Research method

The San Daniele 2 Project: protocol description and population characteristics

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In 1990 we studied the prevalence and determinants of carotid atherosclerosis in an Italian general asymptomatic population (630 males and 718 females aged 18-99 years) living in the San Daniele district of the Friuli-Venezia Giulia Region.

The global prevalence of subclinical carotid atherosclerosis was 25.4% in men and 26.4% in women. We considered intima-media thickness, non-stenotic plaque (< 40%), and stenotic plaque (> 40%).

In the multiple logistic regression, the cross-sectional analysis of subjects aged 40 years showed a positive significant association between plaques/stenosis and age ($p < 0.001$), systolic blood pressure ($p < 0.01$), cigarette smoking ($p < 0.0001$), and the protective effect of high-density lipoprotein cholesterol ($p < 0.037$).

In 2002 we decided to re-examine the initial cohort with the following objectives: prospectively evaluating the modifications of the previous carotid findings, their relationship with known and less documented cardiovascular risk factors and the predictive power of those variations on incident coronary and cerebrovascular events. We plan to evaluate the association of carotid plaque and carotid intima-media thickness with the genetic polymorphisms involved in atherosclerosis in survivors and finally to study the incidence and the determinants of atrial fibrillation in a general population.

In this paper, we will describe the methodology of the screening and the cohort population characteristics.

We have compared the San Daniele Project survivor cohort's characteristics to the current general population of the same age living in the Friuli-Venezia Giulia Region so as to extend the study's conclusions to the whole regional population.

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Introduction

Well-documented modifiable risk factors are considered those with clear, supportive epidemiological evidence in addition to evidence of risk reduction with modification as documented by randomized trials. Asymptomatic carotid stenosis is considered a well-documented modifiable risk factor for ischemic stroke in a recent statement of the American Heart Association[1].

Even though the intima-media thickness (IMT) of carotid arteries is associated with the prevalence and incidence of cerebrovascular and cardiovascular ischemic events, it is not yet considered a validated, well-documented risk factor, mainly because of the lack of trials documenting the role of IMT reduction *per se* in decreasing incident coronary and cerebrovascular events.

The carotid IMT is easily and precisely evaluated at non-invasive B-mode imaging, but the measurement procedures are not standardized and are still subject of debate. The atherosclerotic plaque and carotid IMT are different elements of atherosclerosis and reflect diverse aspects and phases of the inflammatory process.

Therefore, we considered whether the separate characterization of plaque and IMT may provide better information for the determination of disease risk.

In 1990, we studied the prevalence and determinants of carotid atherosclerosis in an Italian general population living in the San Daniele area of the Friuli-Venezia Giulia Region[2]. In this study, called the San Daniele Project, we examined 630 men and 718 women aged 18-99 years (participation rate 74.9%): the global prevalence of asymptomatic clinical and subclinical

carotid atherosclerosis was 25.4% in men and 26.4% in women. Intima-media thickening, defined as a thickness > 1 mm, was found in 9.4% of men and 11.7% of women; the plaque prevalence was 13.3% in men and 13.4% in women; the prevalence of stenotic plaques in the two genders was 2.7 and 1.5% respectively. In the multiple logistic regression, the cross-sectional analysis of subjects aged 40 years showed a positive significant association between the severity of carotid atherosclerotic lesions (plaques and stenosis) and age ($p < 0.001$), systolic blood pressure ($p < 0.01$), cigarette smoking ($p < 0.0001$), and the protective effect of high-density lipoprotein (HDL) cholesterol ($p < 0.037$). This analysis did not provide evidence of a clear-cut association between risk factors and IMT. After 12 years we decided to re-examine the initial cohort to prospectively evaluate the modifications of the previous carotid findings, their relationship with known and less documented cardiovascular risk factors and the predictive power of those variations on incident coronary and cerebrovascular events. Furthermore, we plan to evaluate the association of carotid plaque and IMT with the genetic polymorphisms involved in atherosclerosis in survivors and finally to study the incidence and the determinants of atrial fibrillation in a general population. The first step to organize the new examination is to assess the original cohort's survival and this was done by comparing this population with the general population of the same age living in the Friuli-Venezia Giulia Region.

In this paper we will describe the protocol of the new examination, after evaluating the initial cohort's survival. The determination of the incidence of cardiovascular fatal and non-fatal events and their possible correlation with the initial and final carotid situation will be possible at the end of the new study, when the relevant information will be available and validated.

Methods

Subjects. Of the 1348 subjects, 630 men and 718 women aged 18-99 years at entry in 1990, 150 (81 men and 69 women) died during the follow-up period, while 1 male and 4 females migrated out of the Friuli-Venezia Giulia Region. Therefore the cohort of the San Daniele 2 Project consists of 1193 subjects (548 males and 645 females). In order to evaluate the possibility of extending the results of the new project to the whole Region, we performed a distribution analysis of the original cohort and compared it with that of the general population of the Friuli-Venezia Giulia Region of the same age range, resident in 1990. We were then able to evaluate the survival of the original cohort in comparison with that of the regional population. Both analyses were performed by sex. All these elaborations were possible because the Friuli-Venezia Giulia Region has a computerized roll of all the resident citizens covered by the National Health System, as well as computerized mortality and hospital discharge registries. Every citizen has a unique identification code which is mandatory for all National Health System services and it is recorded also in the death certificate. The code with the relevant demographic and health information is blocked only if the citizen migrates definitively out of the Region.

General methodology. The study protocol was submitted to and approved by the local Health Authority (Azienda per i Servizi Socio-Sanitari N. 4 "Medio Friuli", Udine), the Medical Chamber of Udine and the National Institute of Health, which is responsible for the serum and DNA banks produced by the Project. The San Daniele 2 Project was presented to the local Councils, to the general practitioners and to the community through various meetings and media events. All the subjects of the San Daniele 2, as well as their general practitioners, received a letter in which the study coordinator (PP) explained the reason for attending the surveys, the ways in which the subjects would receive a telephone call and arrange their visit to the San Daniele Hospital, clearly stating the responsibilities of the Centers involved through their directors (PP, DV, LM). A research nurse was hired and specifically trained for the project by the Cardiovascular Prevention Center, ASS 4, Udine, which is a WHO Collaborating Center for the Monitoring and Prevention of Cardiovascular Diseases.

Measurements of cardiovascular risk factors. The study adopts procedures for data collection and measurement assessment in strict adherence to the WHO MONICA manual [WHO MONICA Project. MONICA Manual (1998-1999), available at http://www.ktl.fi/publications/monica/manual/index.htm, URN:NBN:fife19981146.], as used in the Italian Cardiovascular Epidemiological Surveillance System[3].

First of all, the invited subjects sign a written informed consent on a form provided by the National Institute of Health. Briefly, the research nurse takes the blood pressure with the subject seated and at rest for at least 10 min, using a standard mercury sphygmomanometer. The systolic and diastolic blood pressures are taken at the first and fifth phases of the Korotkoff sounds, respectively. Two measurements, taken 5 min apart, are obtained for each subject. The sphygmomanometer is equipped with two-side cuff bladders (13 and 17 cm). The height and weight are measured on subjects without shoes and wearing light clothing. The body mass index is then computed as the weight in kilograms divided by the square of the height in meters. The waist circumference is measured at a level midway between the lower rib margin and iliac crest with the tape extended horizontally all around the body. The hip circumference is measured as the maximal circumference over the buttocks. A standard 12-lead ECG is recorded as described in Cardiovascular Survey Methods[4] and read in a simplified standardized

form by a single cardiologist (LM). Venous blood specimens are taken from the antecubital vein with the subject seated and after a 12-hour fast. A tourniquet is used only if necessary. The specimens are immediately transferred to the Laboratory of the San Daniele Hospital for basic metabolic assays (total and HDL cholesterol, triglycerides, glucose, uric acid) and serum separation and then stored at -20°C for the subsequent determination of lipoprotein(a) and for highly-sensitive C-reactive protein assay[5] and total EDTA blood refrigeration at -20°C for future genetic research. Total cholesterol and HDL cholesterol determinations are made on sera using an automatic enzymatic method and the HDL cholesterol subfraction is separated using the phosphotungstate-Mg^{++} PEG 6000 method. Triglycerides are determined using the GPO-PAP enzymatic colorimetric method and low-density lipoprotein cholesterol is calculated using the Friedewald formula[6]. The glycemia is determined using the kinetic UV-test with the glucose dehydrogenase method and uric acid by uricase-PAP. National and regional-based external and internal quality control programs are periodically performed at the San Daniele Hospital Laboratory.

A computerized standardized questionnaire is used to collect information relevant to risk assessment and incident event evaluation. The questionnaire includes aspects regarding smoking habits, the use of antihypertensive, lipid-lowering and antidiabetic medications within the past 2 weeks, the use of aspirin for cardiovascular prevention, and the use of oral contraceptives and hormone replacement therapy in women. A family history of first-degree premature myocardial infarction (MI), coronary heart disease (CHD), stroke and peripheral artery disease is investigated by means of differently posed questions regarding the age of onset in men (< 55 years) and women (< 65 years). A family history of hypertension, dyslipidemia and diabetes is also ascertained. Prevalent diabetes mellitus is defined using self-reported physician diagnosis of diabetes, insulin and oral hypoglycemic treatments, or fasting blood glucose levels > 126 mg/dl when available. A positive history of acute coronary syndromes is attributed for documented MI or unstable angina pectoris or cardiac revascularization procedures, or in case of ECG changes suggestive of MI (Minnesota code 1-1 or 1-2, except 1-2-6). Stable angina pectoris as well as stroke and transient ischemic attacks are confirmed on the basis of medical records.

Ascertainment of incident major coronary and cerebrovascular events. Incident major coronary and cerebrovascular events are validated on the basis of the MONICA protocol and of the new criteria of the European Society of Cardiology (ESC) and the American College of Cardiology (ACC)[7]. Briefly, according to the MONICA criteria, non-fatal coronary events may be classified as definite and possible.

A definite non-fatal event is defined as:
1) progression of Minnesota codes on serial ECGs: a) progression from no Q wave to a definite Q wave; or b) a lesser Q wave progression combined with progressive ST-segment depression, developing ST-segment elevation, or progressive T wave inversion; or c) persistent ST segment elevation with progressive T wave inversion in sequential daily ECGs, or
2) cardiac enzyme levels 2 times the normal cut-off point, either with typical symptoms and an abnormal ECG, or with lesser symptoms and an ECG progression labeled as probable.

Non-fatal events are placed in the category possible if a typical prolonged chest pain (20 min) occurs together with lesser or no ECG and enzyme findings.

Fatal coronary events are classified as definite, possible, and unclassifiable.

Events are definite if they satisfy the following criteria: 1) definite criteria reported for non-fatal events; or 2) autoptic evidence of a recent MI or coronary thrombosis.

Possible coronary death includes suggestive terminal symptoms, or a CHD history in the absence of chronic occlusive CHD, or an old MI without pathological findings suggestive of a fatal disease.

Recently, sensitive and specific serologic biomarkers have become available for the identification of very small MIs that could not have been detected earlier. The biomarker of myocardial damage is cardiac troponin, which has almost absolute myocardial tissue specificity, as well as a high sensitivity. This fostered the release of the new criteria of the Joint ESC/ACC Committee for the Redefinition of Myocardial Infarction[7].

• Criteria for an acute, evolving or recent myocardial infarction. Either one of the following criteria satisfies the diagnosis for an acute, evolving or recent MI:
1) a typical rise and a gradual fall (troponin) or a more rapid rise and fall (creatine kinase-MB) of the biochemical markers of myocardial necrosis associated with at least one of the following: a) ischemic symptoms; b) the development of pathologic Q waves on the ECG; c) ECG changes suggestive of ischemia (ST-segment elevation or depression); or d) coronary artery intervention (e.g., coronary angioplasty);
2) pathologic findings of an acute MI.

• Criteria for an established myocardial infarction (past). Any of the following criteria satisfies the diagnosis for an established MI:
1) the development of new pathologic Q waves on serial ECGs. The patient may or may not remember previous symptoms. The biochemical markers of myocardial necrosis may have normalized, depending on the time elapsed since the infarct developed;
2) pathologic findings of a healed or healing MI.

According to the MONICA criteria, definite stroke is a rapid development of focal (or global) signs or dis-

turbance of cerebrovascular function lasting > 24 hours (unless interrupted by surgery or death), with no apparent cause other than that of a vascular origin; this category includes patients presenting with clinical signs and symptoms suggestive of subarachnoid hemorrhage, intracerebral hemorrhage, or a cerebral ischemic infarct. The term "global" refers to patients with subarachnoid hemorrhage or deep coma but it excludes coma of a systemic vascular origin such as shock, Stokes-Adams syndrome or hypertensive encephalopathy.

The suspected events are identified during the standardized interviews for participants and by means of a phone call interview to the subjects or his/her relatives. Moreover, a systematic search of the Hospital Discharge Register is performed. Fatal events have already been identified.

To evaluate the modifications of the previous carotid findings in relation to known and less documented cardiovascular risk factors, univariate and multivariate logistic regression analyses will be performed, while the predictive power of those variations on incident coronary and cerebrovascular events will be analyzed using Cox proportional hazard models.

Ultrasonographic methods. The ultrasound measurements were performed by the same trained neurologist (MC) who performed the exams in 1990 employing a 7.5 MHz probe frequency 4500/5500 HP machine (Philips Medical System, Andover, MA, USA) (axial resolution of 0.4 mm and a bidirectional 4 continuous wave Doppler probe). The ultrasonographic examination was performed according to a standardized protocol described by Touboul and Prati[8]. Subjects are examined in the supine position in a darkened room: the common carotid, its bifurcation and internal and external carotid arteries are bilaterally insonated with longitudinal (anterior, lateral, posterior) and transverse scans. A plaque is defined as localized focal echo structure encroaching into the vessel lumen and dilations of the arterial wall > 1.5 mm. The plaques are classified in non-stenotic and stenotic according to the percentage obstruction of the arterial lumen (inferior or superior to 40%). The plaques are visualized in the transverse scan in case of non-stenotic plaques and in the projection showing the greatest hemodynamic impairment at Doppler spectral analysis in case of stenotic plaques. The plaques are described according to the same standardized ultrasonographic form of the previous survey. All participant subjects have intima-media thickening measurements of the far wall of the common carotid artery, of at least 10 mm of continuous boundary between the intima-media and media-adventitia clearly distinguishable and free of plaque, faraway from the bifurcation and at the end of the P wave of the ECG with a digital acquisition/measurement software (M'ath, Metris, France)[9]. On average, a total of 100 measures are automatically performed on the right and left far walls and the mean value is considered. The maximal IMT, the SD and the number of measures taken, were available. All subjects have at least two stored common carotid artery digital images and, when plaques were found, the videotape is recorded. The reproducibility of the ultrasonographic procedures has been previously described[10]. The intraobserver variability (correlation coefficient, r) for the automatic or manual procedure was 0.61 vs 0.77 and the interobserver variation was 0.58 vs 0.71.

Results

The percent class distribution of the population of the San Daniele cohort at entry, aged 18-99 years in 1990, and compared to the one of the general population of the Friuli-Venezia Giulia Region, by sex, is illustrated in figure 1. In the San Daniele population, 630 were males as compared to 467 404 in the regional population; the corresponding figures for females were 718 and 522 201. Both for males and females there were no differences despite the large difference in absolute numbers (χ^2 statistics). Therefore, we could consider the San Daniele initial cohort as being representative of the whole area. Moreover, with regard to the follow-up period, the actuarial life curve method revealed that there were no substantial differences in survival. Figure 2

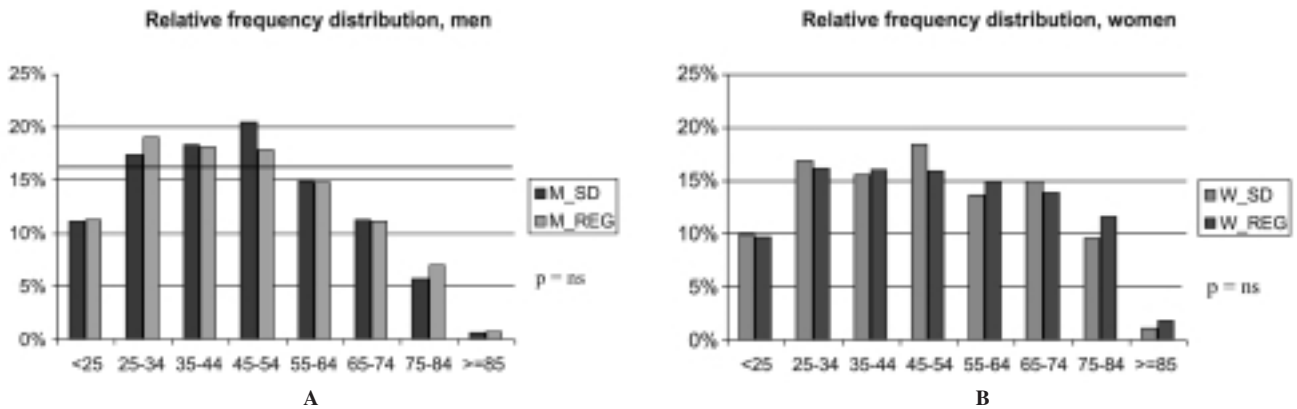

Figure 1. *Age distribution between the San Daniele cohort and the regional population of the same age range in 1990 in males* (A) *and females* (B).

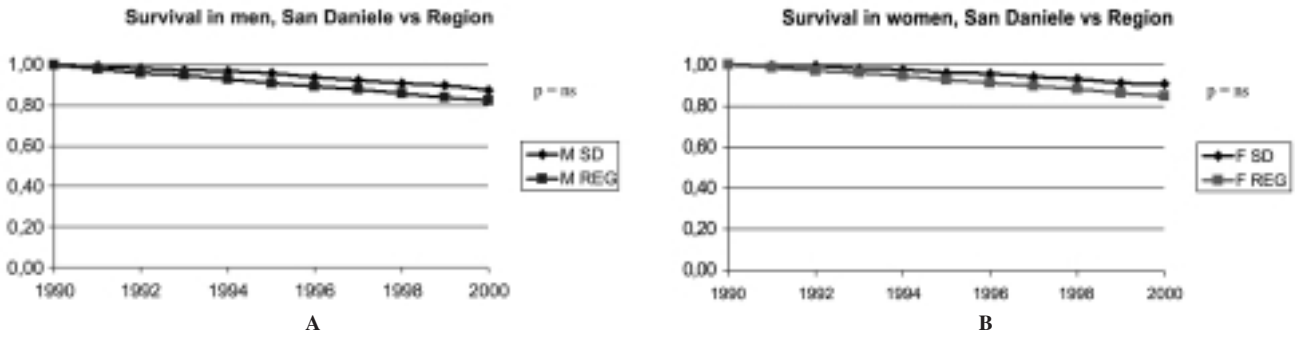

Figure 2. *Survival curves in the San Daniele cohort and in the regional population of the same age range in 1990 in males* (A) *and females* (B).

shows the survival curves for males and females respectively together with a comparison of the San Daniele cohort and the regional population of the same age range at entry (χ^2 statistics). The absence of significant differences allows us to continue to consider the San Daniele cohort as being representative of the regional population.

Discussion

Many clinical and epidemiological studies have found a close association between carotid IMT and a higher prevalence and incidence of ischemic stroke and CHD. In fact, cross-sectional associations between common carotid artery IMT and cardiovascular risk factors have been demonstrated in several studies[11,12] and similarly, common carotid IMT has been associated with an increased prevalence of cardiovascular disease in cross-sectional studies[13-15]. Therefore, it is unclear whether the favorable effect on cardiovascular events detected in small drug intervention trials should be attributed to IMT or to the effects of these drugs on risk factors, considering also other intervention studies with discordant effects on events compared to IMT modifications[16-20]. Besides, it is as yet unclear whether IMT provides an additional/additive information for the prediction of future coronary artery diseases and stroke[21].

However, more than five published studies found that carotid IMT measurement is a viable predictor of the presence of coronary atherosclerosis and of its clinical sequelae[22-26]. Thus, carotid IMT as defined at non-invasive B-mode ultrasound has been shown to be an independent risk factor for CHD and stroke. The strongest data relating IMT measurement with incident cardiovascular events derive from the Atherosclerosis Risk in Communities (ARIC) Study[24]. In this study, the relation of carotid IMT to CHD incidence was studied over 4 to 7 years of follow-up of 4 US communities. The study population consisted of 7289 women and 5552 men aged 45 to 64 years who were free of clinical CHD at baseline. The hazard ratio comparing the extreme mean IMT (≥ 1 mm) to the non-extreme IMT (< 1 mm) was 5.07 for women (95% confidence interval-CI 3.08 to 8.36) and 1.85 for men (95% CI 1.28 to 2.69). The relation was graded, and although the strength of the association was found to be decreased when major CHD risk factors were included in the analysis, it remained elevated at a higher IMT. In the Cardiovascular Health Study[26], associations between the thickness of the carotid artery intima and media and the incidence of new MI or stroke in persons without clinical cardiovascular disease were studied in 5858 subjects aged ≥ 65 years. The relative risk (RR) of MI or stroke increased linearly with IMT. The RR of MI or stroke (adjusted for age and sex) for the quintile with the highest thickness compared with the lowest was 3.87 (95% CI 2.72 to 5.51). The association between cardiovascular events and IMT remained significant even after adjustment for traditional risk factors, showing an increasing risk for each quintile of combined IMT, from the second quintile (RR 1.54, 95% CI 1.04 to 2.28), to the third (RR 1.84, 95% CI 1.26 to 2.67), fourth (RR 2.01, 95% CI 1.38 to 2.91), and fifth (RR 3.15, 95% CI 2.19 to 4.52). Several epidemiological and clinical intervention or prevention trials have illustrated the ability of carotid B-mode ultrasound imaging to monitor changes in the IMT over time, documenting that the average annual IMT progression rates are ≤ 0.03 mm[18-25]. Besides, increasing common carotid artery intima-media thickening independently predicts the development of atherosclerotic plaque in men and women > 59 years[27]. In such IMT and plaque monitoring studies, the quantitative quality control of the physicians who perform the examinations and of the readers who make the measurements was found to be critical[28,29].

The presence of plaque or carotid intima-media thickening may express a different grade of ischemic risk, thus predicting the possibility of a future event in a different way.

Belcaro et al.[30] conducted a study on 2322 asymptomatic subjects classified in four morphology classes as defined by means of ultrasound assessment of the carotid and femoral artery bifurcations: class I IMT < 1 mm, class II IMT > 1 and < 2 mm, class III plaque defined > 2 mm without hemodynamic alterations, and

class IV stenotic plaque as in class III, but with hemodynamic stenosis on duplex scanning (sample volume in the center of the lumen) indicating stenosis > 50%. After a 6-year follow-up, no cardiovascular events were observed in subjects who were in class I (80.05% of the population sample) at inclusion; there were 69 events in classes II, III, and IV (19.95% of the population; incidence 17.3%); 59 events, including the 5 deaths, occurred in classes III and IV (10.85% of the population), producing an event incidence of 27.2%. The increased event rate in classes II, III, and IV was significant (log-rank test; $p < 0.05$, $p < 0.025$, and $p < 0.025$ respectively). However, to our knowledge, no population studies evaluated the progression of both plaques and IMT as well as the risk factor variation vs the incident coronary and cerebrovascular events in a long follow-up. This is the main reason why we decided to set up the San Daniele 2 study.

The similar age distribution and survival of the initial San Daniele cohort in comparison with the general population of the Friuli-Venezia Giulia Region is a major point in favor of the extension of the San Daniele data to a wider area. Therefore, the San Daniele 2 study will be of particular epidemiological interest, considering also the possibility of accurately ascertaining the incident events and comparing them with the ultrasonographic modifications that were observed during follow-up. Our study will again be observational but with at least two risk factors and ultrasonographic measurements taken 12 years apart. We hope to find some evidence about the role of carotid atherosclerosis in determining *per se* incident events, but we are aware that observational evidence is only hypothesis-generating for cardiovascular prevention, which is the real goal of the routine activity of our Centers.

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