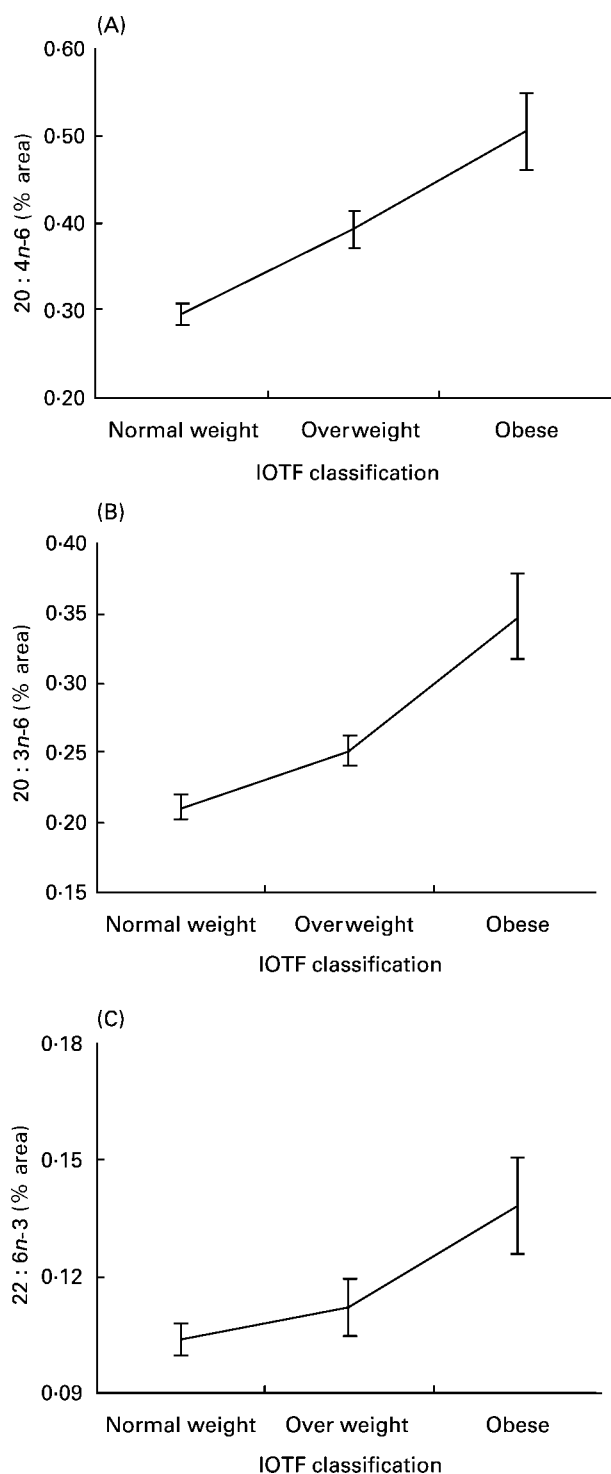
There is evidence, however, that could possibly explain the higher levels of adipose tissue AA in obese children, namely increased serum levels of AA, altered LA metabolism and increased adipose tissue FA-trapping in obese subjects. Obese children have been shown to have higher plasma *n*-6 FA than their normal-weight peers: this difference was attributed to an enhanced  $\Delta^6$ -desaturase activity and increased insulin-induced *n*-6 FA synthesis (Decsi *et al.* 1996, 2000). The uptake, on the other hand, of long-chain NEFA in Zucker rats, which are genetically susceptible to obesity, has been shown to be enhanced in adipocytes but not in other cell lines such as hepatocytes or cardiomyocytes (Berk *et al.* 1997). Similarly, the uptake of FA by subcutaneous adipose tissue adipocytes has been found significantly greater in obese women compared with lean (Kalant *et al.* 2000). The composition of adipose tissue in PUFA varies depending on the particular FA. LA, an essential FA not produced *in vivo*, is highly correlated

with dietary intake of LA, not only in the short term (Plakke *et al.* 1983), but also in the long term where multiple longitudinally dietary assessments showed a strong correlation between dietary intake and adipose tissue concentration of LA (Katan *et al.* 1986; Van Staveren *et al.* 1986). There is no similar correlation between adipose tissue AA content and dietary intake of AA (Garland *et al.* 1998), since adipose tissue AA is associated with dietary supply of AA and to *in vivo* desaturation and elongation of essential FA (Zhou & Nilsson, 2001). Serum levels of AA vary depending on diet composition and also on supplementation of diets with certain *n*-3 and *n*-6 FA. Thus, a diet supplemented with EPA and  $\alpha$ -linolenic acid does not increase serum AA levels in human subjects, as a diet supplemented only with AA does (Barham *et al.* 2000).

There is also evidence that AA mediates adipogenesis. A recent study showed that AA enhances adipogenesis *in vitro*, whereas mice fed a diet rich in LA were heavier compared with mice fed a standard diet enriched with a mixture of LA and  $\alpha$ -linolenic acid. Fat mass was also increased in the group fed the LA-enriched diet (Massiera *et al.* 2003). Several prostaglandins, i.e. AA derivatives, were found to mediate adipogenesis (Darimont *et al.* 1994; Long *et al.* 1996; Reginato *et al.* 1998). Prostaglandins J<sub>2</sub> and F<sub>2 $\alpha$</sub>  promote and block adipogenesis respectively, mediating this effect by activating or inhibiting the nuclear hormone receptor PPAR. The balance, therefore, of these eicosanoids may be important in the development of obesity (Reginato *et al.* 1998). Another derivative of AA, prostaglandin E<sub>2</sub>, suppresses GLUT4 mRNA, which was also found to be mediated by PPAR; thus, AA may be important in exacerbation of diabetes (Long *et al.* 1996).

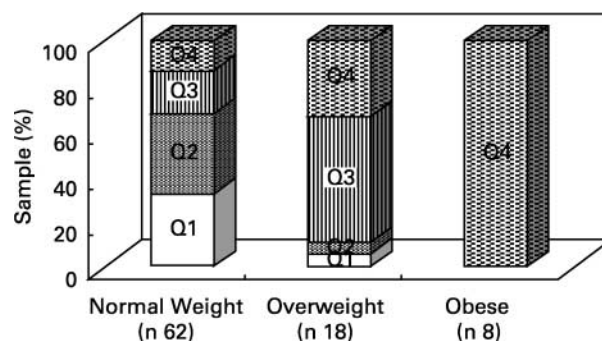
The results of the present study are important in the setting of an increasing prevalence of obesity. There is a high prevalence of obesity in the two Mediterranean islands in the present study; the estimates of the incidences of obesity and overweight in the present study are similar to those reported in the two islands by Mamalakis *et al.* (2000)





**Fig. 1.** Subcutaneous adipose tissue fatty acid composition in relation to the International Obesity Task Force (IOTF; Cole *et al.* 2000) classification for overweight and obesity in children from Crete and Cyprus (*n* 88). For details of subjects and procedures, see Table 1 and pp. 644–645. Values are means with standard errors of the means shown by vertical bars. (A),  $P < 0.001$ ; (B),  $P < 0.001$ ; (C),  $P < 0.022$ .

and Savva *et al.* (2002). The dietary habits in these two islands as reflected in their adipose tissue FA composition, however, are different; subcutaneous adipose tissue MUFA were significantly higher in Cretan children. This finding



**Fig. 2.** Adipose tissue arachidonic acid quartiles in relation to weight status in children from Crete and Cyprus. For details of subjects and procedures, see Table 1 and pp. 644–645. Q, quartile.  $\chi^2$  44.99, 6 df,  $P < 0.001$ .

**Table 3.** Stepwise multiple regression analysis with BMI as the dependent variable\*†‡

Predictors	B***	SEB	R <sup>2</sup> change	P value
<b>Model 1§</b>				
Constant	26.7	1.0	—	<0.001
Log AA	14.3	2.0	0.382	<0.001
<b>Model 2  </b>				
Constant	28.9	1.7	—	<0.001
Log DGLA	18.7	2.7	0.300	<0.001
Country	2.0	0.7	0.056	0.008
<b>Model 3¶</b>				
Constant	29.2	1.6	—	<0.001
Log AA	9.4	2.4	0.382	<0.001
Log DGLA	10.6	3.3	0.045	0.002
Country	1.5	0.7	0.028	0.041
<b>Model 4**</b>				
Constant	29.2	1.6	—	<0.001
Log AA	9.4	2.4	0.382	<0.001
Log DGLA	10.6	3.3	0.045	0.002
Country	1.5	0.7	0.028	0.041

AA, arachidonic acid; DGLA, di-homo- $\gamma$ -linolenic acid.  
 \* For details of subjects and procedures, see Table 1 and pp. 644–645.  
 † Only models with significant predictors are presented.  
 ‡ Gender: male 1, female 2; country: cyprus 1, crete 2.  
 § Independent variables: age, gender, country, log AA;  $R^2$  0.382.  
 || Independent variables: age, gender, country, log DGLA;  $R^2$  0.356.  
 ¶ Independent variables: age, gender, country, log AA, log DGLA;  $R^2$  0.454.  
 \*\* Independent variables: age, gender, country, log linoleic acid, log  $\alpha$ -linolenic acid, log  $\gamma$ -linolenic acid, log AA, log DGLA, log EPA, log docosahexaenoic acid;  $R^2$  0.454.  
 \*\*\* B, unstandardized coefficient; SEB, standard error of B.

was not unexpected, since the Cretan population consumes a high-fat diet (45 % energy as fat) that is rich in oleic acid (27 % energy; Aravanis *et al.* 1988). Cypriot children have lower total fat consumption (about 35 % energy) and lower MUFA consumption (about 15 % energy, based on 24 h dietary recall) than Cretan children (Child Health Foundation, Cyprus database 1997–2002, unpublished results). In summary, the present study has identified a significant strong association between adipose tissue *n*-6 FA and BMI. AA explained BMI variance better than any other PUFA. These associations are reflected in the higher adipose tissue levels of PUFA, especially of AA, in overweight and obese subjects. Further research, however, is needed to estimate whether this association is causal, that is, if a high concentration in adipose tissue

AA predisposes to obesity. Further research could also link dietary habits to adipose tissue PUFA composition.

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