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ORIGINAL CONTRIBUTION

Paraoxonase activity in Greek migrants and Anglo-Celtic persons in the Melbourne Collaborative Cohort Study: relationship to dietary markers

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Summary *Background* Greek migrants to Australia have low all-cause and cardiovascular disease (CVD) mortality. This may be partly due to maintenance of a traditional Mediterranean diet and its interaction with CVD risk factors. The enzyme paraoxonase-1 (PON1) is thought to contribute to the anti-atherogenic properties of high density lipoproteins (HDL) by metabolizing lipid peroxides. PON1 activity is subject to modulation by dietary and genetic factors. *Aims* To determine PON1 activity in Greek migrants and Anglo-Celtic subjects recruited from the Melbourne Collaborative Cohort Study, and its relationship to coronary risk factors and dietary markers. *Methods* Greek (n = 127) and Anglo-Celtic (n = 128) participants in the MCCS were recruited. By design, there were approximately equal numbers of men and women and of diabetic and non-diabetic subjects. Subjects were screened for glucose tolerance, dyslipidaemia, hypertension and coronary heart disease. Plasma markers of diet (carotenoids, retinol, tocopherol, homocysteine) and inflammation (C-reactive protein) were assessed. Serum PON1 activity was determined spectrophotometrically using two substrates: paraoxon (paraoxonase) and phenylacetate (arylesterase). *Results* PON1 activity was significantly higher in the presence of hyperlipidaemia but otherwise did not vary by ethnicity, presence of coronary heart disease, diabetes, hypertension or smoking. Among subjects with the high activity phenotype (defined by the ratio of paraoxonase:arylesterase activity), paraoxonase activity correlated directly with circulating diet-derived carotenoid concentrations for Greeks, and inversely with homocysteine and C-reactive protein for Anglo-Celtics. No such associations were seen among subjects with the low activity phenotype. *Conclusions* The data suggest that dietary modulation of atherosclerotic risk may vary according to PON1 phenotype.

Key words paraoxonase – carotenoids – Greek migrants – Anglo-Celtic Australians – cardiovascular risk factors – C-reactive protein – homocysteine

Introduction

Despite having a cardiovascular mortality advantage, Greek migrants to Australia have similar or higher prevalences of the major risk factors for coronary heart disease (CHD) compared with Australian-born persons: smoking, obesity, hypertension and a 2–3 times higher prevalence of known diabetes in the 45–69 year age group [1]. There is considerable interest in the role of the Mediter-

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ranean diet in protection from cardiovascular disease [2], and this dietary regimen has been maintained by Greek migrants in Australia [3]. Hence an explanation for the relatively low CHD mortality of this ethnic group may lie partly in dietary factors and their potential interactions with novel risk factors for vascular disease, such as insulin resistance and oxidative stress.

The dyslipidaemia of insulin resistance includes low concentrations of HDL cholesterol and elevated LDL particles of the small, dense phenotype that are more susceptible to oxidation. HDL has important protective functions which include anti-oxidant and anti-inflammatory activity. The enzyme paraoxonase (PON1), a 43 kDa protein located specifically on HDL particles in the circulation, is thought to prevent accumulation of oxidised lipids in LDL particles, a known contributor to atherogenesis [4]. PON1 activity is modulated by dietary factors and smoking [5–7]. A genetic polymorphism at position 192 of the PON1 molecule confers low, high or intermediate activity against the substrate paraoxon in individuals homozygous for a glutamine substitution (192-QQ), homozygous for arginine (192-RR) or heterozygous (192-QR) respectively. Studies of genotype in prediction of CHD risk are inconsistent [8] but CHD has been associated with low PON1 activity [9], as well as in the presence of diabetes, peripheral neuropathy and familial hypercholesterolaemia [10, 11]. We have previously reported positive associations of diet-derived antioxidants with PON1 activity in an Australian population group with poor dietary quality [12].

The aim of the present study was to examine PON1 activity in Greek migrants and Australian-born (largely Anglo-Celtic) persons, and its relationship to other risk markers, including dietary antioxidants.

Materials and methods

This study was approved by the Deakin University Ethics Committee (the institution responsible for the study during the recruitment phase), the St Vincent's Hospital Human Research Ethics Committee, and the Melbourne Collaborative Cohort Study (MCCS) Scientific Advisory Committee. Participants in this study provided written, informed consent to participate. The study was conducted in accordance with the NHMRC National Statement on Ethical Conduct in Research Involving Humans.

Subject recruitment and sample collection

Between 1995 and 1997, Greek and Australian-born (largely Anglo-Celtic), diabetic and non-diabetic subjects from the MCCS [13] who had previously indicated a willingness to participate in further studies were recruited to a survey of CHD risk factors [14]. Diabetic status was confirmed by 2 hour oral glucose tolerance test and the presence of CHD by ECG, self-reported history and administration of the Rose questionnaire. Hypercholesterolaemia was defined as plasma total cholesterol ≥ 5.5 mmol/L, hypertriglyceridaemia as fasting plasma triglycerides ≥ 2.0 mmol/L. Smoking status was determined by standardised questionnaire. Screening included collection of a fasting blood sample into a serum tube that was left at room temperature for 1 hour, then held on ice until centrifugation and separation of serum. Serum samples were stored at –70 °C thereafter (until assay for PON1 activity in 2001). A sample of 214 stored serum samples collected from these subjects was selected by taking each alternate sample in the series. In addition, samples from all further subjects with CHD (n = 41 additional subjects) were included in order to maximize the statistical power of comparisons with non-CHD subjects.

Biochemical methods

PON1 activity was measured against two substrates: paraoxon (1 mM; paraoxonase activity) and phenyl acetate (5 mM; arylesterase activity). These two substrates are routinely used to assess PON1 activity [4]. Serum samples were first incubated with 5 mM of eserine for 10 minutes at room temperature to inhibit butylcholinesterase activity. Paraoxonase activity was determined spectrophotometrically in 50 mM Tris/1 mM $CaCl_2$ pH 8.0 buffer at 25 °C and calculated from change in absorbance at 412 nm over 6 minutes, expressed as nmol converted/minute/mL of plasma (U/L). Arylesterase activity was determined in 30 mM Tris/1.5 mM $CaCl_2$ pH 8.0 buffer at 25 °C and calculated from change in absorbance at 270 nm over 3 minutes, expressed as μmol converted/minute/mL of plasma (U/mL) [15, 16]. All samples were run in duplicate with blanks to correct for spontaneous hydrolysis of substrate and with a quality control in each run. Reagents were obtained from a commercial supplier (Sigma-Aldrich, Castle Hill, NSW, Australia). Inter-assay coefficient of variation was 12 % for paraoxonase and 6 % for arylesterase activities. Low and intermediate/high activity phenotypes (the latter being a surrogate marker for the presence of the 192-R allele) were identified from the ratio of paraoxonase to arylesterase activity [16]. In a separate series of healthy persons (n = 46) for whom PON1–192 genotype was determined by restriction fragment length polymorphism techniques, this ratio predicted the presence of the 192-R allele with sensitivity of 94 % and specificity of 83 % in our hands. Sensitivity was lower in a series of diabetic patients (n = 81) at 85 % (E. Glare, C. L. Nelson, A. J. Jenkins et al., unpublished data).

Plasma antioxidants were assayed using HPLC as

previously described [17]. Homocysteine was measured by fluorescence polarisation immunoassay [18], CRP by a high sensitivity immunonephelometric assay (Dade-Behring Diagnostics, Lane Cove, NSW), and plasma glucose and lipids by standard enzymatic techniques with commercial reagents (Boehringer-Mannheim Australia, Sydney NSW). HbA_{1c} was measured using a commercial HPLC method (Diamat™ analyzer; BioRad Laboratories, North Ryde, Australia).

Dietary intake

Dietary intake was measured by a semi-quantitative food frequency questionnaire (FFQ) developed and validated by Ireland and colleagues [19]. Subjects were asked to complete the FFQ and consider each food as if it was in season. The frequency responses were converted to serves/day and multiplied by subject-specific portion sizes to obtain intake amounts in grams/day for each food in the 121-food frequency questionnaire. Olive oil and vegetable oil intakes were calculated from usage per month per household: the monthly household oil usage was converted to monthly per head consumption using MCCS baseline data on number of people in the household.

Statistical analyses

Variables with skewed distributions were log transformed prior to analysis and are reported as geometric mean (95% confidence interval). For comparisons of clinical characteristics of ethnic groups, continuous variables were tested by unpaired t-tests and categorical variables by Chi-square tests. Dietary intake was compared using Mann-Whitney U-test. General Linear Modelling was used to compare PON1 activities between ethnic groups and clinical categories, with age as a covariate and gender as a fixed factor. Bivariate associations of PON1 activity with other continuous variables were tested using Pearson product-moment correlations. Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago IL, USA).

Results

Subject characteristics

Numbers of diabetic persons (n = 64 and 65 in Anglo-Celtic and Greek groups respectively) and of men and women (n = 63 men and 65 women in the Anglo-Celtic group, n = 68 men and 59 women in the Greek group) in each ethnic group were similar by study design. There were 42 CHD cases in the Anglo-Celtic group and 36 cases in the Greek group. The ethnic groups did not differ significantly with respect to mean age and HDL cholesterol, nor with respect to prevalence of smoking, hypertension or hypercholesterolaemia (Table 1). Greek subjects had significantly higher mean BMI and lower prevalence of hypertriglyceridaemia (Table 1). Greek subjects had higher dietary intakes of vegetables and fruit, which was reflected in their higher plasma carotenoid concentrations, and higher olive oil intake (Table 1).

Distribution of PON1 phenotypes

PON1 phenotype was characterised according to the paraoxonase:arylesterase activity ratio. As in other populations [16], there was a clear partition between low activity (assumed to correspond to the 192-QQ genotype) and intermediate/high activity phenotypes (QR and RR; Fig. 1). Taking a paraoxonase:arylesterase activity ratio of 0.40 as a cut point (Fig. 1), the prevalence of the low activity phenotype in the Anglo-Celtic group (55%) did not differ significantly from that in the Greek migrants (61%; $\chi^2 = 0.922$, P = 0.337). This result was reflected in the similar cumulative frequency distribution curves of paraoxonase:arylesterase activity ratio in the two ethnic groups (Fig. 1 inset).

Associations of PON1 activity with clinical and behavioural characteristics

Average paraoxonase activity did not vary significantly by ethnicity, diabetes, CHD, hypertension or smoking

Table 1 Clinical and dietary characteristics of subjects

	Anglo-Celtic (n = 128)	Greek (n = 127)	P
Age (year)	64 (6)	62 (7)	0.071
BMI (kg/m^2)	27.8 (4.5)	29.7 (4.4)	0.001
Current smokers (%)	8	10	0.576
Hypertension (%)	64	61	0.624
Hypercholesterolaemia (%)	56	50	0.350
Hypertriglyceridaemia (%)	31	18	0.012
HDL cholesterol (mmol/L)	1.25 (0.40)	1.20 (0.43)	0.386
Plasma carotenoids (μg/dL)	75 (68–83)	97 (89–105)	< 0.001
Dietary intake:			
Vegetables (g/day)	358 (238, 484)	534 (361, 661)	< 0.001
Fruit (g/day)	327 (187, 466)	450 (284, 625)	< 0.001
Olive oil (g/day)	11 (0, 17)	33 (22, 44)	< 0.001
Vegetable oil (g/day)	17 (0, 17)	0 (0, 22)	0.304

Continuous data are mean (SD) except for plasma carotenoids which are geometric mean (95% confidence interval) and dietary intake data which are median (interquartile range)

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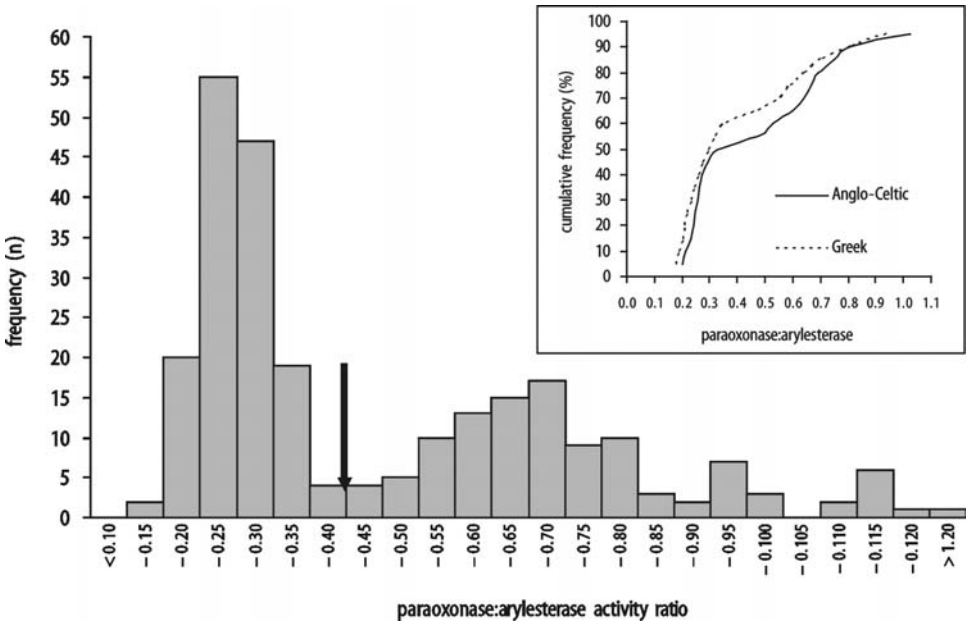

Fig. 1 Frequency distribution of PON1 phenotypes (defined by ratio of paraoxonase to arylesterase activity) and (inset) cumulative frequency of phenotypes stratified by ethnicity

status (Table 2) but it was significantly higher in the presence of hypercholesterolaemia or hypertriglyceridaemia. Stratification by high or low PON1 activity phenotypes (as defined above) did not substantially alter these relationships except that there were no longer any significant differences between categories of plasma triglycerides (data not shown). Arylesterase activity did not vary significantly by ethnicity, diabetes, smoking or hypertriglyceridaemia status (Table 2). CHD was associated with higher mean arylesterase activity while hypercholesterolaemia was also associated with an increase in arylesterase activity that approached statistical significance. None of these associations varied by ethnicity as indicated by non-significant P-values for interaction of clinical status with ethnicity (Table 2).

Associations of PON1 activity with lipids, glycaemia and inflammation

Paraoxonase and arylesterase activities were significantly correlated for Greek ($r = 0.270$, $P = 0.002$) and An-

Table 2 PON1 activity stratified by ethnicity and selected clinical characteristics

	Paraoxonase activity (U/L)					Arylesterase activity (U/mL)			
	n	Anglo-Celtic	Greek	$P_{category}$[1]	$P_{interaction}$[2]	Anglo-Celtic	Greek	$P_{category}$	$P_{interaction}$
All subjects	255	40 (36, 44)	37 (33, 41)	0.408[3]	–	102 (98, 107)	106 (102, 111)	0.217[3]	–
Non-diabetic	126	42 (36, 48)	40 (34, 46)			106 (100, 113)	107 (100, 114)		
Diabetic	129	37 (32, 43)	35 (30, 41)	0.147	0.913	98 (91, 104)	106 (99, 112)	0.147	0.272
Non-CHD	177	40 (36, 46)	38 (33, 43)			100 (94, 105)	103 (98, 109)		
CHD	78	38 (31, 46)	36 (30, 44)	0.529	0.893	107 (99, 115)	113 (105, 122)	0.020	0.745
Non-smokers	232	39 (35, 43)	37 (33, 42)			102 (98, 107)	106 (102, 111)		
Current smokers	18	49 (32, 74)	34 (24, 50)	0.695	0.276	100 (81, 118)	101 (84, 117)	0.448	0.818
Normotensive	95	44 (37, 52)	39 (33, 47)			105 (97, 113)	103 (95, 111)		
Hypertensive	155	37 (33, 43)	36 (32, 42)	0.180	0.550	101 (95, 107)	108 (102, 114)	0.957	0.185
Cholesterol < 5.5 mmol/L	120	35 (30, 41)	36 (31, 42)			98 (91, 105)	103 (96, 109)		
Hypercholesterolaemia	133	44 (38, 51)	38 (33, 45)	0.049	0.267	105 (99, 112)	109 (102, 115)	0.057	0.801
Triglycerides < 2.0 mmol/L	191	36 (32, 41)	36 (32, 41)			103 (97, 108)	106 (101, 112)		
Hypertriglyceridaemia	62	48 (40, 58)	39 (33, 53)	0.008	0.389	101 (93, 109)	103 (92, 114)	0.565	0.800

Mean (95% confidence interval), adjusted for age and gender; [1] P-value for difference between clinical categories; [2] P-value for interaction between ethnic group and clinical category; [3] P-value for difference between ethnic groups

glo-Celtic persons ($r = 0.367$, $P < 0.0001$). Paraoxonase activity was not significantly correlated with HDL cholesterol concentration for either group, whereas it was correlated with LDL cholesterol only for Anglo-Celtic subjects (Table 3). No significant correlations with triglycerides, HbA_{1c} or CRP were apparent. Arylesterase activity was significantly correlated with HDL cholesterol for Greek subjects, and inversely with CRP for Anglo-Celtic subjects (Table 3). No other significant correlations were observed.

Arylesterase activity was significantly correlated with HDL cholesterol concentration only in Greek subjects, and inversely with CRP in Anglo-Celtic subjects (Table 3). No significant relationships were apparent with LDL cholesterol in either ethnic group. Triglycerides and HbA_{1c} were not correlated with arylesterase activity in either ethnic group.

Associations of PON1 activity with dietary markers

Of the circulating dietary markers examined (Table 3), paraoxonase activity correlated positively with β-carotene, lycopene and lutein + zeaxanthin for Greek but not Anglo-Celtic subjects. Arylesterase activity correlated inversely with homocysteine in Anglo-Celtic subjects.

Stratification of data by PON1 phenotype showed that the positive relationship of paraoxonase activity with plasma carotenoid concentration was significant only for the Greek subjects with the high activity phenotype ($r = 0.437$, $P < 0.01$; Fig. 2). Also within the higher activity phenotype group, there were significant inverse associations of homocysteine ($r = -0.291$, $P < 0.05$) and CRP ($r = -0.277$, $P < 0.05$) with paraoxonase activity only for Anglo-Celtic subjects (Fig. 2). None of these associations were apparent for the low PON1 activity phenotype group.

Table 3 Bivariate correlations of PON1 activity with risk factors and dietary and inflammatory markers

	Paraoxonase activity[a]		Arylesterase activity	
	Anglo-Celtic	Greek	Anglo-Celtic	Greek
HDL cholesterol	−0.084	0.136	0.136	**0.238**[c]
LDL cholesterol	**0.202**[b]	0.081	0.067	0.051
Triglycerides[a]	0.164	0.024	−0.019	−0.053
HbA_{1c}[a]	−0.085	−0.140	−0.094	−0.110
Retinol	0.032	−0.046	−0.001	−0.105
α-tocopherol[a]	0.118	0.039	0.011	0.115
β-carotene[a]	−0.001	**0.208**[b]	0.083	0.078
α-carotene[a]	0.018	0.082	0.079	0.035
Lycopene[a]	−0.062	**0.244**[c]	−0.129	−0.029
Cryptoxanthin[a]	0.138	0.120	−0.017	0.044
Lutein + zeaxanthin[a]	0.134	**0.187**[b]	−0.037	0.146
Total carotenoids	0.039	**0.251**[c]	0.006	0.133
Homocysteine[a]	−0.041	−0.046	**−0.228**[b]	−0.045
CRP[a]	−0.029	−0.154	**−0.188**[b]	0.151

Pearson correlation coefficients; [a] log-transformed variables; [b] $P < 0.05$; [c] $P < 0.01$

Discussion

In this study we did not find differences in average PON1 activity between Greek and Anglo-Celtic ethnic groups that might explain differences in CHD mortality between these populations. Furthermore, we did not identify differences in PON1 phenotype distribution that would account for lower CHD mortality despite higher reported levels of some CHD risk factors in the Greek population of Australia. We have used phenotype categories as a surrogate for the genotype distribution, with the low activity phenotype representing PON1–192 QQ and the intermediate/high activity phenotype representing subjects with the R-allele [16]. A recent review by Mackness et al. suggests that PON1 genetic polymorphism contributes to only a minor degree in protection from atherosclerosis [20, 21]. Other recent evidence suggests that polymorphisms of PON1 promoter regions may be more important in mediating CHD risk [22].

We did not detect any significant variation in PON1 activity between categories of hypercholesterolaemia, diabetes, smoking or prevalent CHD (defined by clinical history and ECG). Other studies have shown lower PON1 activity in the presence of CHD (perhaps of a more severe nature than was included in the present community-based sample) [9], type 2 diabetes [23], hypercholesterolaemia [11] and in smokers (of which there were only a very small number in the present study) [5].

The major observation of interest from the present study was a correlation of PON1 activity with diet-derived antioxidants in the form of plasma carotenoids. This relationship was observed only for subjects with the higher activity phenotypes (corresponding to those subjects with the R-allele of PON1–192), and only for Greek subjects. We have previously observed a similar association for another Australian population among whom the lower activity phenotype was absent and average antioxidant levels were low [12]. Mean plasma concentration of carotenoids was 25% higher for the Greek migrants than for Australian-born persons in this study (consistent with their greater intake of fruit and vegetables), although there was considerable overlap in the range of plasma carotenoid concentrations in the two groups (Fig. 2). This suggests that we are not merely observing an effect on PON1 activity of carotenoids only at very high plasma concentrations, but rather an interaction with ethnicity. In this regard, the food sources of the carotenoids and their preparation methods differ

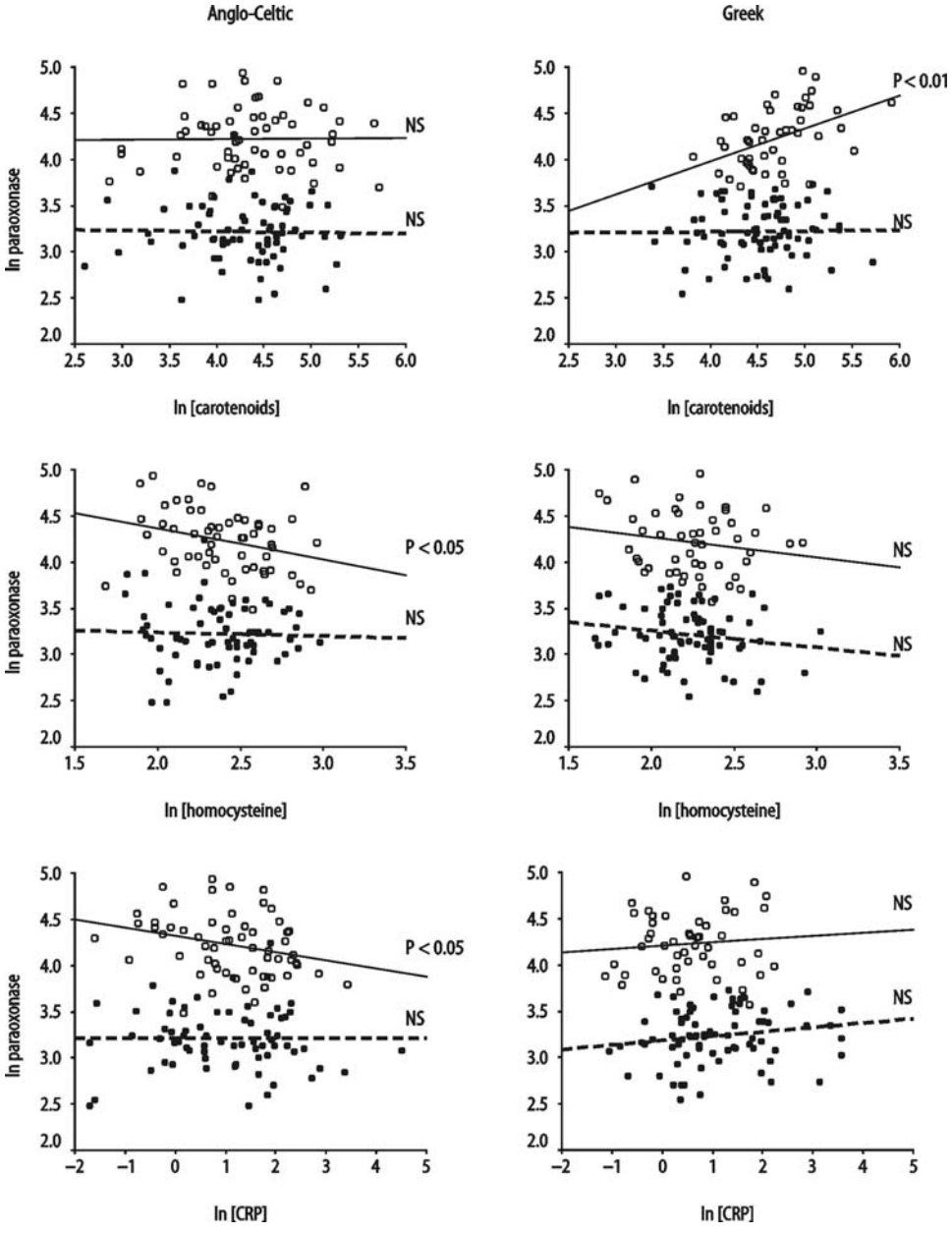

Fig. 2 Relationships of paraoxonase activity to circulating carotenoids, homocysteine and CRP, stratified by PON1 phenotype and ethnicity. Filled circles, dashed line: low activity PON1 phenotype; open circles, solid line: intermediate/high activity PON1 phenotype

greatly between the two ethnic groups, particularly with respect to green leafy vegetables, fruit, tomatoes and the use of olive oil [24]. Hence the biological significance of carotenoids *per se* may be secondary to that of the foods from which they are derived and their preparation (for example greater use of olive oil). In addition, *in vivo* interactions with other compounds (including fatty acids) may contribute to variation in the influence of the dietary antioxidants on PON1 activity.

While there have been several studies examining the effects of diet on PON1 activity, the results remain ambiguous. One study showed that PON1 activity had a negative correlation with fruit and vegetable intake [25], while others showed modest reduction [26] or no effect [27] in response to short-term increase in fruit and vegetable consumption. In contrast, others have found the following: red wine or its isolated components preserve or increase PON1 activity [7, 28]; fruit juice enhanced PON1 activity in mice [29]; PON1 activity was positively related to intake of vitamins C and E by men [30]; and that meals high in oxidised fat decreased PON1 activity [6]. Tomás et al. found that, in a Spanish population, higher intake of oleic acid was associated with greater paraoxonase activity but only for the highest activity PON1 genotype group (RR) [31]. Antioxidant protection by a tomato-rich diet was also found to be greater for

subjects with the R-allele [32]. These studies and the different relationship of PON1 activity to antioxidants between the two phenotype categories seen here may be important in interpreting studies of the relationship of PON1 genotype to CHD risk: the potential for dietary protection from atherosclerosis through modulation of HDL function is greater for those persons with the R allele, and that elevated risk for these people is unmasked in the presence of poor dietary quality.

Activity of PON1 enzyme has been found to vary in different lipid environments. Rats fed with monounsaturated fatty acids have been shown to have a higher paraoxonase activity than did rats fed with saturated and polyunsaturated fatty acids [33]. Olive oil consumption is one of the factors that differs markedly between these two ethnic groups [Table 1, 24]. As noted above, Tomás et al. have also shown a positive association between oleic acid intake and PON 1 activity levels in the presence of the 192-R allele. Homocysteine and CRP both predict CHD, at least in some populations, and this effect may derive from pro-oxidant mechanisms. In the present study, both were inversely related to PON1 activity in Anglo-Celtic subjects (again, only for those subjects assumed to have the 192-R allele). This implies that the Greek diet protects PON1 from detrimental effects associated with CRP and homocysteine. In this context, the influence of CRP and homocysteine as risk factors may be differentially expressed between populations. Further studies of the relationships of PON 1 activity to dietary factors are warranted.

In conclusion, although we did not find significant differences in PON1 activity between Greek and Australian-born subjects from a community-based survey sample that might explain observed ethnic differences in CHD mortality, we have observed associations of PON1 activity with certain dietary markers (carotenoids and homocysteine) and with CRP. The data suggest that dietary modulation of atherosclerotic risk may vary according to PON1 phenotype.

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