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Non-esterified fatty acid levels and physical inactivity: the relative importance of low habitual energy expenditure and cardio-respiratory fitness

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The fasting concentration of non-esterified fatty acids (NEFA) and the degree to which it declines during an oral glucose tolerance test are closely associated with insulin resistance and glucose intolerance. However, relatively few studies have described possible environmental determinants of NEFA concentrations. Physical activity is likely to be related to NEFA levels, but habitual activity level is difficult to quantify in epidemiological studies. In particular, it is unclear whether NEFA is more closely related to cardio-respiratory fitness or to habitual energy expenditure. In order to quantify these relationships, we analysed data from the Ely prospective population-based study in which 931 subjects underwent a glucose tolerance test with measurements of cardio-respiratory fitness and 4 d energy expenditure by heart-rate monitoring, a technique previously validated against whole-body calorimetry and doubly-labelled water. In order to estimate the latent variables of usual fitness and energy expenditure, a subset of 190 subjects underwent repeat testing on three further occasions over 1 year. In analyses adjusting only for age and sex, energy expenditure and cardio-respiratory fitness were both negatively correlated with the total area under the NEFA curve following the oral glucose load (standardised β coefficients −0·030 and −0·039 respectively; both $P<0·001$) However, further adjustment for degree of obesity and bivariate measurement error suggested that the effect of energy expenditure was significantly greater than that for fitness (−0·047 and −0·005 respectively). These results suggest that the area under the NEFA curve in the oral glucose tolerance test, a measure of insulin sensitivity, is strongly associated with the habitual level of physical activity.

Bivariate error correction: Energy expenditure: Cardio-respiratory fitness: Non-esterified fatty acid: Heart-rate monitoring

Insulin resistance is a key element in the pathophysiology of type 2 diabetes and heart disease (Reaven, 1988). Elevated fasting non-esterified fatty acid (NEFA) levels are observed in individuals with diabetes and these levels are not suppressed in response to insulin to the same degree as in individuals with normal glucose tolerance (Byrne *et al.* 1994). Similar but less marked abnormalities of NEFA metabolism are observed in subjects with insulin resistance (Byrne *et al.* 1995). Some, but not all, studies have shown that elevated NEFA levels predict the development of diabetes, suggesting that the lipid abnormality may be a part of the causal pathway leading to hyperglycaemia and diabetes (Charles *et al.* 1991; Paolisso *et al.* 1993; Byrne *et al.* 1995).

Physical inactivity is strongly predictive of type 2 diabetes (Helmrich *et al.* 1991; Manson *et al.* 1991) and the causal nature of this relationship is indicated by the impact of physical activity interventions in people at risk of developing diabetes such as those with impaired glucose tolerance (Pan *et al.* 1997; Tuomilehto *et al.* 2001). Physical activity also impacts upon lipid metabolism (Wolfe, 1998), but most previous studies have concentrated on the acute rather than chronic effects of activity, and in population cohorts NEFA has been rarely measured.

Abbreviations: AUC, area under the curve; EE, energy expenditure; NEFA, non-esterified fatty acid; PAL, physical activity level; $\dot{V}O_2$max, maximum oxygen uptake.

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Those studies that have examined the effects of chronic activity and NEFA tend to be small-scale and involve comparisons of trained and untrained individuals (Martin, 1996; Halle *et al.* 1999). These studies leave major uncertainties about the strength of the association and which dimension of physical activity is most strongly associated with lipid metabolism.

Physical activity is not a simple exposure to measure in epidemiological studies, particularly when it is sub-divided into its underlying dimensions. In the context of lipid metabolism, it is uncertain whether the effects of activity are more closely related to energy expenditure (EE) or cardio-respiratory fitness. The latter is conventionally defined as: 'the health-related component of physical fitness that relates to the ability of the circulatory and respiratory systems to supply oxygen during sustained physical activity' (United States Department of Health and Human Services, 1996). It is usually measured by assessing maximum O_2 uptake ($\dot{V}O_2max$) Cardio-respiratory fitness is thus one element of physical fitness in general, which is defined as: 'a set of outcomes or traits that relate to the ability to perform physical activity' (United States Department of Health and Human Services, 1996) Quantifying the effects of habitual EE on metabolic outcomes is difficult because of the complexity of measuring usual activity in free-living individuals (Wareham & Rennie, 1998). It is also difficult to separate the aetiological effects of the different dimensions of activity such as EE or fitness. These partially correlated phenomena are measured with different degrees of measurement error and therefore inferences about their respective aetiological importance cannot be made without understanding how they relate to each other and the precision with which they are measured (Wareham *et al.* 2000). Advances in statistical methods have recently allowed correction for this form of bivariate measurement error in epidemiological studies (Wong *et al.* 1999). However, these analyses are dependent upon accumulating a dataset with objective measures of both variables of interest with a repeated measures sub-cohort to allow measurement error to be quantified. One such objective method for quantifying EE is the FLEX heart-rate method with individual calibration, a technique which has previously been validated against whole-body calorimetry and doubly-labelled water (Spurr *et al.* 1988; Ceesay *et al.* 1989; Livingstone *et al.* 1991), and is suitable for use in epidemiological studies (Wareham *et al.* 1997). We describe here a study designed to explore the relationship between habitual EE and NEFA concentrations and to address whether NEFA is affected more by cardio-respiratory fitness, as measured by the O_2 uptake capacity of the individual, or by their usual EE.

Methods

The volunteers were participants in the Ely study, a continuing population-based cohort study in Ely, Cambs., UK, the design of which has been described previously (Wareham *et al.* 1997). The original sample of 1122 individuals without known diabetes were recruited in 1990–2 at random from a population-based sampling frame consisting of all people in Ely aged 40–65 years in 1990 (Williams *et al.* 1995). The initial response rate was 74 %. In 1994–7, a 4·5-year follow-up study was undertaken of all those individuals who did not have diabetes by WHO criteria at baseline (*n* 1071; WHO, 1985) Twenty subjects had died in the interim and 937 of the remaining volunteers attended for follow-up (restudy rate 89 %). These individuals constituted the sample for this particular study, of which 83 % agreed to participate. Sixty-four of the 162 individuals who did not undertake all the tests were excluded for medical reasons including angina or dysrhythmia, treatment with β-blocking agents or the presence of a pacemaker. A further 156 individuals, who had been recruited following the same criteria described earlier, were drawn from an extension of the original sampling frame (aged 31–72 years). These people, as well as the 775 people from the original cohort (*n* 931), attended the clinic at 08.30 hours having fasted since 22.00 hours the previous evening. At this visit, height and weight were measured in light clothing and body circumference was measured in duplicate using a metal tape. Body fat (%) was obtained using a standard impedance technique (Bodystat, Isle of Man, UK). Blood pressure was measured using an Accutor automatic sphygmomanometer (Datascope, Montvale, NJ, USA) while the participant was seated.

Assessment of blood lipids: non-esterified fatty acid concentration following an oral glucose tolerance test

Following the collection of standard anthropometric data detailed earlier, all participants provided informed consent and received an explanation of the procedure for the collection of blood. A sample of fasting blood was taken, and participants drank 75 g anhydrous glucose (BMS Laboratories, Beverley, Yorks., UK) dissolved in 250 ml water over 2–5 min. Further blood samples were then taken at 30 and 120 min. Participants completed a self-report questionnaire enquiring about habitual diet and alcohol consumption, smoking, and current and past employment, based on the Health and Lifestyle Survey (Cox *et al.* 1993). Ethical permission for the study was granted by the Cambridge Local Research Ethics Committee.

Assessment of resting and exercise oxygen consumption: heart-rate relationship

Following the measurement of anthropometry and blood pressure, a standard protocol for individually calibrating heart rate and EE was used (Ceesay *et al.* 1989). This method relies on the computation for each individual of resting EE, and the slope and intercept of the regression line describing the linear relationship between heart rate and EE during exercise. The final variable that is measured is the FLEX heart rate, which is the level used in the method to distinguish between resting and activity. When heart-rate data is collected over the following 4 d, EE when the heart rate is lower than FLEX is assumed to be equal to resting EE, and when it is greater then FLEX it is considered to represent activity. The FLEX point and level of activity is predicted by the simple linear regression computed during the calibration. In the present study, O_2 consumption, EE and heart rate were measured at rest with

the participant lying supine and then seated. Participants bicycled on a cycle ergometer at several different workloads to provide the slope and the intercept of the line relating EE to heart rate. Each participant cycled at 50 rpm and the workload was increased progressively from 0·0 to 37·5, 75·0 and 125·0 W in 5 min stages. At each workload three separate readings were made of heart rate, minute volume and expired air O_2 concentration using indirect calorimetry (PK Morgan Ltd, Gillingham, Kent, UK). The volunteer wore a mouthpiece and nose clip enabling ambient air to be freely breathed and expired air to be collected for minute-by-minute on-line analysis. Mean resting EE was taken as the mean value of the lying and sitting values. The FLEX heart rate was calculated as the mean of the highest resting pulse rate and the lowest on exercise. Finally, the slope and intercept of the least squares regression line of the exercise points were calculated. $\dot{V}O_2$max was measured from the linear regression as predicted O_2 consumption at maximal heart rate (220 – age (years)) and was expressed per unit body weight in the results. The volunteers wore the heart-rate monitor (Polar Electro, Oy, Kempele, Finland) continuously during the waking hours over the 4 d following their visit to the clinic.

Heart-rate readings were downloaded directly into a computer via a serial interface and the individual calibration data were used to predict minute EE for each person. Sleeping EE was calculated as 95 % BMR where this was derived from published prediction equations (James & Schofield, 1990). A physical activity level (PAL), which is total EE: BMR, was computed for each day and averaged over the 4 d period.

Repeated-measures sub-study

A random group of 190 participants in the cohort re-attended for measurements on a further three occasions at intervals of 4 months for 1 year, when height, weight and impedance were also re-measured. The calibration between heart rate and resting and exercise EE was repeated and the volunteers then underwent 4 d heart-rate monitoring.

Statistical analyses

The within-subjects and between-subjects mean squares and the reliability coefficients for PAL, height, weight, BMI, % body fat and $\dot{V}O_2$max/kg were estimated using the formulas described by Armstrong *et al.* (1994). By this method, each of n subjects is measured k times with X_{ij} being the jth measure of subject i. $\bar{X}_i$ is the mean of k measurements in subject i, and $\bar{X}$ is overall mean. The reliability coefficient is given by:

$$(\mathrm{BMS} - \mathrm{WMS})/(\mathrm{BMS} + (\mathrm{k} - 1)\mathrm{WMS}),$$

where BMS is the between–subjects mean equal to $\mathrm{k}\sum\mathrm{i}(\bar{X}_i - \bar{X})2/(n - 1)$ and WMS is the within-subjects mean square equal to $\sum\mathrm{i}\sum\mathrm{j}(X_{ij} - \bar{X}_i)2/n(\mathrm{k} - 1)$. Simple and multiple linear regressions were undertaken using SAS (version 8 for Windows; SAS Insititute Inc., Cary, NC, USA) and the regression coefficients per standard deviation for each variable were calculated. The univariate correction was undertaken using the reliability coefficient (Armstrong *et al.* 1994). The multivariate correction factors for PAL and $\dot{V}O_2$max/kg were estimated using the method described by Wong *et al.* (1999). Both the univariate and bivariate correction factors were calculated under the assumption that the errors associated with repeated measures on the same individual were independent.

Results

Table 1 shows the characteristics of the 400 men and 531 women who took part in this study. Men and women were of similar age, but BMI and waist:hip ratio were significantly greater in the men than the women. The mean anthropometric characteristics were comparable with those values observed in nationally representative samples, suggesting that this cohort is not selected on the basis of degree of overweight (Colhoun & Prescott-Clarke, 1996). The mean values for the PAL and $\dot{V}O_2$max were both significantly ($P < 0{\cdot}001$) greater in men. Comparison with national cohorts to determine the magnitude of possible selection bias is not possible for EE as there are no comparable data on a population level. The values of $\dot{V}O_2$max are similar to those observed in participants in the Allied Dunbar National Fitness Survey (The Sports Council and The Health Education Authority, 1992). As described previously (Keins, 1998), the fasting and 30 min non-esterified fatty acid (NEFA) concentrations were higher in women than men, as was the total area under the NEFA suppression curve (AUC).

The focus of the present analysis was on the relationship between NEFA concentrations and measures of physical activity and fitness. We have demonstrated previously that the NEFA AUC is associated with glucose intolerance and features of the metabolic syndrome (Byrne *et al.* 1994, 1995). Therefore, in order to simplify further analyses, the NEFA AUC during the oral glucose tolerance test was considered as the outcome variable. Table 2 describes the pattern of the relationship between this NEFA AUC and the key potential determinants. In both men and women, NEFA AUC was positively correlated with age, % body fat and the waist:hip ratio. However, it was negatively correlated with the PAL and $\dot{V}O_2$max. The correlation with NEFA AUC was significant for $\dot{V}O_2$max and for PAL, although the magnitude of the relationship was greatest for $\dot{V}O_2$max, a finding displayed in Figs 1 and 2. This might lead some to conclude that $\dot{V}O_2$max was a more important determinant of the NEFA AUC than PAL. However, such a conclusion would ignore possible confounding errors and the different degrees of measurement error with which these variables are estimated.

In order to take confounding and measurement error into account, we developed a series of multivariate regression models, the results of which are shown in Table 3. So that strength of association could be compared, the regression coefficients have been standardised for both the dependent and the independent variable. They can therefore be interpreted as the proportion of a standard deviation change in the outcome variable for a standard deviation change in the exposure variable. The simple

Table 1. Characteristics of the participants: the Ely study 1994–7
(Mean values and standard deviations or geometric mean values and 95 % confidence intervals)

	Men (*n* 400)		Women (*n* 531)		Statistical significance of difference between sexes (*t* test)
	Mean	SD	Mean	SD	
Age (years)	54	11·03	53	10·40	
Body fat (%)	24·0	5·09	37·0	6·59	***
Waist:hip ratio	0·95	0·07	0·80	0·07	***
BMI (kg/m^2)	26·8	3·50	26·1	4·79	*
PAL	1·95	0·39	1·77	0·36	***
$\dot{V}O_2$max (ml/kg per min)	33	8·02	26	6·93	***
Fasting NEFA (mmol/l)§	0·45	0·19	0·54	0·24	***
NEFA at 30 min (mmol/l)§	0·30	0·14	0·32	0·18	**
NEFA at 120 min (mmol/l)§	0·08	0·05	0·06	0·03	***
NEFA AUC (mmol/l per h)§	0·47	0·19	0·51	0·24	**
HDL-cholesterol (mmol/l)	1·33	0·36	1·64	0·41	***
LDL-cholesterol (mmol/l)	4·03	0·95	3·87	1·04	*
Triacylglycerol (mmol)	1·35†	1·29, 1·42‡	1·11†	1·07, 1·16‡	***
Fasting glucose (mmol/l)§	5·13	0·93	4·80	0·54	***
Glucose at 30 min (mmol/l)§	8·43	1·84	7·29	1·60	***
Glucose at 120 min (mmol/l)§	5·82	2·33	5·67	1·67	NS
Fasting insulin (pmol/l)§	43·06†	40·45, 45·60‡	38·34†	36·53, 40·25‡	**
Insulin at 30 min (pmol/l)§	306·6†	290·0, 323·8‡	288·6†	275·9, 301·6‡	*
Insulin at 120 min (pmol/l)§	211·1†	194·4, 230·0‡	230·1†	224·1, 237·2‡	NS

PAL, physical activity level; $\dot{V}O_2$max, maximum oxygen uptake; NEFA, non-esterified fatty acid; AUC, area under the curve.
*$P<0·05$, **$P<0·01$; ***$P<0·001$.
† Geometric mean value.
‡ 95 % CI.
§ Values determined during oral glucose tolerance test: for details of procedures, see p. 308.

regression coefficient adjusted only for age and sex is more strongly negative for the fitness variable ($\dot{V}O_2$max) than for PAL, supporting the observation seen in the unadjusted analysis. However, further adjustment for confounding by % body fat attenuates the effect of fitness to a greater extent than physical activity. In the simple model that included each other as a potential covariate, the attenuation of fitness was increased, whereas the variable estimate for physical activity was largely unchanged. The adjustment for measurement error using the results of the repeated measures sub-study suggests that the coefficient for PAL would be at least twofold that which was observed without correction for error and it remains statistically significant ($P<0·05$) Conversely, the term for fitness is markedly attenuated and becomes non-significant. These results would suggest that habitual PAL is a more important determinant of NEFA concentrations than fitness, a conclusion directly opposite to that which have been deduced from the simple analysis.

Discussion

The present study investigated the relationships of habitual EE and cardio-respiratory fitness with plasma NEFA concentrations following an oral glucose tolerance test in 931 men and women from the population-based Ely study in Cambs., UK. The design of the study, incorporating a sub-study with repeated measures of fitness and EE using objectives methods, has allowed the relative importance of these two exposures to be determined. Our present

Table 2. Unadjusted correlations between exposure covariates for men (*n* 400) and women (*n* 531) in the Ely study 1994–7†
(Correlation coefficients)

	Age (years)	Body fat (%)	Waist:hip ratio	PAL	$\dot{V}O_2$max	NEFA AUC (mmol/l per h)	Fasting insulin (pmol/l)	2 h glucose (mmol/l)
Age (years)		*0·44****	*0·31****	*0·10**	*−0·43****	*0·19****	*0·06*	*0·21****
Body fat (%)	0·50***		*0·71****	*−0·07*	*−0·47****	*0·36****	*0·40****	*0·29****
Waist:hip ratio	0·19***	0·48***		*−0·10*	*−0·36****	*0·35****	*0·41****	*0·24****
PAL	−0·02	−0·11*	−0·01*		*0·29****	*−0·14***	*−0·03*	*−0·10***
$\dot{V}O_2$max (ml/kg per min)	−0·36***	−0·48***	−0·24***	0·44***		*−0·24****	*−0·15****	*−0·50**
NEFA AUC (mmol/l per h)	0·10**	0·21***	0·20***	−0·12**	−0·16***		*0·16****	*0·30****
Fasting insulin (pmol/l)	0·55	0·54***	0·45***	−0·12***	−0·20***	0·26***		*0·27****
2 h glucose (mmol)	0·20***	0·39***	0·28***	−0·20***	−0·21***	0·50***	0·35***	

PAL, physical activity level; $\dot{V}O_2$max, maximum oxgen uptake; NEFA, non-esterified fatty acid; AUC, area under the curve.
* $P<0·05$, ** $P<0·01$, *** $P<0·001$.
† For details of subjects and procedures, see Table 1 and p. 308.
Men: values non-italic.
Women: values in italics.

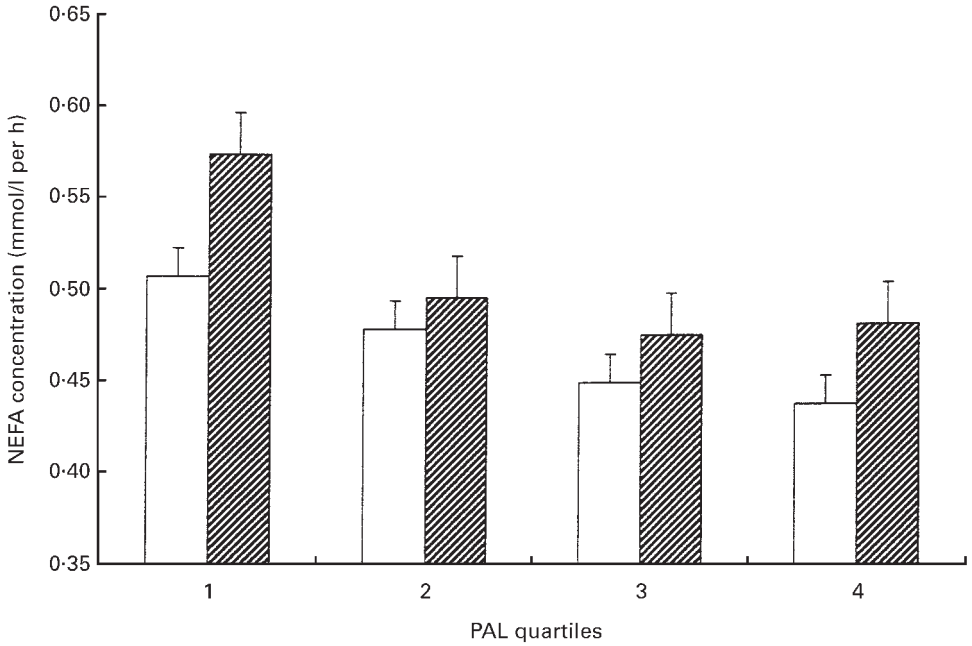

Fig. 1. Total area under the non-esterified fatty acid (NEFA) suppression curve by quartile of physical activity level (PAL): the Ely study, 1994–7. □, Men (*n* 400); ▨, women (*n* 531). For details of subjects and procedures, see Table 1 and p. 308. Values are means with standard errors shown by vertical bars. Interquartile ranges for men: 1, <1·66; 2, 1·66–1·90; 3, 1·91–2·17; 4, >2·17. Interquartile ranges for women: 1, <1·52; 2, 1·52–1·72; 3, 1·73–1·93; 4, >1·93.

results indicate that although the simple univariate or age- and sex-adjusted results suggest that cardio-respiratory fitness is the more strongly negatively correlated with NEFA concentrations, this conclusion is reversed when allowance is made for the precision with which habitual EE and fitness are estimated from a single measurement. This conclusion highlights the importance of considering measurement error when determining the magnitude of association in epidemiological studies.

Although we included the potential confounding factors

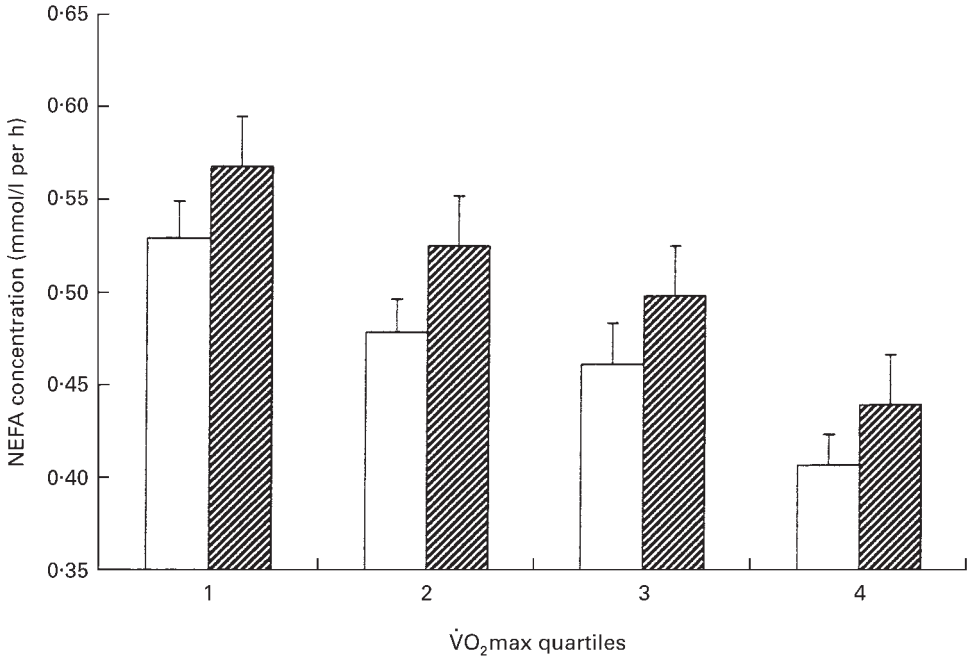

Fig. 2. Total area under the non-esterified fatty acid (NEFA) suppression curve by quartile of $\dot{V}O_2$max: the Ely study, 1994–7. □, Men (*n* 400), ▨, women (*n* 531). For details of subjects and procedures, see Table 1 and p. 308. Values are means with their standard errors shown by vertical bars. Interquartile ranges for men: 1, <27·5; 2, 27·5–32·4; 3, 32·5–37·9; 4, >37·9. Interquartile ranges for women: 1, <21·3; 2, 21·3–25·0; 3, 25·1–29·0; 4, >29·0.

Table 3. Effect of adjusting for confounding and measurement error on the regression coefficients for physical activity level and maximum oxygen uptake predicting the total area under the non-esterified fatty acid curve following an oral glucose tolerance test; the Ely study 1994–7 (*n* 931)†‡

(Data shown are standardised β coefficients and standard errors)

	PAL		$\dot{V}O_2$max (ml/kg per min)	
	Coefficient	SE	Coefficient	SE
Adjusted for age, sex	−0·0298	0·0075***	−0·0387	0·0086***
Adjusted for age, body fat (%), sex	−0·0238	0·0074**	−0·0210	0·0091*
Adjusted for age, body fat (%), sex, and PAL or $\dot{V}O_2$max as appropriate	−0.0202	0.0080*	−0.0109	0.0099
Adjusted with univariate correction	−0·0553	0·0228*	−0·0167	0·0151
Adjusted with bivariate correction	−0·0466	0·0195*	−0·0045	0·0153

PAL, physical activity level; $\dot{V}O_2$max, maximum oxygen uptake.
*$P<0·05$, **$P<0·01$, ***$P<0·001$.
† For details of subjects and procedures, see Table 1 and p. 308.
‡ In the first two models, PAL and $\dot{V}O_2$max are treated separately. In the final three models, both variables are included in the model.

of age, sex and % body fat, residual confounding may still exist. It is possible, for example, that NEFA concentrations following an oral glucose load may be affected by dietary fat intake. As fat intake could also be associated with physical activity patterns, this would introduce the potential for confounding. However, our present study design did not include repeated measures of dietary intake, and so it would be difficult to adjust appropriately as the degree of measurement error would be unknown. Even if repeated dietary assessment were available, this would not necessarily allow estimation of habitual intake, because repeated assessment with a subjective instrument may give rise to correlated error. The use of repeated measures of an objective technique such as heart-rate monitoring is fundamentally different, because there is no reason to suppose that the error in one measurement is systematically related to that on a different occasion.

Volunteers for the present study were randomly selected from a population sampling frame. The allocation of days on which volunteers attended our laboratory and on which EE was assessed was random and involved week and weekend days. We have previously demonstrated that there is no overall difference in energy expended on week and weekend days (Wareham *et al.* 1997). In view of these points, it is unlikely that selection bias could explain our results.

Previous studies that have examined the effect of training on uptake of NEFA have almost exclusively involved the comparison of trained and untrained subjects (Keins, 1998). Because these studies are cross-sectional and involve the comparison of two fundamentally different populations, one is unable to determine whether differences in NEFA levels are due to training and hence greater cardio-respiratory fitness, higher levels of fitness irrespective of activity or overall EE. Moreover, the between group differences in lipid characteristics reported in these studies may also be due to factors that are not related to fitness or activity and which are not necessarily dealt with by matching. However, the general conclusion of these studies is that physical activity is negatively associated with plasma NEFA levels, a notion that is supported by the results in our present study.

Understanding how complex behaviours such as physical activity relate to intermediate metabolic variables such as NEFA levels is important to identify the strength of association and the specific sub-component that is most closely related to the outcome. This specificity becomes particularly important when considering the impact of genetic variants that may alter the relationship between physical activity and lipid levels. Such a gene–environment interaction would be virtually undetectable in a study in which physical activity was measured imprecisely (Luan *et al.* 2001).

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