Carcinogenesis vol.34 no.6 pp.1244–1250, 2013
doi:10.1093/carcin/bgt045
Advance Access publication February 6, 2013

**Hemochromatosis (HFE) gene mutations and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC) study**

Antonio Agudo,1,4,6 Catalina Bonet1, Núria Sala1,2, Xavier Muñoz1,2, Núria Aranda1, Ana Fonseca-Nunes1, Françoise Clavel-Chapelon4,5, Marie Christine Boutron-Ruault4,5, Paolo Vineis6,7, Salvatore Panico6, Domenico Palli6, Rosario Tumino6,8, Sara Grioni6,9, J.Ramón Quirós7, Esther Molina10,13, Carmen Navarro11,14, Aurelio Barricarte14,16, Saioa Chamosa17, Naemi E. Allen18,19, Kay-Tee Khaw18, H.Bas Bueno-de-Mesquita1,2,21,22, Peter D. Siersema23,24, Mattijs E. Numan25,26, Antonia Trichopoulou27,28, Pagona Lagiou29,30, Dimitrios Trichopoulos31,32,33, J.Ramón Quirós31,34, Kay-Tee Khaw8,9, Anne Tjonneland11,12, Elisabete Weiderpass7,4,5, Mary Christine Boutron-Ruault4,5, Marie Christine Boutron-Ruault4,5, Antonia Trichopoulou31,34, J.Ramón Quirós31,34, Kay-Tee Khaw8,9, Anne Tjonneland11,12, Elisabete Weiderpass7,4,5, Mary Christine Boutron-Ruault4,5, Antonia Trichopoulou31,34, J.Ramón Quirós31,34, Kay-Tee Khaw8,9, Anne Tjonneland11,12, Elisabete Weiderpass7,4,5, Mary Christine Boutron-Ruault4,5, Antonia Trichopoulou31,34, J.Ramón Quirós31,34, Kay-Tee Khaw8,9, Anne Tjonneland11,12, Elisabete Weiderpass7,4,5

1Unit of Nutrition, Environment and Cancer, Catalan Institute of Oncology-ICO, IDIBELL, L’Hospitalet de Llobregat, Barcelona 08908, Spain, 2Molecular Epidemiology Group, Translational Research Laboratory, Catalan Institute of Oncology-ICO, IDIBELL, L’Hospitalet de Llobregat, Barcelona 08908, Spain, 3Department of Preventive Medicine and Public Health, Faculty of Medicine and Health Sciences, Rovira i Virgili University, Reus, Spain, 4Inserm, Centre for Research in Epidemiology and Population Health, U1018, Institut Gustave Roussy, Villejuif, France, 5MRC/HPA Centre for Environment and Health, School of Public Health, Imperial College, London, 6HuGeF Foundation, Torino, Italy, 7Department of Clinical and Experimental Medicine, Federico II University, Naples, Italy, 8Molecular and Nutritional Epidemiology Unit, Cancer Research and Prevention Institute – ISPO, Florence, Italy, 9Cancer Registry and Histopathology Unit, ‘Civile M.P.Arezzo’ Hospital, ASP Ragusa, Italy, 10Epidemiology and Prevention Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy, 11Public Health Directorate Asturias, Spain, 12Andalusian School of Public Health, Granada, Spain, 13CIBER Epidemiology and Public Health (CIBERESP), Spain, 14Department of Epidemiology, Regional Health Authority, Murcia, Spain (CN), 15Navarre Public Health Institute, Pamplona, Spain (AB), 16Public Health Division of Gipuzkoa, Epidemiology Unit, Basque regional Health Department and Biodonostia, Spain, 17Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, Oxford, UK, 18Cancer Epidemiology Unit, University of Oxford, Oxford, UK, 19Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK, 20National Institute for Public Health and the Environment, Bilthoven, The Netherlands, 21Department of Gastroenterology and Hepatology, University Medical Center, Utrecht, The Netherlands, 22Department of Primary Care Julius Center UMC, Utrecht, The Netherlands, 23Department of General Practice and Elderly Care, VUMC, Amsterdam, The Netherlands, 24WHO Collaborating Center for Food and Nutrition Policies, Department of Hygiene, Epidemiology and Medical Statistics, University of Athens Medical School and 25Hellenic Health Foundation, Athens, Greece, 26Department of Epidemiology, Harvard School of Public Health, Boston, MA 02115, USA, 27Bureau of Epidemiologic Research, Academy of Athens, Athens, Greece, 28Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany, 29Genomic Epidemiology Group, German Cancer Research Center (DKFZ), Heidelberg, Germany, 30Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Germany, 31Public Health and Clinical Medicine, Nutritional Research, Umeå University, Sweden, 32International Agency for Research on Cancer (IARC-WHO), Lyon, France, 33Department of Surgical and Perioperative Sciences, Surgery, Umeå University, Sweden, 34Department of Surgery, Skane University Hospital Malmö, Lund University, Malmö, Sweden, 35Department of Epidemiology, School of Public Health, Aarhus University, Aarhus, Denmark, 36Danish Cancer Society, Rambouillet, France, 37Department of Medicine, Nutritional Research, Umeå University, Sweden, 38Department of Public Health, Faculty of Medicine and Health Sciences, Rovira i Virgili University, Reus, Spain, 39Department of Surgery, Skane University Hospital Malmö, Lund University, Malmö, Sweden, 40Epidemiology, School of Public Health, Imperial College London, St Mary’s Campus, Imperial College, London, UK

*To whom correspondence should be addressed. Tel: +34 932607401 ext. 3075; Fax: +34 932607787; Email: a.agudo@iconcologia.net

**Hereditary hemochromatosis (HH) is a strong risk factor for hepatocellular cancer, and mutations in the HFE gene associated with HH and iron overload may be related to other tumors, but no studies have been reported for gastric cancer (GC).** A nested case-control study was conducted within the European Prospective Investigation into Cancer and Nutrition (EPIC), including 365 incident gastric adenocarcinoma and 1284 controls matched by center, sex, age and date of blood collection. Genotype analysis was performed for two functional polymorphisms (C282Y/rs1800562 and H63D/rs1799945) and seven tagSNPs of the HFE genomic region. Association with all gastric adenocarcinoma, and according to anatomical localization and histological subtype, was assessed by means of the odds ratio (OR) and 95% confidence interval (CI) estimated by unconditional logistic regression adjusted for the matching variables. We observed a significant association for H63D with OR (per rare allele) of 1.32 (CI = 1.03–1.69). In subgroup analyses, the association was stronger for non-cardia anatomical subsite (OR = 1.60, CI = 1.16–2.11) and intestinal histological subtype (OR = 1.82, CI = 1.27–2.62). Among intestinal cases, two tagSNPs (rs1572982 and rs6918586) also showed a significant association that disappeared after adjustment for H63D. No association with tumors located in the cardia or with diffuse subtype was found for any of the nine SNPs analyzed. Our results suggest that H63D variant in HFE gene seems to be associated with GC risk of the non-cardia region and intestinal type, possibly due to its association with iron overload although a role for other mechanisms cannot be entirely ruled out.

**Introduction**

Previous studies have reported that non-cardia gastric cancer (GC) risk was significantly associated with increasing intakes of total, red and processed meat (1). This could be due to a high exposure to N-nitroso-compounds (NOC), such as nitrosamines and nitrosamides. Meat may have a high content of such compounds, but it is also a source of precursors of NOC (nitrates, nitrites and proteins) and of heme iron that could act as nitrosating agent; thus, the observed association could be due to endogenous nitrosation (2). Following these results, we have also reported that GC risk was associated with increasing dietary intake of heme iron, mainly among subjects with low plasmatic levels of vitamin C (3).

Iron has long been suggested to play a role in carcinogenesis, based mainly on animal model studies, and to a smaller extent, observational studies in humans (4). The main putative mechanism is believed to be iron-induced oxidative stress (5). Redox cycling of iron is closely related with the production of reactive oxygen species able to induce lipid peroxidation and oxidative damage to DNA. Furthermore, reactive oxygen species produced by iron have been shown to specifically target some tumor suppressor genes (5). As mentioned above, heme...
iron also plays an important role in endogenous nitrosation; the group nitrosyl iron of heme acts as a nitrosating agent and in the presence of amines or amidcs this could lead to the formation of NOC, including known human carcinogens (6).

If iron plays a role in carcinogenesis, individuals with elevation of total body iron stores or iron overload could be at higher risk of developing cancer. Hereditary hemochromatosis (HH) is the most severe clinical expression of iron overload, leading to dysfunction of liver, pancreas, heart and other organs (7). The commonest clinical form is HH type 1, an autosomal recessive disease caused by mutations in the HFE gene (8). Most individuals affected by HH are homozygous for the polymorphism C282Y in HFE. The variant form is relatively common in European populations, with allelic frequencies 7–10% in Great Britain, 4–5% in Central and Northern Europe and 3% or below in Spain and Italy. Another common polymorphism is H63D, with allelic frequency 10–20% in European populations. The prevalence estimates in US population (non-Hispanic whites) are 6% for C282Y and 15% for H63D (9). H63D is also considered a mutation associated with HH, but its penetrance is much lower than for C282Y. Compound heterozygotes for C282Y and H63D or homozygous variant H63D rarely develop clinical disease, but they have moderate degree of overload, with high serum ferritin and transferrin saturation (8).

From observational studies in humans, there is little doubt that HH is a strong risk factor for hepatocellular carcinoma (HCC). Two recent meta-analyses (10,11) reported a strong association between variant C282Y (homozygous or allelic) and risk of HCC, but the evidence is limited for the association with non-hepatic localizations. For colorectal cancer (CRC), three studies (12–14) have shown a positive association for homozygous C282Y or carriers of at least one mutation in C282Y or H63D, whereas others did not find any association with CRC (15,16) or colorectal adenomas (17). Women with C282Y were found to have an increased risk of breast (12) and epithelial ovarian cancer (18), but no associations were reported for C282Y or H63D variants with prostate or breast cancer in males (12,19), pancreatic cancer (20) or endometrial cancer (18). The presence of H63D mutation was associated with an increased risk of acute lymphoblastic leukemia, but not with other types of acute leukemia (21).

To our knowledge, no studies have been published on the association between gastric cancer and hemochromatosis. The purpose of this work was to assess the potential effect of polymorphism in the HFE gene on the risk of gastric adenocarcinoma, according to anatomical localization and histological subtype, in a prospective study in European populations.

Materials and methods

Study design and participants

The study subjects were participants from the European Prospective Investigation into Cancer and Nutrition (EPIC), following a nested case–control design. Methods and rationale of the EPIC study have been reported elsewhere (22). Briefly, the EPIC cohort includes 521 457 participants recruited between 1992 and 2000 in 23 centers from 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom). At enrollment, each subject provided information about usual diet over the previous year as measured at recruitment by country-specific validated questionnaires (22). Using this information, dietary iron intake was computed using country-specific food composition tables (3).

Statistical analysis

For each polymorphism, HWE and pair-wise linkage disequilibrium were tested separately for cases and controls. Association between each SNP and GC risk was assessed by the odds ratio (OR) and 95% confidence interval (CI) estimated by unconditional logisitic regression, adjusted for the matching variables sex, age (5 years categories), center and date of blood collection (quarters of year). Given the matched design of the study, we checked that this approach provided approximately the same results obtained by means of conditional logistic regression. Other covariates potentially related with GC risk were expected to be independent of genetic variation and were not included in the model. The potential confounding of these variables was considered within stratified analyses, and interaction with the SNP of interest was assessed by means of a likelihood ratio test. The possibility of population stratification was considered within the context of the main study. The observed distribution of
The main analysis to explore the potential relationship between each SNP and GC risk was based on the log-additive (per allele) model, meant to be the most sensitive to detect an association. In subsequent analyses, other genetic models such as dominant, recessive and codominant were further explored. These analyses were carried out in the whole data set and for each tumor localization and histological type. To account for multiple testing related to assessing several SNPs, a gene-based permutation test was performed. After 10,000 permutations, the distribution of minimum P values of each of the nine SNPs analyzed was well fitted by a uniform distribution. Compared with controls, cases had a significantly higher proportion of current smokers (32 versus 23%), ate significantly less fruit than controls (216 and 236 g/day). A more detailed analysis exploring other genetic models was carried out for the three SNPs with significant association according to the log-additive model (Table III). The variant alleles were relatively common for the seven tagSNPs (MAF ranging from 10 to 37%), as well as for the variant form of H63D (allele G at nucleotide 266), the variant A had frequency 37%, as well as for the variant form of H63D (allele G at nucleotide 266).

Lauren’s classification (126 intestinal for 128 diffuse type), whereas 8 had a mixed type and 103 could not be classified. The nine genotyped SNPs were in HWE among controls, and the allelic frequencies were in good agreement with the expected prevalence in Caucasian populations (Table II). The variant alleles were relatively common for the seven tagSNPs (MAF ranging from 10 to 37%), as well as for the variant form of H63D (allele G at nucleotide 266), the variant A had frequency 37%, as well as for the variant form of H63D (allele G at nucleotide 266). The nine genotyped SNPs were in HWE among controls, and the allelic frequencies were in good agreement with the expected prevalence in Caucasian populations (Table II). The variant alleles were relatively common for the seven tagSNPs (MAF ranging from 10 to 37%), as well as for the variant form of H63D (allele G at nucleotide 266), the variant A had frequency 37%, as well as for the variant form of H63D (allele G at nucleotide 266).
Bayesian model, with OR = 1.73 (P = 0.004) and OR = 1.93 (P = 0.004) for the non-cardia and intestinal cases, respectively. A similar pattern was observed for the two tagSNPs associated with increased risk of intestinal GC (rs1572982 and rs2691586).

According to Haploview, H63D (rs1799945) is located in the recombination point of two blocks, one at 5’ of the gene tagged by the tagSNP rs4529296 and another tagged by the remaining 7 SNPs (including C282Y). Because rs4529296 was not associated with GC in previous analyses, we performed a haplotype analysis including H63D (rs1799945) plus the seven SNPs in the linkage disequilibrium block at 3’ of the HFE gene (Table V). Only six haplotypes had frequency >5%; the commonest (34.2%) was formed by the wild-type allele of each SNP and was taken as the referent in the analysis. Only one haplotype with frequency 14% was significantly associated with GC risk, whereas the effect of H63D was greater among those with higher iron intake than among those with lower iron intake. On the other hand, as iron from meat (mostly heme iron) is more readily absorbed than iron from plant foods, we also assessed the effect of H63D according to the level of heme iron intake (below or above the median of controls of 1.15 mg/day). The ORs (log-additive model) were 1.63 (1.11–2.39) and 1.14 (0.80–1.61) for those with lower and higher heme iron intake, respectively. Again these two ORs were not significantly different (P value for interaction 0.18), and it cannot be stated whether the effect of H63D is modified by heme iron intake.

Discussion

We have observed that the variant G at nucleotide 187 in exon 2 of HFE (H63D, rs1799945) is associated with increased GC risk in European populations. This association seems to be restricted to cases located in the non-cardia anatomical subsite (distal stomach) and those of intestinal histological subtype. No association was observed for the cardia cases or those of diffuse subtype. This is in agreement with our previous results on the association of GC risk with meat and heme iron intake: red and processed meat were associated with increased OR (95% CI = 1.04–1.73). The ORs for H63D were 1.63 (1.11–2.39) and 1.14 (0.80–1.61) for those with lower and higher heme iron intake, respectively. Again these two ORs were not significantly different (P value for interaction 0.18), and it cannot be stated whether the effect of H63D is modified by heme iron intake.
Table V. Haplotype analysis of SNPs of HFE gene and risk of gastric adenocarcinoma

<table>
<thead>
<tr>
<th>Haplotypea</th>
<th>Frequencyb (%)</th>
<th>ORc</th>
<th>(95% Cl)</th>
<th>P valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGGGTTTG</td>
<td>34.7</td>
<td>1.00</td>
<td>(Reference)</td>
<td></td>
</tr>
<tr>
<td>CGGTGTGA</td>
<td>14.5</td>
<td>1.00</td>
<td>(0.77–1.30)</td>
<td>0.99</td>
</tr>
<tr>
<td>GGACGGCC</td>
<td>14.3</td>
<td>1.34</td>
<td>(1.04–1.73)</td>
<td>0.024</td>
</tr>
<tr>
<td>CGACGGCG</td>
<td>12.3</td>
<td>1.01</td>
<td>(0.76–1.33)</td>
<td>0.96</td>
</tr>
<tr>
<td>CGACCCGG</td>
<td>10.5</td>
<td>1.05</td>
<td>(0.79–1.40)</td>
<td>0.74</td>
</tr>
<tr>
<td>CGACGGTG</td>
<td>6.2</td>
<td>1.05</td>
<td>(0.74–1.49)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Each haplotype is formed by the alleles corresponding to each of eight SNPs, including H63D (rs1799945) and seven tagSNPs of a linkage disequilibrium block in the HFE genomic region, ordered according to the localization in the gene: rs1799945, rs1800562, rs1572982, rs707889, rs1045537, rs17596719, rs6918586 and rs1543680. The first haplotype corresponds to the wild-type allele for each SNP. Marked with bold type, the alleles corresponding to the three SNPs are significantly associated with the univariate analysis. rs1799945, rs1572982 and rs6918586.

Only haplotypes with frequency 5% or above are presented; among haplotypes not shown, no significant associations were observed.

Recessive and Dominant ORs and P values compared with the most frequent haplotype estimated by unconditional logistic regression, adjusted for sex, age and center.

C282Y is associated with hepatocellular carcinoma (10,11), but a causal relationship between HFE mutations and other tumors is still debated. Moreover, the specific polymorphisms involved in such associations may differ. For instance, among the three studies that reported association of HFE mutations with CRC, one study observed such association with both C282Y and H63D (13), another found the association for compound C282Y/H63D heterozygotes (14) and the third observed the increased risk only for homozygous C282Y (12). Regarding other tumor sites, acute lymphoblastic leukemia was found to be associated with H63D (21), whereas epithelial ovarian cancer was associated with C282Y only (18). We found that GC risk is associated with H63D but not with C282Y; because our hypothesis was that increases in body iron status may promote gastric carcinogenesis, the reasons for this finding are unclear. Some HFE variants associated with HII have relatively high frequencies in some populations, probably due to selection because of their protective effect from iron deficiency. Some have proposed that, given this positive selection, if C282Y is not frequent in a population its role is assumed by H63D (26). In our study, the frequency of variant alleles for H63D and C282Y was 12.7 and 4.2%, respectively, in agreement with the expected (9). However, C282Y has variable frequency across European countries, with prevalence of 7–10% in Great Britain, 4–8% in Central and Northern Europe and 3% or less in Southern Europe. In our study, leaving out France and Norway given the small number of cases, the allelic frequency of this variant was 2.6% in Southern Europe (Greece, Italy and Spain), 4.1% in Northern Europe (Denmark and Sweden) and 6% in Central Europe (Germany, The Netherlands and the United Kingdom).

One possible explanation of our findings is that H63D is associated with chronic subclinical increases in body iron stores, which in turn promotes increased oxidative stress and induces DNA damage (5). Increased body iron status may also promote endogenous nitrosation resulting in the formation of NOC (6). We found an increased risk for H63D among subjects with high iron intake although there was no significant interaction between both factors. Moreover, intake is not a good indicator of body iron stores. Other mechanisms may also contribute to the association between HFE gene mutations and GC risk observed in our population. The peptide hormone gastrin, originally identified as a stimulant of gastric acid secretion, has been demonstrated to act as a growth factor in the gastrointestinal mucosa (27). Therefore, elevated plasma gastrin concentrations can be considered an indicator of GC risk, particularly in the presence of Helicobacter pylori although there is a complex interplay between H. pylori-induced gastritis, gastrin levels and GC risk. H. pylori infection in the antral portion of the stomach usually induces chronic gastritis.
without atrophy, with pronounced hyperchlorhydria but normal or slight increase in gastrin levels, often leading to peptic ulcer but no increased cancer risk, whereas the corpus–dependent atrophic gastritis is associated with low acid secretion and hypergastrinemia, and may result in increased GC risk (28,29). Interestingly, it has been demonstrated that circulating concentrations of both amidated and non-amidated forms of gastrin were significantly greater in patients with hemochromatosis compared with a group of normal controls with a similar mean age and sex ratio (30). The potential effect of H63D mediated by gastrin is also consistent with the finding of a significant association of H63D among non-cardia GC; moreover, this mechanism could be shared with H. pylori. However, in recent analysis we have shown that eventually all non-cardia GC cases have been previously infected, suggesting that H. pylori infection is a necessary cause of sporadic non-cardia GC (31). Therefore, it would be very difficult to examine whether there is an interaction of H63D with H. pylori infection on cancer risk.

One limitation of our study is that we have not considered polymorphisms in other genes involved in iron metabolism and homeostasis (8,32). However, these polymorphisms are less common than H63D or C282Y and their clinical significance is uncertain. On the other hand, although H63D has a functional effect on increasing iron stores, it is not clear to what extent it is directly involved in GC risk, or whether it is a marker of other variant. For instance, C282Y had been found to be associated with childhood acute lymphoblastic leukemia. Because HFE is located within the extended HLA complex, several variants have been analyzed from the histone gene HIST1H1C to HIST1H1T, and an intergenic SNP (rs807212) was identified as tagging most common haplotypes of this region. This SNP has been shown to be strongly associated with lymphoblastic leukemia, and accounted for the original C282Y association, which became weaker after adjustment for rs807212 (33). Finally, it should be considered that the reported association may be observed by chance owing to the multiple comparisons performed in this analysis. To take this into account, we carried out a permutation test for the log-additive model, adjusted for the matching variables. The estimated minimum adjusted P values from the permutation test were 0.27 for the whole data set, but remained significant for the GC of intestinal type (P = 0.02) or marginally significant (P = 0.05) for the non-cardia cases.

In conclusion, in our prospective study, the mutation H63D in HFE gene was found to be associated with increased risk of GC in European populations. This finding is consistent with previous results in the same population showing a relationship between GC and meat and iron intake. The association seems to be restricted to tumors located in the distal region of the stomach (non-cardia cases) and tumors of intestinal type. This effect could be due to a potential role of chronic iron overload associated with H63D, but other mechanisms could also be involved. These results should be replicated in order to confirm a role of HFE mutations in GC risk, and extensive analysis of determinants of body iron homeostasis is needed to gain insight on the potential role of iron in gastric carcinogenesis.

Funding

World Cancer Research Fund (WCRF) (WRCF Ref. 5842) and the Health Research Fund (FIS) of the Spanish Ministry of Health (PI11/01486). The EUR Gast study project was supported by the Fundación ‘La Caixa’ (BM06-130-0); Health Research Fund (FIS) of the Spanish Ministry of Health (PI070130, PI081420). The EPIC project received support from the European Commission (EU/6F32005), ‘Europe Against Cancer’ Programme of the European Commission (SANCO); Deutsche Krebshilfe; German Cancer Research Center; German Federal Ministry of Education and Research; Danish Cancer Society; Spanish Ministry of Health (RETIC R06/0020009131; Spanish Regional Governments of Andalucía, Asturias, Basque Country, Murcia, Navarra; and the Catalan Institute of Oncology; Cancer Research UK; Medical Research Council, UK; Stroke Association, UK; British Heart Foundation; Department of Health, UK; Food Standards Agency, UK; Wellcome Trust, UK; Italian Association for Research on Cancer (AIRC); Compagnia di San Paolo; Progetto Integrato Oncologia–PIO, Regione Toscana; Dutch Ministry of Public Health, Welfare and Sports (VWS); Netherlands Cancer Registry (NKR). LR Research Funds, Dutch Prevention Funds, Dutch SON (Zorg Onderzoek Nederland), World Cancer Research Fund (WCRF), Statistics Netherlands, The Netherlands; Stavros Niarchos Foundation; Hellenic Health Foundation; Greek Ministry of Health and Social Solidarity; and the counties of Skane and Västerbotten and the Swedish Research Council/BBMRI.SE, Sweden.

Acknowledgement

The authors acknowledge the technical contribution of Nadia García from the Catalan Institute of Oncology, and Magda Monfort and Sebastián Morán from the Spanish National Genotyping Center (CEGEN), and Antonio Berenguer and Victor Moreno for their advice in the statistical analysis.

Conflict of Interest Statement: None declared.

References


Received December 4, 2012; revised January 23, 2013; accepted January 30, 2013