27.3% lower than in the native cards: median (25th–75th percentiles), 5.6 (3.6–8.9) nmol/L vs 7.7 (5.3–11.1) nmol/L; P = 0.001.

Thus, 17-OHP in dried blood spots on autoclaved or native filter-paper cards decreased 2–3% annually, and the decrease was significant on native cards but not on autoclaved cards. However, given an interassay CV of 8%, this small decrease may be unimportant for interpretation of samples stored for a few years. If samples are kept for a decade or more, however, it may be wise to correct for the loss, certainly at the expense of an increase in the error of estimate. Thus, in a moderate climate such as in Middle Europe, 17-OHP is basically stable in native or autoclaved filter-paper cards stored at room temperature over a prolonged period. The stability of 17-OHP on filter paper for up to 1 year has been ascertained in previous studies (5, 12). Similarly, progesterone has been shown to be stable in filter paper for 15 weeks at 25°C and for 9 weeks at 37°C (13). Steroids are known to be stable in serum stored at −25°C for up to a decade (14).

We conclude that 17-OHP estimates in dried blood on native or autoclaved filter paper stored for more than a decade can reliably be used for retrospective examinations and population studies.

Chromatographic separation and enzymatic determination of progesterone in dried blood spots were achieved by conventional risk factors (1). Various polymorphisms, such as MTHFR C677T, which causes hyperhomocysteinemia in the TT form (2), PON M55L, which causes increased paraoxonase (PON) activity in the LL form (3), and APOE, in which the presence of the ε4 allele adversely affects lipoprotein metabolism (4), have been associated with CAD. Most research to date has focused on the individual effects of each polymorphism on the specific metabolic pathways in which they are known to participate. Discrepancies as to the importance of the effects of these polymorphisms on cardiovascular risk factors exist (5–7). The aim of this study was to investigate the combined effect of the presence of two or more of these polymorphisms on their corresponding cardiovascular risk factors.

The study was performed in accordance with the guidelines of the institutions involved and was approved by the Hospital de Sant Joan and Jordi Gol Gorina Foundation ethics committees. All participants gave informed consent.

Fasting blood samples were obtained from 400 volunteers between 0800 and 0900, following an overnight fast. Blood was collected (10 mL in each tube) in two EDTA-containing Vacutainer Tubes for plasma and leukocyte preparation and in an untreated tube for serum preparation. The tubes were kept at 4°C until processing (within 2 h). Serum and plasma were stored in aliquots of 250 μL to 1 mL in cryotubes at −80°C until required for analysis, and leukocytes were prepared from the remaining pellet, from which DNA was extracted.

The genetic polymorphisms of the MTHFR, APOE, and PON55 genes were analyzed according to previously

The MTHFR C677T, APOE, and PON55 Gene Polymorphisms Show Relevant Interactions with Cardiovascular Risk Factors, Michelle M. Murphy, Elisabet Vilella, Santiago Cervero, Lidia Figuera, Maria Sanchez, Jordi Camps, Gemma Cuco, Natàlia Ferré, Antonio Labad, Natasha Tasevska, Victoria Arija, Jorge Jouven, and Joan Fernandez-Ballart, on behalf of the HOMFOL Study. Unitat de Medicina Preventiva i Salut Pública, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, 43201 Reus, Spain; Grup d’Investigació en Psiquiatria, Hospital Psiquiàtric Universitari Institut Pere Mata, 43201 Reus, Spain; Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, 43201 Reus, Spain; * address correspondence to this author at: Unitat de Medicina Preventiva, Facultat de Medicina, Universitat Rovira i Virgili, C/Sant Llorenç, 21, 43201 Reus, Spain; fax 34-977-759-322, e-mail jdfb@fmcs.urv.es

Coronary artery disease (CAD) often cannot be explained by conventional risk factors (1). Various polymorphisms, such as MTHFR C677T, which causes hyperhomocysteinemia in the TT form (2), PON M55L, which causes increased paraoxonase (PON) activity in the LL form (3), and APOE, in which the presence of the ε4 allele adversely affects lipoprotein metabolism (4), have been associated with CAD. Most research to date has focused on the individual effects of each polymorphism on the specific metabolic pathways in which they are known to participate. Discrepancies as to the importance of the effects of these polymorphisms on cardiovascular risk factors exist (5–7). The aim of this study was to investigate the combined effect of the presence of two or more of these polymorphisms on their corresponding cardiovascular risk factors.

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The genetic polymorphisms of the MTHFR, APOE, and PON55 genes were analyzed according to previously.

References
Table 1. Effect of the MTHFR C677T polymorphism on total cholesterol, triglycerides, and HDL-cholesterol in the APOE ε3/ε3 individuals.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol, mmol/L</th>
<th>Triglycerides, mmol/L</th>
<th>HDL-C, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B**</td>
<td>95% CI</td>
<td>B</td>
</tr>
<tr>
<td>CT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27</td>
<td>(0.01 to 0.53)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34</td>
</tr>
<tr>
<td>TT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23</td>
<td>(-0.12 to 0.58)</td>
<td>-0.001</td>
</tr>
<tr>
<td>F&lt;sub&gt;B,2,265&lt;/sub&gt;P</td>
<td>4.84; &lt;0.0001</td>
<td>10.79; &lt;0.0001</td>
<td>16.72; &lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total cholesterol.
<sup>b</sup> HDL-C, HDL-cholesterol.
<sup>c</sup> Regression coefficient.
<sup>d</sup> With respect to CC genotype. Multiple linear regression model adjusted for sex, age, BMI, WHR, smoking, and alcohol consumption.
<sup>e</sup> Statistically significant observations.

risk factors investigated. When more than one genotype was significantly associated with a cardiovascular risk factor, we explored the combined genotypic effect in a separate multiple linear regression analysis. Diagnosis of residuals was used to test whether the assumptions for the regression models were fulfilled. We refused the null hypothesis when the P value was <0.05 or when the confidence interval did not include zero. Ver. 10.0 of the Statistical Package for Social Sciences (SPSS) for Windows was used for data analysis.

The effects of the polymorphisms on the studied risk markers for CAD were explored by multiple linear regression analysis adjusted for sex, age, body mass index (BMI), waist-to-hip ratio (WHR), smoking, alcohol consumption, and HDL-cholesterol in the case of PON activity. Thus, the CC, ε3/ε3, and LL polymorphisms were references for comparative purpose within each model for the MTHFR C677T, APOE, and PON55 polymorphisms, respectively. tHcy was affected only by the MTHFR C677T polymorphism. Mean tHcy was significantly higher, by 1.24 μmol/L [95% confidence interval (95% CI), 1.15–1.35 μmol/L], in TT compared with CC individuals. We found significantly lower total cholesterol, by −0.42 mmol/L (95% CI, −0.72 to −0.12 mmol/L), in ε2 individuals compared with ε3 individuals. Triglycerides were significantly higher, by 0.21 mmol/L (95% CI, 0.03–0.38 mmol/L), in CT compared with CC individuals. The only risk factor investigated that was affected by more than one polymorphism was PON activity, i.e., APOE and PON55. It was significantly higher in ε2 individuals with respect to the ε3 individuals and also in both the PON55 ML and LL genotypes with respect to the MM genotype.

Given the unexpected relationship between the MTHFR C677T polymorphism and triglycerides, we further investigated its effect on triglycerides, total cholesterol, and HDL-cholesterol in the ε3 individuals only. This was to study the effect of the MTHFR C677T polymorphism on these variables, with the least influence of the APOE genotype. The model, adjusted for sex, age, BMI, WHR, smoking, and alcohol consumption, is shown in Table 1. Total cholesterol and triglycerides were significantly higher in the CT with respect to the CC genotype. HDL-cholesterol was significantly lower in the CT and TT genotypes compared with the CC genotype.
The effects of the APOE polymorphisms adjusted for sex, age, HDL-cholesterol, BMI, WHR, smoking, and alcohol consumption (with reference to the e3 individuals) within each PON55 genotype are shown in Fig. 1. There was a significant interaction between APOE and PON55 on PON activity. This is evident because the differences observed in PON activity among the different APOE genotypes are not the same in the different PON55 genotypes. In the MM genotype, e2 and e4 individuals do not differ significantly in PON activity from e3 individuals. In the ML genotype, PON activity was significantly higher in e2 than in e3 individuals. In the LL genotype, PON activity was also higher in e2 individuals compared with e3 individuals, although not significantly. However, the LL genotype in e4 individuals was associated with significantly lower PON activity compared with e3 individuals.

The MTHFR C677T polymorphism has been reported to be a risk factor for various diseases, exclusively because of its adverse effects on homocysteine metabolism. In this study, however, we also found that this polymorphism adversely affects lipid metabolism. To the best of our knowledge, this has not been reported before, but it has been proposed recently (14) that activation of sterol regulatory element-binding protein caused by homocysteine-induced endoplasmic reticulum stress leads to increased expression of genes responsible for cholesterol/triglyceride biosynthesis and uptake. This would lead to an intracellular accumulation of cholesterol and is a possible mechanism by which homocysteine may affect lipid metabolism. Authors of previous studies have reported a relationship between tHcy and total, LDL-, and HDL-cholesterol (15, 16), but the conclusions drawn from each of these studies are debatable. Significantly increased total and LDL-cholesterol in homozygotes and heterozygotes for the T allele was reported (15) in a bivariate analysis, but the analysis was not adjusted for confounding factors such as those considered in our study. When adjusting for sex, age, BMI, WHR, smoking, and alcohol consumption in the e3 individuals, we found that mean total cholesterol and triglycerides were significantly increased in CT with respect to CC individuals but not in TT individuals, which in the case of total cholesterol may have been attributable to an insufficient number of TT participants. A significant negative correlation between tHcy and HDL-cholesterol and positive correlation between tHcy and LDL-cholesterol has been reported previously (16), but neither sex nor age was considered during data analysis from a control group of a wide age range.

The effect of the MTHFR C677T polymorphism on lipid metabolism observed in our study merits further investigation in an appropriately designed study to determine whether it was attributable to chance. If apart from its effect on tHcy it causes increased cholesterol and triglycerides and decreased HDL-cholesterol, it may be important in understanding the puzzling discrepancies in previous conclusions on the effect of the MTHFR C677T polymorphism on cardiovascular risk factors.

Our finding of the effect of the APOE polymorphisms on PON has not been reported before. Because this model was adjusted for HDL, the effect is not attributable to the close relationship of PON and HDL. Furthermore, there was no significant relationship between APOE polymorphisms and HDL in this study. Further analysis demonstrated that there were significant interactions between the APOE and PON55 polymorphisms that affected PON activity in different ways. PON concentrations and activity vary substantially among individuals, but to date, known polymorphisms do not account for the extent of the variation (17). The level of PON activity is a major determinant of its protective function (18). There are conflicting reports in the literature on the effect of PON polymorphisms on cardiovascular risk factors. Although some studies have shown an association between the PON55 L allele and atherosclerosis (19), others have associated the M allele with this disease (20). Increased PON activity would be expected to confer protection against CAD, but this is not the case in the LL genotype. It appears contradictory that the LL polymorphism, which is associated with higher PON activity, should be a cardiovascular risk factor. However, our study shows that a combination of the LL genotype with the presence of the e4 allele was associated with a significant decrease in PON activity compared with e3 individuals. Because there is such large interindividual variation in PON concentrations and activity, other genetic and phenotypic influences on the PON polymorphisms may contribute to the association of this polymorphism with cardiovascular risk factors. The need to investigate the effects of nutritional differences on PON has been suggested (21). Given the interaction observed between the e4 allele and the PON55 LL genotype in our study, we propose that the APOE genotype should be considered when interpreting the association between PON55 and cardiovascular risk factors.

This project was supported in part by a grant from Fondo de Investigación Sanitaria (FIS:00/0954), Spain.

References
20. Tarragó LM, Joven J, Camps J. Evaluation of a neuron-specific enolase (NSE) ELISA, was used to retrospectively assess serum GS in healthy controls, AD patients, and other dementia patients.

Recombinant human GS (rhGS) cDNA was purchased from ATCC (ATCC No. 409071). The full-length rhGS open reading frame was obtained by PCR and subcloning in pET28a (Ndek/Xhol). The construct included a polyhistidine tag at the N-terminal domain of the rhGS open reading frame and no extra sequence at the C-terminal domains. The protein was expressed in E. coli BL21 (DE3) (7). We prepared extracts and solubilized products as described (8). Affinity purification was performed by nickel-nitritotriacetic acid chromatography.

Female Balb/c mice (7–8 weeks of age) were immunized by subcutaneous injection of 50 μg of rhGS emulsified in complete Freund’s adjuvant. An intraperitoneal booster injection was given 3 weeks later (50 μg in incomplete Freund’s adjuvant) and then at 2- to 3-week intervals (three times or more) with a final injection of 50 μg of antigen in buffer via intraperitoneal route 3 days before cell fusion. Hybridoma cultures were screened by ELISA, and positive cultures were cloned at least twice with limiting dilutions as described (9). Monoclonal antibodies (MAbs) were purified by protein G chromatography (Phamacia Biotech), and subclass was determined (Mouse Type®; Bio-Rad).

Serum S100B and NSE, were analyzed by two ELISAs (SMART S100B and SMART NSE; Syn-X Pharma, Inc.). For GS, Maxisorp™ plates (NUNC) were coated for 16–24 h at 4–8°C with 10 mg/L MAb 1G3 in 0.125 mL/well of coating buffer (20 mmol/L NaHPO4 buffer, pH 7.4). The plates were washed once with wash buffer (phosphate-buffered saline containing 0.5 g/L Tween 20, pH 7.4), followed by another wash with phosphate-buffered saline, pH 7.4, and the free binding sites were blocked at room temperature for 1 h with 0.25 mL of ELISA that is rapid, highly sensitive, and GS specific. This ELISA, together with the S100B and neuron-specific enolase (NSE) ELISAs, was used to retrospectively assess serum GS in healthy controls, AD patients, and other dementia patients.

**Immunoassay for Serum Glutamine Synthetase in Serum: Development, Reference Values, and Preliminary Study in Dementias, Miyoko Takahashi, Eric Stanton, J. Ignacio Moreno, and George Jackowski (Syn-X Pharma Inc., 6354 Viscount Rd., Mississauga, Ontario, L4V 1H3 Canada; * author for correspondence: fax 905-677-1674, e-mail empty@ICA.net)**

Glutamine synthetase (GS) is a ubiquitous enzyme present at high concentrations in liver, muscle, kidney, and brain (1). GS in the brain is astrocyte specific, is involved in the detoxification of ammonia and glutamate, and is overexpressed after brain injury (2). Monomeric GS protein was found in 38 of 39 cerebrospinal fluid samples obtained from Alzheimer disease (AD) patients (3), and the concentration of GS in the lumbar cerebrospinal fluid of AD patients was increased significantly but nonspecifically (4). GS has also been reported in serum (4).

Markers for AD have poor sensitivity and specificity for overt or preclinical AD (5,6). We have developed an