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Luc, G., Bard, J., Lesueur, C., Arveiler, D., Evans, A., Amouyel, P., Juhan-Vague, I., Fruchart, J., Ducimetiere, P., & Kee, F. (2006). Plasma cystatin-C and development of coronary heart disease: the prime study. *Journal of Atherosclerosis*, 185(2), 375-380. <https://doi.org/10.1016/j.atherosclerosis.2005.06.017>

Published in:
Journal of Atherosclerosis

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Plasma cystatin-C and development of coronary heart disease: The PRIME Study

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Received 21 December 2004; received in revised form 4 May 2005; accepted 15 June 2005

Available online 25 July 2005

Abstract

The pathogenesis of ischemic coronary events involves degradation of the extracellular matrix in atherosclerotic lesions. The cysteine protease inhibitor cystatin-C may be involved in this phenomenon. The association of plasma cystatin-C with the incidence of myocardial infarction—coronary death and angina, was examined in a nested case-control (two controls per case) design within the prospective cohort study (Prospective Epidemiological Study of Myocardial Infarction (PRIME Study)) which included 9758 men aged 50–59 years who were free of coronary heart disease (CHD) on entry and followed for a 5-year period. Three hundred and thirteen participants suffered myocardial infarction or coronary death ($n = 159$) or angina pectoris ($n = 154$) during follow-up. Cystatin-C was positively correlated with body mass index (BMI), low-density lipoprotein (LDL)-cholesterol, triglycerides and several inflammatory markers such as fibrinogen ($r = 0.18$), C-reactive protein (CRP) ($r = 0.24$), interleukin-6 ($r = 0.20$), tumor necrosis factor- α (TNF α) ($r = 0.27$) and two TNF α receptors: TNFR1A ($r = 0.43$) and TNFR1B ($r = 0.41$); and negatively with high-density lipoprotein (HDL)-cholesterol ($r = -0.25$). After adjustment for traditional risk factors (age, diabetes, smoking, hypertension, BMI, triglycerides, LDL- and HDL-cholesterol), cystatin-C was significantly associated with the occurrence of the first ischemic coronary event. However, this association was no longer significant when CRP was included in the analysis. A decrease in glomerular filtration rate did not explain higher cystatin-C in cases than in controls. Cystatin-C appears to participate in the inflammatory phenomenon observed in the atherosclerotic process. Cystatin-C is not a more predictive risk marker of CHD than CRP or interleukin-6, but could be useful in detecting moderate chronic renal disease.

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Keywords: Cystatin-C; Angina; Myocardial infarction; Epidemiology; Risk factors

Abbreviations: CHD, coronary heart disease; GFR, glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction; PRIME Study, Prospective Epidemiological Study of Myocardial Infarction; RR, relative risk; TNF, tumor necrosis factor

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¹ See Appendix A.

1. Introduction

Atherosclerosis is an inflammatory disease that involves extracellular matrix degradation and vascular wall remodeling. Coronary ischemic events which are mainly due to rupture or fissuring of atherosclerotic lesions are thought to be the result of an imbalance between proteases which degrade extracellular matrix and protease inhibitors. Of the latter, cystatin-C, a 13-kDa cysteine protease inhibitor, is believed to play a pivotal role in tissue remodeling because of its high concentration in biological fluids. Cystatin-C is normally expressed in vascular wall smooth muscle cells and inhibits elastase secreted by these cells. While cystatin-C is present in normal arteries, immunostaining of cystatin-C has shown low expression in atherosclerotic plaques [1], suggesting that low levels of cystatin-C could be a risk factor for ischemic events. Absolute or relative low levels of cystatin-C in the artery wall injured by the inflammatory process of atherosclerosis would not counterbalance the increase in cysteine protease induced by pro-inflammatory cytokines.

The present study has examined the association between plasma cystatin-C levels and the incidence of coronary heart disease (CHD) in a prospective cohort study, the Prospective Epidemiological Study of Myocardial Infarction (PRIME Study). The PRIME Study is a cohort study set up to investigate prospectively the association of different risk factors and CHD among initially healthy men simultaneously recruited in France and Northern Ireland, two geographically close countries but characterized by contrasting risk of CHD unexplained by traditional risk factors [2]. Prospective cohort studies have usually evaluated the association between putative risk markers and myocardial infarction (MI) or coronary death but very few have used angina pectoris as an end-point. We have studied the efficiency of cystatin-C levels in predicting CHD risk in the PRIME prospective cohort according to the type of first clinical event during follow-up: MI-coronary death, but also angina pectoris.

2. Materials and methods

The PRIME Study has been described in great detail elsewhere [3]. Briefly, it is a prospective cohort study which was set up to investigate risk factors for ischemic heart disease. From 1991 to 1994, 10,600 men aged 50–59 years living in France and in Northern Ireland were recruited to broadly match the social class structure of the background population. On entry, questionnaires relating to medical history, tobacco consumption were obtained and physical measurements were taken. Venous blood samples were collected after a 12-h fast. Plasma was immediately processed for long storage in liquid nitrogen. Additional data were collected on all participants every year over 5 years of follow-up (98.5% response). For subjects reporting a possible clinical event, clinical information was sought directly from the hospital or general practitioners' files. All details of ECG, hospital

admissions, enzymes, surgical operations, angioplasty, treatment, etc., were collected. Death certificates were also used to complete information on the cause of death.

A Medical Committee was established to provide independent validation and classification of coronary events as previously described [2]. CHD categories retained for analysis were non-fatal MI or coronary death and angina as the first event [4]. The first category included subjects who had at least one non-fatal MI or who died from CHD during follow-up. Angina pectoris was defined by the presence of chest pain at rest and/or on exertion and at least one criterium indicating angiographic or electrical coronary abnormality.

The number of lost-to-follow-up subjects, i.e. those who could not be contacted in the fifth year of surveillance or who refused to participate in the study at any time during the follow-up, was 228. As low-density lipoprotein (LDL)-cholesterol used in the statistical analysis was calculated according to the Friedewald formula [5], subjects with triglycerides up to 400 mg/dl ($n=217$) were excluded. Furthermore, only subjects without any history of CHD on entry were included in this study. The number of subjects free of CHD on entry and not lost-to-follow-up was therefore 9758: 7359 living in France and 2399 in Northern Ireland.

To evaluate cystatin-C as a marker of coronary risk in the PRIME Study, baseline plasma samples were assayed from the 313 study participants who subsequently developed a coronary ischemic event during follow-up and from 2 controls per case. Matched controls were study participants recruited in the same center and on the same day (± 3 days) as the corresponding case and were free of CHD on the date of the ischemic event of the case. Cystatin-C and C-reactive protein (CRP) were measured by immunonephelometry (CRP: Dade Behring, Reuil-Malmaison France), creatinine by colorimetric method (DiaSyst GmbH, Holzheim, Germany) and fibrinogen as previously described [6]. Interleukin-6 (IL-6), tumor necrosis factor α (TNF α) and its two receptors, TNFR1A and TNFR1B, were determined by ELISA using commercial kits (R&D Systems, Minneapolis, MN). Methods used to evaluate baseline lipid parameters and fibrinogen have been described elsewhere [3]. Laboratory personnel were unaware of case or control status. Glomerular filtration rate (GFR) was calculated according to the MDRD Study formula [7]. Similar results were obtained using the Cockcroft–Gault equation [8] and are not shown because they are accurate than those with the MDRD formula [7].

2.1. Statistical analysis

All statistical analyses were carried out using the statistical SAS package (SAS Institute, Cary, NC). Values of continuous variables are expressed as means $\pm$ S.D., but the median and inter-quartile values of cystatin-C, triglycerides, fibrinogen, CRP, TNF α , TNFR1A and TNFR1B are given because of their rightward skewed distribution. Correlations between continuous variables were calculated using Spearman's rank correlation coefficients in the control group. A

Table 1
Baseline patient characteristics of participants (The Prime Study)

	Cases (<i>n</i> = 313)	Controls (<i>n</i> = 626)	<i>p</i>
Age (years)	55.4 ± 2.9	55.2 ± 2.	ns
Body mass index (kg/m ²)	27.1 ± 3.4	26.5 ± 3.4	0.02
Cholesterol (mg/dl)	231 ± 37	221 ± 37	0.001
LDL-cholesterol (mg/dl)	155 ± 35	145 ± 35	0.001
HDL-cholesterol (mg/dl)	45 ± 13	48 ± 13	0.001
Triglycerides (mg/dl)	136 (102–1.91)	124 (92–179)	0.04
Current smokers (%)	56	41	0.008
Hypertension (%)	27	15	0.001
Diabetes mellitus (%)	10	6	0.005
Cystatin-C (mg/l)	0.81 (0.71–0.89)	0.78 (0.69–0.87)	0.003
Creatinine (mg/l)	10.9 ± 2.7	10.9 ± 2.7	ns
GFR (ml/min 1.73 m ²)	79.6 ± 23.5	80.5 ± 25.8	ns
Fibrinogen, (mg/dl)	340 (287–415)	314 (275–372)	0.001
CRP (mg/l)	2.00 (0.74–3.55)	1.31 (0.62–2.62)	0.001
Interleukin-6 (pg/ml)	1.58 (1.04–2.60)	1.25 (0.84–1.97)	0.001
TNFα (pg/ml)	3.76 (3.07–4.65)	3.60 (2.97–4.43)	ns
TNFR1A α (pg/ml)	1044 (873–1255)	1041 (869–1233)	ns
TNFR1B α (pg/ml)	2104 (1764–2445)	2094 (1731–2426)	ns

Means ± S.D. are presented for age, body mass index (BMI), cholesterol, LDL-, HDL-cholesterol and cystatin-C, medians (25–75th percentile) for triglycerides, fibrinogen, CRP, interleukin-6, TNFα, TNFR1A and TNFR1B, and percentages for smokers, hypertension and diabetes mellitus. The comparison between cases and controls was performed using conditional logistic regression analysis (univariate analysis). ns: not significant (*p* > 0.05).

conditional logistic regression analysis suitable for a nested case–control design was performed to identify discriminating predictive parameters. The same type of analysis was used to determine the relative risk (RR) of future CHD event after controlling for the presence of diabetes, hypertension or smoking, and possibly for LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride and CRP levels. Homogeneity of the effect of cystatin-C between populations was tested by the Z-score. Log-transformation was carried out for non-normally distributed parameters before analysis. All tests were considered significant at the 0.05 level.

3. Results

The characteristics and biological values of 313 cases and 626 controls included in the nested case–control study are presented in Table 1. Compared with their matched controls without CHD events, the subjects with incident CHD during the 5-year follow-up were of similar age. As expected, body mass index (BMI), total cholesterol, LDL-cholesterol, and also triglycerides, were significantly higher in cases, while HDL-cholesterol was lower. Furthermore, the prevalence of smoking, hypertension and diabetes was also higher in cases. Creatinine and GFR were no different between cases and controls. Most subjects had normal creatinine levels (95th per-

Table 2
Spearman's rank correlation coefficients between cystatin-C and age, BMI, lipids, creatinine, glomerular filtration rate (GFR) and inflammatory parameters in the control group (The Prime Study)

	Cystatin-C
Age	0.09*
BMI	0.01
Cholesterol	0.03
LDL-cholesterol	0.05
HDL-cholesterol	−0.26**
Triglycerides	0.20**
Fibrinogen	0.14**
Creatinine	0.06
GFR	−0.03
CRP	0.23**
Interleukin-6	0.19**
TNFα	0.24**
TNFR1A	0.44**
TNFR1B	0.42**

** *p* < 0.001.

* *p* < 0.05.

centile: 16 mg/l). In univariate analysis, cystatin-C plasma levels were significantly higher in cases than in controls (*p* = 0.003). This result is in contrast with our initial hypothesis. As cystatin-C is related to tissue remodeling and so potentially with the inflammatory process present in the artery wall, we checked relations between cystatin-C and inflammatory markers previously measured in the PRIME Study. As already described, fibrinogen, CRP and IL-6 were significantly higher in cases than in controls [9], whereas TNFα and its two soluble receptors, TNFR1A and TNFR1B, were not significantly different. Results of correlations between cystatin-C and anthropometric and other biological parameters in the control cohort are shown in Table 2. Cystatin-C was positively and significantly correlated with age, triglycerides and all inflammatory markers, whereas a significantly negative correlation was observed with HDL-cholesterol. These coefficients were clearly high for triglycerides (*r* = +0.20), HDL-cholesterol (*r* = −0.26) and all inflammatory markers measured in the present study. These coefficients were even relatively high for TNFR1A (*r* = +0.43) and TNFR1B (*r* = +0.41). In particular, cystatin-C was similarly correlated with CRP in controls (*r* = 0.19; *p* ≤ 0.001) and also in subjects with future MI or coronary death (*r* = 0.22; *p* = 0.02) and subjects with future angina (*r* = 0.19; *p* = 0.02). No significant correlation was observed between cystatin-C and renal function markers such as creatinine or GFR in controls (Table 2).

To assess the predictive value of cystatin-C, this parameter was included in a logistic regression analysis after controlling for age (model 1), non-lipid covariates (diabetes, hypertension and smoking) (model 2) and non-lipid and lipid parameters (LDL-, HDL-cholesterol and triglycerides) (model 3). The results presented in Table 3 show that cystatin-C was significantly associated with CHD after controlling for age, this association being attenuated when non-lipid and lipid covariates were included (models 2 and 3), but remaining statistically significant (*p* = 0.04). The relative risk, which

Table 3
Conditional logistic regression of CHD risk upon cystatin-C (The Prime Study)

Cystatin-C (mg/l)	Wald χ^2	<i>p</i>	Relative risk per S.D. (95% CI)
Model 1	8.4	0.004	1.25 (1.07–1.45)
Model 2	6.0	0.01	1.21 (1.04–1.42)
Model 3	4.4	0.04	1.19 (1.01–1.40)
Model 4	2.1	0.15	1.13 (0.95–1.34)

Subjects with MI or coronary death or angina pectoris as the first coronary ischemic event were compared to controls. The analysis included age (model 1), age, presence of diabetes, hypertension and smoking (model 2), the same covariates and lipid parameters (model 3) and the covariates included in model 3 and CRP (model 4). Non-normally distributed parameters were log-transformed.

was moderately attenuated when non-lipid and lipid factors were included in the model, increased by about 20–25% per 1S.D. change of cystatin-C. However, cystatin-C was no longer significantly ($p = 0.15$) associated with a future coronary ischemic event when CRP was included in the model (Table 3, model 4), while CRP remained highly significant ($p = 0.003$). Adding IL-6 instead of CRP in the model gave similar results.

The PRIME Medical Committee enabled us to divide cases into two categories: MI and coronary death ($n = 159$), and angina pectoris ($n = 154$) according to the first occurrence of the disease (the total number of those suffering MI and coronary death, and angina is higher than the total number of cases because 4 subjects who first had angina, then MI in the following month were included in both clinical categories). Medians of cystatin-C plasma levels were similar, 0.79 and 0.82 mg/l for MI-coronary death and angina, respectively.

No significant interaction was observed between cystatin-C and the clinical category—MI-coronary death and angina (Z -score = 0.88; $p > 0.05$ for model 3).

Similarly, no interaction was noted between cystatin-C and the country, France or Northern Ireland (Z -score; $p = 0.67$).

4. Discussion

The present analysis of cystatin-C levels in a prospective cohort study on CHD was undertaken under the hypothesis that cystatin-C would be lower in cases than in controls, because it had been observed that cystatin-C was considerably reduced in atherosclerotic lesions [1]. It was therefore surprising to note a significantly higher cystatin-C level in subjects developing ischemic coronary event than in controls who did not. Cystatin-C seems to have an important role in the artery wall because its deficiency, reflected by its decrease in plasma and in artery lesions, is seen in patients with aortic aneurysms [1,10]. The appearance of these aneurysms could be due to a low cystatin-C level which does not counterbalance the elevated expression of cysteine proteases [1,10]. Plaque rupture or fissuring leading to a coronary event is due to an imbalance between synthesis and degradation of the

fibrous cap. A hypothesis is that factors inhibiting extracellular matrix degradation decrease, so favoring plaque rupture. Cystatin-C is an important inhibitor of proteases and its decrease could have a role in the onset of an ischemic event. The present results do not eliminate the possibility that cystatin-C expression decreases in the atherosclerotic plaque before an ischemic event because samples were taken several months or years before it occurred. However, the analysis of cystatin-C in cases taking into account the time interval between blood sampling and the date of ischemic event did not show any difference: in particular, cystatin-C levels were similar in subjects suffering a coronary event in the 3 or 12 months after blood drawing and in those who presented such an event after this delay. Low cystatin-C expression in atherosclerotic lesions at the moment of plaque rupture or fissuring leading to a coronary event is not excluded by the present results because samples were taken several months or years before it (data not shown).

The higher plasma concentration of cystatin-C in cases than in controls detected in the present study contrasts with the report of the absence of association between cystatin-C and the risk of peripheral arterial disease [11]. This difference could be consequent to variable pathophysiology and depend on the localization of atherosclerosis. Indeed, if risk factors for peripheral vascular disease are the same as for CHD, a different set of variables is predictive of outcomes for the various vascular beds. The effects of cigarette smoking and diabetes are especially important for the development of peripheral arterial disease [12]. A difference in risk markers between CHD and peripheral disease is therefore not surprising. Recently, results obtained in a German cohort including subjects in secondary prevention concord with the present results confirming the positive association between the incidence of CHD and cystatin-C [13].

Animal studies show that the balance between protease activities and their inhibitors have an important role in vascular disease [14]. Our results suggest that ischemic events would be the consequence of relative insufficiency of protease inhibitors, such as cystatin-C, the main one, in relation to the increased expression of matrix proteinases in inflammatory atherosclerotic lesions, even if the inflammatory process increases cystatin-C expression. Deficiency in cystatin-C could be partially genetically determined, a relation between mutant cystatin-C haplotype having been associated with a decrease in cystatin-C levels [15].

The apparent inconsistency between the reduction in cystatin-C in coronary plaques [1] and our results showing higher plasma levels in subjects with future coronary ischemic events could indicate an overexpression of cystatin-C in cells outside the artery wall, which could create a balance with decreased arterial expression. But the high level of cystatin-C in cases could also be the result of the inflammatory process in the arteries. The present study has found strong correlations between levels of cystatin-C and those of several inflammatory markers (Table 2). High levels of cystatin-C have already been observed in diseases such as

asthma [16] and neoplasia [17], associated with moderate, local and chronic inflammation. As markers such as CRP and IL-6 are considered as surrogates of the inflammatory process within the artery wall [18,19], it is possible that inflammatory cytokines secreted by inflammatory cells present in atherosclerotic lesions stimulate the secretion of cystatin-C. The particularly strong correlation between cystatin-C and soluble TNF receptors ($r \sim 0.40$) (Table 2) suggests that cystatin-C expression is partly controlled by the TNF α pathway. Other experimental studies are needed to determine if elevated levels of cystatin-C are due to a high secretion rate by arterial cells, such as smooth muscle cells, or by extravascular cells in response to a high level of inflammatory cytokines.

Mild or severe chronic renal insufficiency is associated with atherosclerosis [20–22] and cystatin-C is recognized as an accurate endogenous marker of the glomerular filtration rate, probably superior to creatinine [23]. We therefore assessed whether higher cystatin-C in cases than in controls was the consequence of a decrease in GFR (calculated using creatinine levels) in cases. The absence of correlation between creatinine and cystatin-C levels was not surprising considering the narrow range of both these parameters in this population [24]. The absence of association between GFR and CHD risk excluded moderate renal insufficiency at the origin of higher cystatin-C in cases than in controls in the PRIME Study. Furthermore, Koenig et al. [13] noted that cystatin-C was associated with the risk of future coronary vascular disease in patients with CHD independently of creatinine levels or GFR. As for the high correlation coefficients between cystatin-C and inflammatory markers, it is therefore likely that higher levels of cystatin-C in cases than in controls reflect a more marked inflammatory process rather than a lower glomerular rate.

There are potential limitations to our study. First, we cannot exclude the possibility that protein degradation appeared during storage and affected the results, even if plasma was stored at a very low temperature (-196°C). However, cystatin-C levels measured in the present study are similar to those reported in previous ones which used fresh plasma samples [23,24]. Second, cases were subjects who suffered MI-coronary death as well as angina and subjects were recruited in two different countries with a different incidence of CHD. Angina has not usually been used as a clinical end-point in prospective epidemiological studies, but, in the PRIME Study, angina was diagnosed not only clinically but through the evidence of coronary lesions. Finally, there are no differences in the association between cystatin-C and the clinical form of CHD on the one hand and the country on the other hand.

Different methods were used to measure cystatin-C and if reference intervals determined by different methods were globally similar [23], the cystatin-C measurement was not standardized, which means it was difficult to compare not only the studies themselves but the use of cystatin-C as a marker of GFR. However, results of the two only prospective studies on the interest of cystatin-C, the PRIME Study and the

German cohort [13], used the same commercial kit. Cystatin-C appeared moderately higher in the German cohort than in the PRIME cohort, this difference probably being due to that of the subjects included: CHD and CHD-free, respectively. As shown in the present study and in the German cohort, cystatin-C was positively correlated with CRP. So, the presence of a subclinical inflammatory process in subjects with present or future CHD partially explained why CHD subjects had a higher cystatin-C level than those who remained CHD-free.

In conclusion, a high cystatin-C level could also be a response to the inflammatory process in atherosclerotic lesions as documented by strong relations with other inflammation markers such as CRP, IL-6 and markers of the TNF α pathway. Prospective cohort studies are unable to determine the mechanisms causing elevated cystatin-C in CHD patients and more studies are needed to explain them. Finally, cystatin-C is determined using similar methods and tools to those used for CRP, which needless to say, appears to be more discriminative, indicating that its measurement is preferable for defining CHD risk. However, as chronic kidney disease appears to be associated with high CHD risk, cystatin-C could be measured rather than creatinine to detect renal failure because of its higher sensitivity.

Acknowledgements

We are indebted to Ms. Latifa Elkhailil, Emmanuelle Lee and Emmanuelle Moitrot for their technical assistance with this project. We thank the following organizations which allowed the recruitment of the PRIME subjects: the Health Screening Centers organized by the Social Security of Lille (Institut Pasteur), Strasbourg, Toulouse and Tourcoing; Occupational Medicine Services of Haute-Garonne, of the Urban Community of Strasbourg; the Association Inter-entreprises des Services Médicaux du Travail de Lille et environs; the Comité pour le Développement de la Médecine du Travail; the Mutuelle Générale des PTT du Bas-Rhin; the Laboratoire d'Analyses de l'Institut de Chimie Biologique de la Faculté de Médecine de Strasbourg; the Department of Health (NI) and the Northern Ireland Chest Heart and Stroke Association.

We also thank the members of the Event Validation Committees: Pr L. Guize, Dr. C. Morrison, Dr. M.-T. Guillauneuf, Pr M. Giroud; and the Alliance Partnership Programme for its financial support. We thank Dade Behring for providing cystatin-C reagents.

This work was supported by grants from INSERM, Pasteur Institute of Lille and Fondation de France.

Appendix A

A.1. The PRIME Study Group

The PRIME Study is organized under an agreement between INSERM and the Merck, Sharpe and Dohme-

Chibret Laboratory, with the following participating laboratories:

- The Strasbourg MONICA Project, Department of Epidemiology and Public Health, Faculty of Medicine, Strasbourg, France (D. Arveiler, B. Haas).
- The Toulouse MONICA Project, INSERM U558, Department of Epidemiology, Paul Sabatier-Toulouse Purpan University, Toulouse, France (J. Ferrières, J.B. Ruidavets).
- The Lille MONICA Project, INSERM U508, Pasteur Institute, Lille, France (P. Amouyel, M. Montaye).
- The Department of Epidemiology and Public Health, Queen's University, Belfast, Northern Ireland (A. Evans, J. Yarnell, F. Kee).
- The Department of Atherosclerosis, INSERM UR545, Lille, France (G. Luc, J.M. Bard).
- The Laboratory of Haematology, La Timone Hospital, Marseilles, France (I. Juhan-Vague).
- The Laboratory of Endocrinology, INSERM U326, Toulouse, France (B. Perret).
- The Vitamin Research Unit, The University of Bern, Bern, Switzerland (F. Gey).
- The Trace Element Laboratory, Department of Medicine, Queen's University Belfast, Northern Ireland (Jayne Woodside, Ian Young).
- The DNA Bank, INSERM U525, Paris, France (F. Cambien).
- The Coordinating Center, INSERM U258, Paris-Villejuif, France (P. Ducimetière, A. Bingham).

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