Increased plasma total homocysteine (tHcy) levels have been shown to be a risk factor for coronary artery disease (CAD). The common methylenetetrahydrofolate reductase C677T (MTHFR C677T) polymorphism has been reported to be a strong predictor of mild hyperhomocysteinaemia (HHcy). We assessed whether this mutation was associated with increased risk of CAD and plasma levels of tHcy. We also evaluated interactions between this polymorphism, mild elevated tHcy levels and conventional risk factors of CAD.

**Method**

Using PCR-RFLP analysis, we studied the frequency of the C677T genotypes and its effect on CAD and on tHcy concentrations in 400 subjects without and with CAD angiographically confirmed. There were 210 subjects with CAD and 190 subjects without CAD.

**Results**

The frequencies of the C677T genotypes were 53% (59.5% in controls versus 48.1% in cases), 34.8% (32.1% in controls versus 37.1% in cases), and 11.8% (8.4% in controls versus 14.8% in cases), respectively, for 677CC, 677CT, and 677TT. The genotype frequencies were significantly different between case and control groups (P < 0.05). The 677T allele enhances the risk of CAD associated to HHcy (P < 0.01). In multivariate analysis models, MTHFR C677T polymorphism effect on CAD was masked by other risk factors. HHcy was only and independently influenced by MTHFR polymorphism and smoking habits, and it is a strong predictor of CAD independently of conventional risk factors. Conclusion. Our data suggest that HHcy is strongly and independently associated to CAD risk increase; and MTHFR C677T polymorphism and smoking habits were the main predictors of tHcy levels. The CAD risk increase is mainly associated with mild HHcy in 677TT, whereas in 677CT and 677CC it is mainly associated with the conventional risk factors.

**Conclusion**

Our data suggest that HHcy is strongly and independently associated to CAD risk increase; and MTHFR C677T polymorphism and smoking habits were the main predictors of tHcy levels. The CAD risk increase is mainly associated with mild HHcy in 677TT, whereas in 677CT and 677CC it is mainly associated with the conventional risk factors.
Hcy is a sulphur-containing amino acid, formed by demethylation of the essential amino acid methionine. It can be either degraded by transsulfuration, involving the vitamin B_{12}-dependant enzyme cystathionine β-synthase, or remethylated to methionine, involving the cobalamin (vitamin B_{12}-dependant enzyme) methionine synthase. Elevations of tHcy might result from nutritional deficiencies, including folate, pyridoxal phosphate (vitamin B_{6}), and methylcobalamin (vitamin B_{12}), or from genetically determined abnormalities of Hcy metabolism (e.g., MTHFR), or a combination of these [5, 6].

MTHFR is an enzyme that reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the main circulating form of folate and the methyl donor for the remethylation of Hcy to methionine. The MTHFR gene has been mapped to the chromosomal region 1p36.3. A thermolabile form of MTHFR has been identified [7, 8] and found to be caused by a missense mutation in its encoding gene, with the cytidine residue at nucleotide position 677 being replaced by thymidine (MTHFR C677T), resulting in the substitution of valine for alanine in the enzyme. The homozygous 677TT genotype of this mutation has been found to specify a variant enzyme with reduced activity and to be associated with elevated tHcy levels, particularly in the setting of low folate levels, as compared to the wild-type (677CC) and heterozygous (677CT) genotypes. Also, data have shown that the frequency of the mutation varies among different populations [9, 10]. These considerations have raised the possibility that the relatively common C677T mutation in MTHFR might be an important genetic risk factor for cardiovascular disease through its effects on homocysteine metabolism [11, 12].

The aim of this study was to investigate the frequency of MTHFR C677T mutation and its association to CAD and to fasting plasma tHcy concentrations in Moroccan population. We also evaluated interactions between mild elevated tHcy levels, MTHFR C677T polymorphism, and conventional risk factors of CAD.

2. MATERIALS AND METHODS

2.1. Subjects

The study sample consisted of individuals undergoing coronary angiography because of either symptoms of suspected CAD or unrelated conditions requiring angiographic evaluation (e.g., cardiomyopathy, valvular disease). Patients were designated as having CAD if they had ≥50% stenosis of at least one coronary artery, and having no CAD if <10% stenosis was present in all major vessels.

The protocol was ethically approved by the Review Board of the National League of Cardiology, Rabat, Morocco. The study consisted of 400 unrelated individuals (age mean = 50 ± 10) in whom coronary angiography was clinically indicated (218 men, 182 women). The group of cases was formed by 210 (52.5%) patients with angiographically confirmed CAD. The group of controls included 190 (47.5%) subjects with normal coronary angiograms. Angiograms were analyzed by cardiologists blinded to risk factors and genetic study. Patients with an acute illness, such as myocardial infarction or coronary artery bypass graft surgery occurring three months prior the study, or with a chronic disease, such as chronic renal, hepatic or thyroid failure, were excluded of the protocol. None of the subjects took vitamin supplements.

2.2. Definition of conventional cardiovascular risk factors

Arterial hypertension was defined in the presence of active treatment with antihypertensive agents or otherwise as systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg on at least two separate occasions. Hypercholesterolemia was defined as a total cholesterol value of ≥5.70 mmol/L. Smokers were defined as those currently smoking any tobacco. Patients were considered to have diabetes mellitus if they were receiving active treatment with insulin or oral hypoglycemic agents, or if fasting glucose in the serum was ≥126 mg/dL. Dyslipidemia was defined as triglycerides (TG) value of ≥1.50 mmol/L or LDL cholesterol (LDLc) value of ≥3.92 mmol/L. The weight and height were measured and the body mass index (BMI) was obtained from the ratio of weight (Kg) to height squared (m²). Subjects were considered substantially obese when the BMI was >30 Kg/m².

2.3. Biochemical analyses

Twelve hour fasting venous blood samples were obtained from all subjects at the day of the coronary angiography. Venous blood was collected into heparinized tubes and immediately centrifuged at 1,550 g for 10 minutes. Total serum cholesterol, HDL cholesterol, and triglycerides were measured by enzymatic methods on a Hitachi analyzer using standard kits (Roche Diagnostics). LDL cholesterol was calculated with the Friedewald formula in subjects with triglycerides levels below 4.40 mmol/L.

2.4. Determination of homocysteine

Plasma total homocysteine was determined by immunoassay (Axis Biochemicals, Oslo, Norway). The assay method was based on an enzymatic conversion of homocysteine to S-adenosyl-L-homocysteine, followed by quantification of S-adenosyl-L-homocysteine by an enzyme-linked immunoassay [13]. The coefficients of variation within and between days for the assays were 5% or less. Cross reactivity with glutathione, L-cysteine, adenosine, L-cystathionine was below 0.1%. Plasma homocysteine was recorded in units of μmol/L.
2.5. Genetic analysis

DNA was extracted from white cells of fresh or frozen peripheral blood by phenol-chloroform-isooamylic alcohol method. The region surrounding the 677 MTHFR mutation site (a 223 bp fragment) was polymerase chain reaction-amplified, using the following primers (MTHFR 618A: 5′-AGCTTTTAGGCTGACCTGAAG-3′ and MTHFR 14-5B: 5′-AGGACGTTCCGGTGAGTGTG-3′) (Oligo Express) [12]. PCR amplification reactions were performed with 300 ng of genomic DNA in PCR buffer containing 1.5 mM MgCl2, 200 μM dNTPs, 0.1 μM of each primer, and 1 U Taq polymerase (Applied Biosystems). The reaction mixture (20 μL) was denatured at 94°C for 1 minute, annealed at 58°C for 15 seconds and extended at 72°C for 30 seconds for a total of 34 cycles. Since the polymorphism creates a HinfI recognition site, 5 μL of the PCR product was then digested with 5U HinfI, 2 μL 10X reaction NE buffer 2 (New England, Biolabs) and 12.5 μL of water at 37°C for 3 hours. The digestion product was electrophoresed on a 2.5% agarose gel. Restriction fragments were stained with ethidium bromide. The wild-type allele (677CC) corresponds to the presence of one band of 223 bp, and the mutant allele (677TT) corresponds to the presence of two bands of 175 bp and 48 bp. The heterozygous genotype (CT) corresponds to the presence of three bands of 223, 175, and 48 bp.

2.6. Statistical analysis

Normal data distributions were assessed with the Kolmogorov-Smirnov test. Skewed variables were naturally log-transformed to normalize the distribution before any statistical analysis. The Student’s t-test and one-way ANOVA procedures were used for mean comparison between groups. Hardy-Weinberg equilibrium was assessed by Chi-square test, which was equally used for the comparison of categorical variables between two groups. The comparison between three groups was assured by the Kruskal-Wallis test and the adequate post hoc multiple comparisons. Model selection log-linear analysis based on backward elimination was used for highly correlated variables identification, and the discriminant analysis with stepwise method was computed for identifying variables that have significant effects on CAD and on HHCy. On the basis of the previous statistical analysis, logistic regression model with backward likelihood ratio method was used to compute odds ratio (ORs) and their 95% confidence interval (95% CI). All statistical analysis was performed with SPSS for windows (Release 7.5.1; Standard version). All tests were two-tailed and P-value of <.05 was deemed significant.

3. RESULTS

The demographic characteristics of the study population and the main risk factors are presented in Table 1. As expected, patients with CAD had more adverse atherosclerosis profile than controls. The mean age, frequency of occurrence in men, hypertension, smoking, diabetes, dyslipidemia, and obesity were significantly higher in cases than in controls (P < .05). The risk of CAD were significantly increased by the presence of conventional risk factors (all 95% CI not contain the value of 0), and varying from ≈ 1.5 fold for male compared to female (OR = 1.60, 95% CI: 1.07–2.38, P < .05) to ≈ 3 fold for smoking habits compared to no smoking (OR = 2.97, 95% CI: 1.84–4.78, P < .001).

Independently of CAD, no significant difference was found between men and women for MTHFR C677T genotype frequencies (P = .880). In women, the genotype frequencies were 53.3% (21.4% in cases and 31.9% in controls), 55.7% (17.0% in cases and 18.7% in controls), and 11.0% (7.7% in cases and 3.3% in controls) for 677CC, 677CT, and 677TT, respectively. In men, the genotype frequencies were 53.7% (28.4% in case and 25.2% in controls), 33.9% (21.6% in cases and 12.4% in controls), and 12.4% (7.8% in cases and 4.6% in controls) for 677CC, 677CT, and 677TT, respectively. MTHFR C677T polymorphism and CAD were significantly independent in men (P = .302); but in women, the association was at the limit of the significance (P = .049).

MTHFR genotype and allele frequencies are shown in Table 2. For the overall population, the prevalence of 677CC, 677CT, and 677TT genotypes were 53.5%, 34.8%, and 11.8%, respectively. The mutant homozygous, 677TT, was recorded in 8.4% of controls and 14.8% of CAD patients. The wild-type genotype, 677CC, was found in 59.5% of controls and 48.1% of CAD subjects. The mutant allele frequency was 33.3% for CAD patients and 24.5% for subjects without CAD. The difference between controls and CAD patients was statistically significant for MTHFR gene polymorphism and allele frequencies (P < .05). Taking the wild-type genotype as a reference, the mutant genotype increases the risk of CAD by ≈ 2 fold (OR = 2.17, 95% CI: 1.12–4.20, P < .05), while the CAD risk increase associated to the mutant allele (677TT) was ≈ 1.5 fold compared to the wild-type allele 677C (OR = 1.54, 95% CI: 1.11–1.84, P < .01).

Subjects with CAD had significantly (P < .001) higher tHcy levels (mean ± SD = 14.9 ± 3.1 μmol/L) than controls (11.3 ± 3.1 μmol/L). Using 15 μmol/L as the cut-off point to classify mild hyperhomocysteinaemia (HHcy), 49.5% of CAD patients (49.2% men, 50.0% women) and 21.1% of controls (27.2% men, 15.3% women) had HHcy (Table 2). Mild HHcy was significantly associated to CAD (P < .001) in men and women separately. In all subjects, CAD was significantly associated to HHcy, with a risk increase of about 4 fold compared to non-HHcy (OR = 3.68, 95% CI: 2.37–5.72, P < .001). tHcy levels were positively and significantly associated to MTHFR gene polymorphism (Spearman’s rho = 0.38, P < .001). This relationship was observed in case (Spearman’s rho = 0.44, P < .001) and control (Spearman’s rho = 0.32, P < .01) groups. Based on the post hoc multiple comparison appropriate for equal variances not assumed, there is a graded and significant (P < .01) increase in tHcy levels from 677CC to 677TT genotypes of MTHFR gene in both cases and controls groups.

The frequencies of the simultaneous presence of CAD and HHcy were 16.4%, 30.2%, and 57.4% in 677CC, 677CT, and 677TT genotypes, respectively (see Table 2).
Table 1: Characteristics of the studied population and OR of CAD associated to traditional risk factors. $P^2$ is a comparison between case and control groups of mean age (ANOVA), and traditional risk factors (chi-square test). OR: odds ratio; 95% CI: 95% confidence interval.

| Number of subjects | Total population | Control | Case | $P^2$ | OR (95% CI) |
|-------------------|-----------------|---------|------|-------|-------------|
| Age, year         | 400             | 190     | 210  | —     | —           |
| Mean ± SD         | 49.9 ± 9.8      | 47.3 ± 9.4 | 52.3 ± 9.6 | 0.000 | —           |
| Sex, n (%)        | —               | —       | —    | —     | —           |
| Women             | 182 (45.5)      | 98 (51.6) | 84 (40.0) | —     | 1.00        |
| Men               | 218 (54.5)      | 92 (48.4) | 126 (60.0) | —     | 1.60 (1.07–2.38) |
| Smoking, n (%)    | —               | —       | —    | —     | —           |
| No                | 292 (73.0)      | 159 (83.7) | 133 (63.3) | —     | 1.00        |
| Yes               | 108 (27.0)      | 31 (16.3) | 77 (36.7) | —     | 2.97 (1.84–4.78) |
| Hypertension, n (%)| —               | —       | —    | —     | —           |
| No                | 254 (63.5)      | 135 (71.1) | 119 (56.7) | —     | 1.00        |
| Yes               | 146 (36.5)      | 55 (28.9) | 91 (43.3) | —     | 1.88 (1.24–2.84) |
| Diabetes, n (%)   | —               | —       | —    | —     | —           |
| No                | 307 (76.8)      | 156 (82.1) | 151 (71.9) | —     | 1.00        |
| Yes               | 93 (23.3)       | 34 (17.9) | 59 (28.1) | —     | 1.79 (1.11–2.89) |
| Obesity, n (%)    | —               | —       | —    | —     | —           |
| No                | 292 (73.0)      | 148 (77.9) | 144 (68.6) | —     | 1.00        |
| Yes               | 108 (27.0)      | 42 (22.1) | 66 (31.4) | —     | 1.62 (1.03–2.54) |
| Dyslipidemia, n (%)| —               | —       | —    | —     | —           |
| No                | 296 (74.0)      | 151 (79.5) | 145 (69.0) | —     | 1.00        |
| Yes               | 104 (26.0)      | 39 (20.5) | 65 (31.0) | —     | 1.74 (1.10–2.74) |

Discriminant analysis and model selection log-linear analysis were computed prior to the logistic regression analysis based on the backward likelihood ratio method. The final model shows that HHcy is mainly and only significantly ($P < .01$) affected by the MTHFR polymorphism and smoking habits, and their combined effects were not significant (see Table 3). The mutant genotype had the more adverse effect (OR = 11.72, 95% CI: 4.91–27.96, $P < .001$) than smoking habits (OR = 6.92, 95% CI: 3.50–13.70, $P < .001$).

As expected, traditional risk factor frequencies were higher in cases than controls, with exception for dyslipidemia and obesity in the 677TT genotypes. In cases subjects, all conventional risk factor frequencies were lower in 677TT genotype than in the remainders (see Table 4). Those differences lead to think that conventional risk factors do not have the same effect on CAD according to MTHFR genotypes. In fact, in subjects with the wild-type genotype, the CAD risk increase was significantly associated to smoking habits, obesity, Dyslipidemia, and hypertension. In heterozygous individuals, the risk increase was more likely associated to smoking habits and hypertension; while the HHcy was the sole factor that significantly increases the risk of CAD in subjects with the mutant genotype. In addition, it is important to note that HHcy was associated to a significant and graded CAD risk increase from $\approx 2.5$ fold in subjects with 677CC genotype (OR = 2.47, 95% CI: 1.31–4.65, $P < .01$) to $\approx 9$ fold in individuals with 677TT genotype (OR = 8.68, 95% CI: 2.05–36.69, $P < .01$). In consequence, it appears that the 677T allele multiplies by 2 the risk of CAD associated to HHcy (see Table 5).

On the basis of the results from model selection log-linear analysis and discriminant analysis procedures, logistic regression model based on backward likelihood method was used to compute the OR of CAD associated to the studied parameters (see Table 6). Dyslipidemia, smoking habits, hypertension, and diabetes, as well as HHcy, were independent risk factors for CAD. The MTHFR polymorphism effect was masked. Therefore, MTHFR polymorphism and HHcy interactive effect were found to be a strong predictor of CAD risk ($P < .01$). The negative interactive effects between smoking habits and mutant genotype, as well as between Dyslipidemia and HHcy, should be more likely the consequence of the small observed frequencies of smokers with 677TT genotype and the lowest frequency of HHcy among subjects with Dyslipidemia, rather than an eventual protective effect.

4. DISCUSSION

In accordance with a previous study (see [14]), the present report confirms that CAD is strongly and significantly associated to HHcy in Moroccan population. This is in agreement with the meta-analysis by Christen et al. [15], in which 43 studies were analyzed. In another meta-analysis, Boushey et al. [4] evaluated 27 studies and showed that Hcy is an independent and gradual risk factor for CAD. The ORs for CAD ranged from 1.6 for men to 1.8 for women, for each tHcy level elevation of 5 μM. The increase in coronary risk resulting from this increase is similar to an increase of 20 mg/dL in total cholesterol levels. The authors considered that 10%
Table 2: Hyperhomocysteinaemia and MTHFR polymorphism distributions according to CAD, and HHcy distribution according to MTHFR polymorphism and CAD. $P^1$ is an MTHFR polymorphism and HHcy comparison (chi-square test) and Hcy comparison (ANOVA and median tests) between cases and controls. $P^3$ is the significant value of the hypothesis testing that the OR of CAD associated to MTHFR polymorphism and HHcy is 1. $P^4$ is a tHcy comparison between genotypes in cases and controls separately (Kruskal-Wallis test).

| Variable                  | Total population | Control | Case | $P^1$ | OR (95% CI) | $P^2$ |
|---------------------------|------------------|---------|------|-------|-------------|-------|
| MTHFR C677T               |                  |         |      |       |             |       |
| Genotype, n (%)           |                  |         |      |       |             |       |
| 677CC                     | 214 (53.5)       | 113 (59.5) | 101 (48.1)   | 0.038 | —           | —     |
| 677CT                     | 139 (34.8)       | 61 (32.1)  | 78 (37.1)    | —     | 1.43 (0.93–2.20) | 0.102 |
| 677TT                     | 47 (11.8)        | 16 (8.4)   | 31 (14.8)    | —     | 2.17 (1.12–4.20) | 0.022 |
| Allele, n (%)             |                  |         |      |       |             |       |
| 677C                      | 567 (70.9)       | 287 (75.5) | 280 (66.7)   | —     | 1.00        | —     |
| 677T                      | 233 (29.1)       | 93 (24.5)  | 140 (33.3)   | —     | 1.54 (1.11–1.84) | 0.007 |
| tHcy, μmol/L              |                  |         |      |       |             |       |
| Mean ± SD                 | 13.1 ± 3.6       | 11.3 ± 3.1 | 14.8 ± 3.1   | 0.000 | —           | —     |
| Median                    | 13.8             | 11.1      | 14.9         | 0.000 | —           | —     |
| HHcy, n (%)               |                  |         |      |       |             |       |
| No                        | 256 (64.0)       | 150 (78.9) | 106 (50.5)   | —     | 1.00        | —     |
| Yes                       | 144 (36.0)       | 40 (21.1)  | 104 (49.5)   | —     | 3.68 (2.37–5.72) | 0.000 |

Table 3: Logistic regression model (with backward likelihood ratio method) built with HHcy as a dependent variable and all other risk factors as predictors variables. B: logistic regression coefficient; SE: standard error; Df: degree of freedom; Sig.: significant level; OR: odds ratio; 95% CI: 95% confidence interval of the OR.

| Variable                  | B     | SE    | Wald  | Df   | Sig.  | OR    | 95% CI for OR |
|---------------------------|-------|-------|-------|------|-------|-------|---------------|
| MTHFR C677T               | —     | —     | —     | —    | —     | —     | —             |
| MTHFR 677CT               | 0.88  | 0.31  | 8.07  | 1    | 0.005 | 2.40  | 1.31–4.40     |
| MTHFR 677TT               | 2.46  | 0.44  | 30.79 | 1    | 0.000 | 11.72 | 4.91–27.96    |
| Smoking habits            | 1.93  | 0.35  | 30.83 | 1    | 0.000 | 6.92  | 3.50–13.70    |
| MTHFR C677T* smoking habits | —    | —     | —     | —    | —     | —     | —             |
| MTHFR 677CT* smoking habits | —    | —     | —     | —    | —     | —     | —             |
| MTHFR 677TT* smoking habits | —    | —     | —     | —    | —     | —     | —             |
| Constant                  | —1.72 | 0.22  | 60.19 | 1    | 0.000 | —     | —             |

*Indicates the interactive effects between variables.

of the risk of the general population for the development of CAD can be attributed to Hcy.

The homocysteine hypothesis of atherosclerosis was largely based on the clinical and pathological observations in patients with homocystinuria, a metabolic disorder characterized by markedly increased levels of homocysteine [16]. Biologic support of the theory was derived from experimental studies in which homocysteine concentrations far exceeded the levels encountered, even under the most severe pathological conditions, putting doubt on pathophysiological relevance in the clinical setting [17]. The strongest support for a possible causal link between homocysteine levels and atherosclerosis came from retrospective, cross-sectional, or case-control studies.
Table 4: Traditional risk factors distribution according to MTHFR polymorphism and CAD. 677CC, 677CT, and 677TT are the wild-type, the heterozygous, and the mutant genotypes. Comparison of traditional risk factors between MTHFR genotypes (chi-square test), in cases and controls separately (P1) and in all subjects (P2).

|                      | Total population | MTHFR polymorphism | P1  | P2  |
|----------------------|-----------------|--------------------|-----|-----|
| Number of subjects   | 400             | 214                | 139 | 47  |
| Male gender, n (%)   | —               | —                  | —   | —   |
| Case                 | 126 (31.5)      | 62 (29.0)          | 47 (33.8) | 17 (36.2) |
| Control              | 92 (23.0)       | 55 (25.7)          | 27 (19.4) | 10 (21.3) |
| Smoking yes, n (%)   | —               | —                  | —   | —   |
| Case                 | 77 (19.3)       | 41 (19.2)          | 29 (20.9) | 7 (14.9) |
| Control              | 31 (7.8)        | 15 (7.0)           | 7 (5.0)   | 9 (19.1) |
| Hypertension yes, n (%) | —           | —                  | —   | —   |
| Case                 | 91 (22.8)       | 51 (23.8)          | 30 (21.6) | 10 (21.3) |
| Control              | 55 (13.8)       | 39 (18.2)          | 13 (9.4)  | 5 (6.4)   |
| Diabetes yes, n (%)  | —               | —                  | —   | —   |
| Case                 | 59 (14.8)       | 29 (13.6)          | 24 (17.3) | 6 (12.8) |
| Control              | 34 (8.5)        | 22 (10.3)          | 10 (7.2)  | 2 (4.3)   |
| Obesity yes, n (%)   | —               | —                  | —   | —   |
| Case                 | 53 (13.3)       | 42 (19.6)          | 10 (7.2)  | 1 (2.1)   |
| Control              | 31 (7.8)        | 17 (7.9)           | 12 (8.6)  | 2 (4.3)   |
| Dyslipidemia yes, n (%) | —           | —                  | —   | —   |
| Case                 | 65 (16.3)       | 39 (18.2)          | 24 (17.3) | 2 (4.3) |
| Control              | 39 (9.8)        | 20 (9.3)           | 15 (10.8) | 4 (8.5) |

Table 5: OR (95% CI) of CAD associated to the studied risk factors according to MTHFR C677T polymorphism. OR: odds ratio; 95% CI: 95% confidence interval; CAD: coronary artery disease; MTHFR: methylenetetrahydrofolate reductase; P: significant value.

| Methylene tetrahydrofolate reductase C677T genotype | 677CC | 677CT | 677TT | P   |
|-----------------------------------------------------|-------|-------|-------|-----|
| Number of subjects                                  | 214   | 139   | 47    | —   |
| Sex                                                 | 1.68  | 0.063 | 1.91  | 0.97–2.89 |
| Smoking habits                                       | 4.46  | 0.000 | 4.57  | 1.84–11.36 |
| Hypertension                                        | 1.94  | 0.019 | 2.31  | 1.08–4.95 |
| Diabetes                                            | 1.67  | 0.015 | 2.27  | 0.99–5.20 |
| Obesity                                             | 4.02  | 0.000 | 0.60  | 0.24–1.50 |
| Dyslipidemia                                        | 2.93  | 0.001 | 1.36  | 0.64–2.90 |
| Hyperhomocysteinaemia                               | 2.47  | 0.005 | 4.31  | 2.02–9.19 |

Prospective studies reported conflicting results. Some identified Hcy as an independent risk factor [18, 19], but others did not [20, 21]. Recently, Wald et al. [22] published a comprehensive meta-analysis of 16 prospective studies. Data from 144,936 patients with 3144 combined events were analyzed. The OR for a 5 μM increase Hcy level was 1.23 (95% CI = 1.14–1.32). Zylberstein et al. [23] published the results of a 24-year follow-up evaluating Hcy quintile, relative risk was 1.86 (95% CI = 1.06–3.26) for acute myocardial infarction and 5.14 (95% CI = 2.22–11.92) for death due to acute myocardial infarction. Our study was of interest because, despite a high prevalence of coronary morbidity and mortality in Morocco [24], the total cholesterol concentrations are relatively low and are not the major cause for the development of atherosclerosis. In the light of these findings, we thought that plasma tHcy concentrations might be an important risk factor for CAD in Morocco. In fact, we found that plasma tHcy concentrations above 15 μmol/L were a significant and independent risk factor for CAD [25, 26]. This association was persistent in men and women when they were considered separately.

The role of environmental factors in determining tHcy levels was examined first. Cigarette smoking is known to increase plasma homocysteine [27]. Our results confirmed this effect which suggests that smoking increases HHcy, which in turn increases risk of CAD. Several mechanisms might explain the increased risk in smokers with high plasma tHcy. Smoking affects the vascular tree, platelet activation, lipid
peroxidation, enhanced tissue factor activation, and reduced Von Willebrand factor, via several different interactive mechanisms [28, 29]. Nicotine and carbon monoxide separately produce tachycardia, hypertension, and vasoconstriction and both produce direct endothelial damage [30]. Smoking also affects vaso-occlusive factors such as platelet aggregation, plasma viscosity, and fibrinogen levels [31, 32]. The mechanism by which mild HHcy is atherogenic is still not completely understood. Homocysteine may damage endothelial cells and has been shown to be associated with elevated levels of von-Willebrand factor in vivo [33], and with enhanced nitric oxide production in cultured cells [34], and thus would increase risk of atherosclerosis. Also, Woo et al. [35] demonstrated impaired flow-mediated dilatation of the brachial artery in 17 healthy individuals who had no other risk factor except HHcy. Furthermore, tHcy levels and endothelial function were corrected with folic acid supplementation. In addition, some studies have demonstrated an association between homocysteine and an increase risk of initial and recurrent venous thrombosis [36], and although this has not been shown in all studies [37], it has been confirmed in a meta-analysis in patients with fasting HHcy [38].

Several characteristics of the present study deserve to be stressed. First, as in previous studies [39], we found a strong link between the MTHFR gene C677T polymorphism and plasma tHcy levels. Second, our study provides a very reliable genotype characterization of CAD, by performing coronary angiography in all patients and controls. Third, our study is the first and the largest one conducted in Moroccan population, this facilitates the examination of the thermolabile MTHFR genotype as an independent risk factor for CAD and of the relations between genotypes, tHcy, and risk.

In the present study, MTHFR gene C677T polymorphism was significantly associated with CAD (P < .05). Compared with homozygous wild-type, the homozygous mutant of MTHFR gene was associated to a significant CAD risk increase. It is in the same line with Frost et al. [11]. In particular, Morita et al. [40] reported that the frequency of this mutation was correlated with the severity of stenotic lesions and the number of stenotic coronary arteries, suggesting that this mutation is closely associated with the severity of CAD. Therefore, the finding of an increased risk of CAD associated with MTHFR is not surprising because MTHFR is strongly associated with an increased risk of HHcy.

In the original description of thermolabile MTHFR, Selhub and D’Angelo et al. [17] reported that thermolabile MTHFR, which is correlated with the 677TT genotype, can be an inherited risk factor for CAD. They documented a prevalence of 17% in patients with CAD and of 5% in control subjects. van der Put et al. [5] reported decreased plasma folate concentrations in individuals homozygous for the mutation.

In contrast with our study, Anderson et al. [41], in a prospective study of patients with angiographically proved CAD, found no relation between MTHFR gene C677T polymorphism and the risk of CAD. In a health report of US physicians, the frequency of the MTHFR genotypes was similar between patients and control subjects [42]. The discrepancy between the results from all those studies is unclear and may be due to differences in nutritional intake of co-factors required for the MTHFR pathway, such as vitamin B12 or folate, or other ethnic differences (e.g., weight, BMI) [43].

Our study showed a positive association between the homozygous mutant genotype and elevated plasma tHcy levels in both cases and controls. Similar findings were observed in the NHLBI Family Heart Study [10]. This association suggests that MTHFR gene C677T mutation may increase plasma tHcy levels and could be an independent predictor of plasma homocysteine levels, particularly in the setting of low folate status. On this basis, but without implying causality, it is reasonable to suggest that the effect of the 677TT genotype
on CAD may be mediated through high plasma tHcy and low plasma folate concentration.

Therefore, it is important to note that when CAD and sex categories were considered together, MTHFR C677T polymorphism was not associated to CAD \((P > .05)\) in men; but in women this relation was at the limit of the significance \((P = .049)\). Our data do not allow us to conclude clearly about MTHFR C677T mutation and CAD association according to sex differences.

When we assess the distribution of conventional cardiovascular risk factors according to MTHFR C677T polymorphism, we found that homozygous 677TT with CAD has significantly lower frequencies of these risk factors than those having 677CT and 677CC. In 677CC and 677CT subjects, the CAD risk is mediated by traditional risk factors and HHcy, while in 677TT subjects, the CAD risk is conferred only by isolated HHcy.

5. CONCLUSION

In the studied Moroccan population, elevated tHcy level is a strong risk factor for CAD independently of the traditional risk factors, and this CAD risk increase is strongly influenced by MTHFR C677T polymorphism. The MTHFR and smoking habits are the major causes of HHcy. There is a significant interactive effect of MTHFR polymorphism and HHcy on CAD. Traditional risk factors were associated to a significant increased risk of CAD in subjects with 677CT and 677CC genotypes, while HHcy were the main and sole risk factor of CAD in subjects with 677TT. For an eventual strategy of cardiovascular disease prevention in Morocco, it is obvious that conventional cardiovascular risk factors should be viewed in the context of MTHFR genotype and, of course, of other genetic determinants.

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