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Smoking and plasma homocysteine

P. O'Callaghan, R. Meleady, T. Fitzgerald, I. Graham and the European COMAC group*

The Adelaide and Meath Hospital Dublin, incorporating the National Childrens Hospital and Trinity College, Dublin, Ireland

Background Smoking is known to be associated with an increased plasma homocysteine level. Both are associated with an increased risk of cardiovascular disease. B-vitamins modulate plasma homocysteine levels.

Aims To investigate the relationships between smoking, plasma homocysteine, nutrient levels and risk of cardiovascular disease.

Methods The European Concerted Action Project case control study of 750 cases and 800 age- and sex-matched controls aged less than 60 years from 19 centres in 10 European countries.

Results Smokers were at increased risk of vascular disease. This risk was greatly increased in the presence of a raised plasma homocysteine; cigarette smokers with a plasma homocysteine above 12 μmol . l^{-1} had a 12-fold increased risk of cardiovascular disease (OR 12·4 95% CI 7·3 to 21·2) compared with non-smokers with a normal plasma homocysteine. In both cases and controls the current smokers had a higher plasma homocysteine level than the never smokers (11·7 μmol . l^{-1} vs 10·07 μmol . l^{-1}, $P<0·05$ cases; 9·90 μmol . l^{-1} vs 9·53 μmol . l^{-1} P value non significant controls). Current smokers tended to have lower levels of folate, and vitamin B6 and vitamin B12 than never smokers. The risk of cardiovascular disease associated with smoking was not significantly altered by adjustment for levels of B-vitamins using a conditional regression model (OR for current smoker >20 . day^{-1} 8·19, after adjustment for B6, B12, folate OR 7·09).

Conclusions This case control study suggests that smokers with high plasma homocysteine are at greatly increased risk of cardiovascular disease and should therefore be offered intensive advice to help them cease smoking. They also have reduced levels of those B-vitamins (folate, vitamin B6 and vitamin B12) that modulate homocysteine metabolism. While this finding may reflect a direct effect of smoking or reduced B-vitamin intake, supplementation of these nutrients may be appropriate in smokers with high homocysteine levels.
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Introduction

Clinical, case–control and cohort studies indicate a graded relationship between an increasing level of plasma homocysteine and risk of cardiovascular disease[1–13]. Suggestions that the relationship may be causal have been tempered by the observation that the relationship may be stronger in case–control observations than in the methodologically stronger cohort studies[14,15], although the appropriateness of combining a small number of heterogeneous cohort studies has been questioned[16]. It is clear that the very high homocysteine concentrations seen with inborn errors of the enzymes involved in homocysteine metabolism do cause a primary thrombotic disorder. However, whether the moderate hyperhomocysteinaemia, more commonly seen in association with cardiovascular disease, is a cause or an effect of the disease awaits the outcome of ongoing placebo-controlled interventional trials with homocysteine lowering vitamins[17–19].

Smoking is strongly and independently associated with cardiovascular disease and is the biggest single avoidable health habit contributing to chronic disease in the Western world[20]. Up to 50% of avoidable deaths

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*Members of The European Concerted Action Project: 'Homocysteine and vascular disease: from natural history to therapy'. Project members are listed in the Appendix.

Correspondence: Professor Ian Graham, Cardiology Department, The Adelaide and Meath Hospital, Tallaght, Dublin 24, Ireland.

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in the industrialized world have been attributed to smoking, half of which are cardiovascular[21,22].

Cigarette smoking is known to be associated with a raised plasma homocysteine level[23–28]. Smokers also tend to have lower levels of the B-vitamins, folate, vitamin B6 and vitamin B12[24,25,29], all of which affect homocysteine levels by acting as co-factors (vitamins B6 and B12) or co-substrate (folate) for the enzymes controlling homocysteine metabolism[30–35]. Despite these observations, little information is available on the effect of homocysteine on the risk of cardiovascular disease in smokers, apart from a single report from our group[12].

The present study utilized the European Concerted Action Project 'Homocysteinaemia And Vascular Disease' to address the following questions:

- What is the relationship between smoking, plasma homocysteine and cardiovascular risk?
- Is this relationship independent of conventional cardiovascular risk factors and the B-vitamins that affect homocysteine levels?

Subjects and methods

The European Concerted Action Project 'Homocysteinaemia And Vascular Disease' is a case–control study of 750 cases (544 men and 206 women) with vascular disease and 800 control subjects (570 men and 230 women), all aged less than 60 years at recruitment and matched for age and sex. Nineteen centres in nine European countries participated.

The inclusion and exclusion criteria have been described in detail elsewhere[12]. Cases had both defined clinical and objective investigational evidence of coronary, cerebral or peripheral atherosclerotic vascular disease. Vascular disease was diagnosed on the basis of the following criteria: coronary heart disease was defined as clinical angina or a previous heart attack with a twofold rise in cardiac enzymes and serial ST-T changes on the ECG, or pathological Q waves, or else angiographic evidence of at least 70% stenosis of a major coronary artery. Cerebrovascular disease was characterized by clinical evidence of stroke or TIA with stenosis demonstrated on carotid Doppler studies or angiography or by evidence of cerebral infarction on computed tomography.

Peripheral vascular disease was defined as either intermittent claudication or diminished pedal pulses with either obstruction of one major vessel on angiography or an ankle brachial index of less than 0·9.

Recently diagnosed cases were recruited as far as possible. Controls were clinically healthy, community based and from as similar background to cases as possible. Half were recruited from industrial employee registers and most of the remainder came from random population samples.

Study measurements

Details of data recorded have been described previously[12]. Smoking was recorded as non-smoker, ex-smoker or current smoker. For ex and current smokers the following were noted:

Age at starting to smoke
Age at giving up smoking
Number of cigarettes per day

Blood samples for total homocysteine were taken onto ice, protected from light and centrifuged within 1 h. Plasma homocysteine was estimated fasting and after administration of an oral load of methionine (100 mg . kg^{-1} body weight of L-methionine). Total plasma homocysteine was measured centrally in Bergen, Norway using a high performance liquid chromatography assay (HPLC). This assay involved reduction of homocysteine using sodium borohydride, derivitization with monobromobimane, HPLC and then detection using a fluorescent marker[36].

Assays of vitamin B12, folate (red cell and plasma folate) and pyridoxal 5′ phosphate (B6) were performed by Mimelab-AB in Soraker, Sweden. Vitamin B12 and folate were measured using a radioimmunoassay and vitamin B6 using enzymatic photometry with HPLC.

Variable definition

Current smoking was defined as the use of any tobacco at the time of vascular diagnosis in cases or on the day of the methionine-loading test in controls. It was recorded as the number of cigarettes or the amount of tobacco used per day. An ex-smoker was a case or control who had not smoked for 6 months or more. Raised plasma homocysteine was defined as > 80th centile of the controls which was 12·1 μmol . l^{-1} for fasting total homocysteine, 27·0 μmol . l^{-1} for the increase in homocysteine after methionine loading and 38·0 μmol . l^{-1} for absolute post-load homocysteine. For assessing the association between homocysteine and risk, plasma homocysteine was also stratified by fifths of homocysteine distribution as follows: top 20% >12·1 μmol . l^{-1}, middle 20% 10·3–12·1 and bottom 60% below 10·3 μmol . l^{-1}.

The lower 20th centile levels for folate, vitamins B6 and B12 among the controls were used to define absolute vitamin deficiency and were similar to widely used reference ranges. Thus folate deficiency was defined as a red cell folate concentration of <373 nmol . l^{-1}, and B12 and B6 deficiency as plasma levels below 125 pmol . l^{-1} and 20 nmol . l^{-1} respectively.

Traditional risk factors for cardiovascular disease including blood pressure and hypercholesterolaemia were measured as previously described. Hypertension was diagnosed if systolic blood pressure >160 mmHg, or diastolic blood pressure >95 mmHg was observed or if treatment for hypertension was being taken at time of interview. Hypercholesterolaemia was recorded if the subject had a serum cholesterol >6·5mmol . l^{-1} or if he or she was taking a lipid lowering agent.

Table 1 Conditional odds ratio for vascular disease (men and women). Conditional logistic analysis that has been adjusted for age, centre, sex, total cholesterol, HDL, creatinine and hypertension. There is a significant interaction between the effects of smoking and raised tHcy (=0·04)

	Normal tHcy (tHcy <12·1)	Raised tHcy (tHcy >12·1)
Never	1·0	2·1 (1·1, 3·8)
Ex-smoker	4·4 (2·8, 6·9)	4·8 (2·6, 8·9)
Current (fewer than 20 per day)	3·1 (2·0, 4·7)	12·2 (6·4,23·3)
Current (20 or more daily)	7·9 (4·6,13·5)	13·1 (6·0,28·6)

Statistical analysis

Variables that exhibited a positive skew were log-transformed in order to normalize the distribution. For these variables the geometric means are given along with the estimates of the percentage difference in geometric means between never smokers, ex smokers and current smokers. Results were estimated for cases and controls separately and an ANOVA was used to compare levels in the three smoking groups.

Risk analysis was performed using a conditional sex-adjusted logistic regression, stratified by age group (<40, 40–49, and >50 years) and centre. Further analysis adjusted for the effects of total cholesterol, HDL cholesterol, creatinine and hypertension and also for B6, B12 and plasma folate. Interaction effects were tested based on a likelihood ratio test that compared the likelihoods associated with the independent model and the larger model that included an interaction between homocysteine and smoking.

Generalized additive logistic regression was used for constructing the dose–response spline graphs. Dose–response or spline graphs are a useful way to investigate the form of an association between risk and the risk factors of interest, with parallel splines suggesting independent effects of two separate risk factors and non-parallel splines suggesting an interaction effect[37].

Results

To assess the interaction between smoking, homocysteine and cardiovascular disease, smokers were grouped into light smokers (<20 cigs . day^{-1}) or heavy smokers (>20 cigs . day^{-1}) and raised plasma homocysteine was defined as greater than 12 μmol . l^{-1}.

Table 1 shows the OR for cardiovascular disease related to homocysteine level in never, ex and current smokers. After appropriate adjustment, there is a significant interaction between smoking and homocysteine (P=0·04). This interaction was stronger for light smokers (<20 cigs . day^{-1}). Current smokers with hyperhomocysteinaemia demonstrated a markedly increased risk of cardiovascular disease (OR 12·2 in light, OR 13·1 in heavy smokers) compared with a never-smoker with normal homocysteine. The combined odds ratio for all smokers with raised plasma homocysteine was 12·4 (CI 7·3 to 21·2). A model which assumed an independent multiplicative effect of raised homocysteine and smoking would predict an odds ratio of 7·4 (CI 4·5 to 12·1) for a hyperhomocysteinaemic light smoker, which is substantially less than that seen in Table 1 (OR 12·2, CI 6·4 to 23·3).

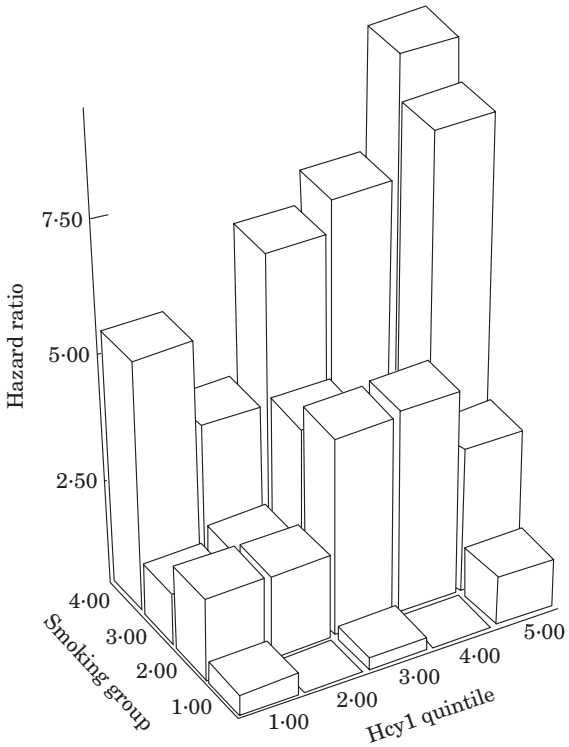

Figure 1 **Three-dimensional histogram of smoking status, homocysteine and risk of cardiovascular disease. Risk is expressed as a conditional odds ratio. Smoking group 1=never smokers, 2=ex-smokers, 3=current <20 . day^{-1} and 4=current >20 . day^{-1}. Homocysteine groups are in quintiles.**

The histogram in Fig. 1 illustrates the findings, with both smoking and homocysteine defined as categorical variables. The increased risk in hyperhomocysteinaemic smokers is again seen. The interaction between smoking, homocysteine and risk was also assessed using a logistic regression model. This is displayed in Fig. 2 as a spline or dose–response curve of homocysteine expressed as a continuous variable. The spline curves demonstrate vascular disease risk rising linearly with homocysteine in all smoking categories and show that ex-smokers resemble current rather than never smokers in terms of risk. For this reason, risk estimates associated with smoking were related to never smokers rather than to never and ex-smokers combined.

To assess the relationship between smoking, vitamins and vascular disease, vitamin levels in different smoking categories were examined. Table 2 shows the geometric means for homocysteine and vitamins in each smoking

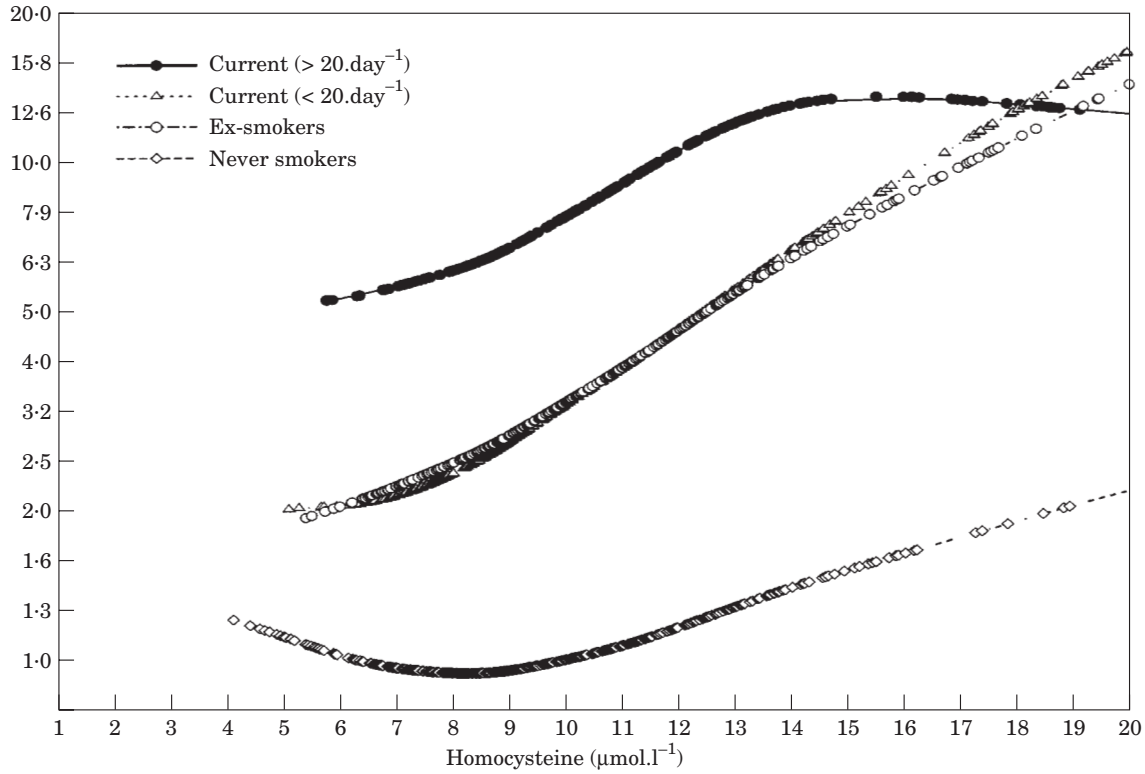

Figure 2 **Spline or dose–response curves of plasma homocysteine and risk of cardiovascular disease for each smoking category.**

Table 2 ***Homocysteine and nutrient levels in cases and controls according to smoking group***

	Cases			Controls		
	Never smoker (n=130)	Ex smoker (n=212)	Current smoker (n=408)	Never smoker (n=368)	Ex smoker (n=165)	Current smoker (n=267)
% Male	55% (n=72)	82% (n=175)	72% (n=297)	64% (n=238)	76% (n=126)	77% (n=206)
Age	44·4	48·1	45·8	41·1	46·4	42·2
Fast tHCY	10·07*	11·10 3 (−5, 12)	11·70 10 (2, 18)	9·53	9·88 −1 (−6, 4)	9·90 2 (−3, 6)
PML tHcy	32·70	35·30 7 (−1, 15)	36·60 8 (1, 15)	29·20	32·30 5 (0, 11)	30·60 5 (0, 9)
B12	242·5	238·1 6 (−4, 18)	233·3 2 (−6, 12)	239·4	223·7 −1 (−8, 7)	235·7 0 (−6, 7)
B6	28·79***	27·28 −6 (−13, 1)	24·85 −14 (−20, −8)	31·40*	32·13 5 (−2, 11)	30·15 −4 (−8, 2)
PL folate	8·88	8·90 6 (−6, 20)	8·03 −5 (−15, 6)	10·05*	9·10 −6 (−14, 3)	8·91 −10 (−17, −3)

*P<0·05; **P<0·01; ***P<0·0001.
P values represent the statistical difference between smoking groups in either cases or controls.
The second value (with confidence intervals) represents the percentage difference in geometric means adjusted for age and sex between each smoking group and the never smokers.

category for cases and controls. Both fasting and post-methionine load homocysteine levels were higher in current than never smokers in cases and controls. Vitamins B6, B12 and folate were lower in current smokers than never smokers. The difference was significant for vitamin B6 levels in both cases and controls (P<0·0001 for cases, P<0·05 for controls) and for plasma folate levels in controls (P<0·05). The difference in B12 levels between current and never smokers did not reach significance in either cases or controls.

A conditional logistic regression analysis was performed to determine whether the odds ratios for

Table 3 Adjusted odds ratio associated with smoking, OR (95% CI) relative to those who never smoked

	Ex-smokers	Current smoker (<20 per day)	Current smoker (20 or more per day)
Adjusted for age, sex and centre	3·79 (2·79, 5·15)	3·69 (2·76, 4·93)	8·19 (5·59, 12·00)
Adjusted for conventional risk factors*	3·74 (2·56, 5·46)	3·49 (2·44, 5·00)	7·38 (4·68, 11·63)
Additional adjustment for nutrients**	3·86 (2·61, 5·70)	3·56 (2·46, 5·15)	7·09 (4·44, 11·32)

*Adjusted for age, sex, centre, total cholesterol, HDL, creatinine, hypertension and homocysteine.
**Additional adjustment for B6, B12 and plasma folate.

vascular disease in separate smoking categories were significantly reduced after adjustment for vitamin levels (Table 3). Odds ratios were adjusted sequentially for age, sex and centre, conventional risk factors and homocysteine and finally for the B-vitamins. Plasma folate and vitamin B12 were dichotomized as top 20% or bottom 80%, whereas vitamin B6 was included as a linear variable as there was a clear linear effect of vitamin B6, in that it was a stronger predictor of risk as a linear than as a dichotomous variable. The regression analysis shows that adjustment for the B-vitamins had no significant effect on the risk associated with smoking. Smoking robustly survived adjustment for conventional and non-conventional risk factors in both light and heavy smoking groups.

Discussion

In this case control study we observed a 12-fold increase in risk in current smokers with modestly increased plasma homocysteine levels compared with subjects who had never smoked and who had plasma homocysteine levels of less than 12 $\mu mol \cdot l^{-1}$. The risk was even greater for men (Odds ratio 17·7, CI 7·4 to 42·6). These risks are greater than previously reported from our group[12]. The reason for this is that the original analysis compared current smokers with combined never and ex smokers. The spline curves in Fig. 2 suggest that, in terms of risk, ex smokers in this study appear to resemble current light smokers rather than never smokers. The comparison of current with never smokers alone results in greater risk estimates associated with smoking. While the OR associated with smoking and a raised plasma homocysteine was similar in both light and heavy smokers (Table 1 and Fig. 2) the proportionate increase in risk was greater in those smoking less than 20 cigarettes per day. While the reason for this is unclear, it may be that light smokers with a raised plasma homocysteine have reached the maximum achievable risk.

Several mechanisms might explain the increased risk in smokers with raised plasma homocysteine. Smoking affects the vascular tree via several different interactive mechanisms[21]. Nicotine and carbon monoxide separately produce tachycardia, hypertension and vasoconstriction and both produce direct endothelial damage. Smoking also affects vaso-occlusive factors such as platelet aggregation, plasma viscosity and fibrinogen levels[21]. Hyperhomocysteinaemia has been associated with impaired endothelial function and abnormal flow mediated vasodilatation has been demonstrated with mild hyperhomocysteinaemia[38–44]. It may also damage the vascular tree via platelet activation, lipid peroxidation, enhanced tissue factor activation, reduced Von Willebrand factor, increased fibrinogen levels and smooth muscle proliferation[45–47]. The fact that both of these risk factors can exert similar effects would suggest strong potential for interaction between them to produce vascular damage.

While both smoking and homocysteine may damage the vascular tree independently, they are also related. The Hordaland[26–28] and other studies[23–25] have shown higher homocysteine levels in smokers, as well as in males and older subjects. While this could be a direct effect of smoking it more likely reflects the different nutritional status of smokers. Subar *et al.*[48] have studied food and nutrient intake differences between smokers and non-smokers based on data from the Second National Health and Nutrition Survey (NHANES II) and found that smokers have a lower intake of most vitamins and were less likely to have consumed fruit, vegetables, vitamin and mineral supplements. A negative linear trend between the number of cigarettes smoked and vitamin intake was also noted. In the Caerphilly Heart Disease Study[49] total nutrient intake was also found to be lower in smokers than non-smokers with ex smokers having similar intake to non smokers.

In the present study, despite lower levels of B vitamins in smokers, adjustment for nutrient levels had an insignificant effect on risk estimates, highlighting the robustness of smoking as a cardiovascular risk factor. It should, however, be considered that smoking may produce vitamin deficiency in individual tissues; Piyathilake *et al.* have demonstrated reduced red cell and buccal mucosal B12 and folate levels in current smokers[50], with evidence of cell damage in these tissues[51]. Such an effect contributing to overall cardiovascular risk in smokers would not be reduced by adjustment for plasma nutrients and must be considered.

The limitations of the present study should be appreciated. Case–control studies of homocysteine yield a higher estimated risk than cohort studies[15]. To our

knowledge this study and our other report[12] remain unconfirmed in observing substantially increased risk in hyperhomocysteinaemic smokers. Examination of this risk in other case–control and especially cohort studies is required. If our findings of a substantially increased risk in smokers with a raised plasma homocysteine are confirmed, the public health implications are substantial. Homocysteine levels should be estimated in smokers and intensive counselling offered to help smokers to cease smoking if their homocysteine levels are raised. B vitamin supplementation might also be considered in such subjects.

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Appendix

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The Concerted Action Project partners are: Lars Brattstrom (*University Hospital, Lund, Sweden*); Leslie Daly (*Dept of Public Health Medicine, University College Dublin, Ireland*); Stephen Whitehead (*Dept of Genetics, Trinity College, Dublin, Ireland*); Ian Graham (*Adelaide and Meath Hospital, Tallaght, Ireland*); Paola Rubba/Generoso Andria (*Institute of Disease of Metabolism and Internal Medicine, University of Naples, Italy*); Per Ueland/Helga Refsum (*Institute of Pharmacology and Toxicology, University of Bergen, Norway*); Frans Kok/Petra Verhoef (*Wageningen Agricultural University, Wageningen, Netherlands*); Godfried Boers (*Dept of Endocrinology, Nijmegen, Netherlands*); H. De Valk (*Utrecht University Hospital, Utrecht, Netherlands*); Roberto Reis/Armando C. Sales Luis (*Lisbon, Portugal*); Alun Evans (*Dept of Community Medicine, Queens University, Belfast, Northern Ireland*); J. C. Wautrecht (*Hopital Erasme, Brussels, Belgium*); F. Parrot-Roulaud (*Hopital Pellegrin, Bordeaux-Cedex, France*); Daniele Garcon (*Laboratoire de Biochemie, Faculte de Pharmacie, Marseille, France*); M. Candito (*Laboratoire de Biochemie, Hopital Pasteur, Nice-Cedex, France*); Killian Robinson (*Wake Forest University Medical Centre, North Carolina, U.S.A.*); Richard Sheahan (*University of Texas, Galveston, U.S.A.*); Bo Israelsson (*Malmo University Hospital, Sweden*); Cuno Uiterwaal/Jacqueline Witteman (*Erasmus University Medical School, Rotterman, The Netherlands*); Helene Bellet (*Institute de Biologie, Montpelier, France*); Kok Soon Tan (*Toa Payoh Hospital, Singapore*).