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Original article

Abnormal maternal biomarkers of homocysteine and methionine metabolism and the risk of congenital heart defects

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ABSTRACT

Background: Recent advances in genetic technology have had a significant impact on the practice of clinical genetics and the diagnosis of genetic syndromes associated with cardiac malformations.
Aim: The present study was aimed to determine whether biomarkers of the folic acid pathway, including homocysteine and methionine metabolism are altered among non pregnant women who have had a previous pregnancy affected by congenital heart defects.
Subjects and methods: The study was conducted on 50 women attending the Medical Genetics center and the Pediatric Cardiology Clinic, Faculty of Medicine, Ain Shams University for follow up. Mothers were subdivided into: *Group 1 (Cases):* 25 mothers with a history of congenital heart defects in previous children. *Group 2 (Controls):* 25 mothers and their children didn't have any birth defects including congenital heart defects. In both groups women will be excluded: If they were pregnant or taking folate antagonist medications (antiepileptic drugs) or vitamin supplementations at the time of the study. Measurement of plasma concentration of: Vitamin B-12, folic acid, Homocysteine, Methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), by using Radio immunoassay kit was done.
Results: There is a significant difference between cases and controls as regards history of early neonatal deaths (28%) in cases versus (4%) in controls ($P < 0.05$). The study also revealed that the most frequent congenital cardiovascular malformation is VSD (32%) followed by ASD (20%).As regards biomarker concentrations all, were significantly different between case and control subjects except for methionine.
Conclusion: An elevated levels of maternal homocysteine is an independent risk factor for congenital heart defects. Finally: There is an increasing need for professionals to apply and interpret genetic testing in a clinically meaningful way for prevention of congenital heart defects.

1. Introduction

Congenital heart defects (CHD) are the most common of all congenital anomalies, representing a major global health problem. Heart morphogenesis is a complex process whose disturbance can produce a range of CHD from harmless to fatal ones [1]. In addition CHD accounts for the majority of deaths from congenital defects in childhood, being six times more common than chromosomal abnormalities and four more common than neural tube defects [2]. Reported birth prevalence of CHD varies widely among studies worldwide [3]. The estimate of 8 per 1000 live births is generally accepted as the best approximation [4]. In Egypt the reported birth prevalence of congenital malformations of the circulatory system is 0.13 per 1000 [5].

The etiologies of the majority of heart defects remain unknown despite their sizable contribution to child morbidity and infant mortality [6]. About 80% of congenital heart disease (CHD) is multifactorial and arises through various combinations of genetic and environmental contributors [7].

Recent advances in genetic technology have had a significant impact on the practice of clinical genetics and the diagnosis of genetic syndromes associated with cardiac malformations as well as sporadic congenital heart disease. These new discoveries have also expanded our understanding of the contribution of genetic

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variation, susceptibility alleles, and epigenetics to isolated congenital heart disease [8]. Scientists are making progress in understanding the genetics of heart defects. Since the 1990s, they have identified about 10 gene mutations that can cause isolated congenital heart disease [9]. In addition environmental factors can contribute to congenital heart defects. Women who contract rubella during the first three months of pregnancy have a high risk of having a baby with a heart defect. Other viral infections, such as the flu, also may contribute. Exposure to certain industrial chemicals (solvents), may play a role. Some studies suggest that drinking alcohol or using cocaine in pregnancy increase the risk of heart defects [10]. Although genetic and environmental factors are involved in the etiology of CHD, only approximately 15% can be attributed to a known cause [7].

Previous epidemiological studies showed that periconceptional use of multivitamins containing folic acid reduces the risk of having a child with CHD [11,12]. Shaw et al., [13] were the first to show that the maternal use of these vitamins during the sensitive period of heart development reduced the risk of conotruncal heart defects in particular. Hernandez-Diaz et al., [14] also suggested that folate is a key factor in cardiovascular development by showing an increased risk of CHD after maternal exposure to folate antagonists during the first trimester of pregnancy. The existing evidence for an association between folic acid and congenital heart defects, however, is still inconclusive [10].

Homocysteine is the product of the intracellular methionine cycle in which methionine is initially activated by ATP to S-adenosylmethionine (SAM), the primary methyl donor for essential methyl transferase reactions. After methyl transfer, SAM is converted to S-adenosylhomocyteine (SAH). The sole source of homocysteine in the body is the hydrolysis of SAH. Interestingly, the equilibrium dynamics favor the reverse reaction, thus elevated homocysteine concentrations cause SAH to accumulate. Increased SAH is a potent product inhibitor of cellular methyl transferases which during organogenesis can alter gene expression, cell differentiation and apoptosis which are associated with congenital heart defects [15].

The aim of the current study is to determine whether biomarkers of the folic acid pathway, including homocysteine and methionine metabolism are altered among non pregnant women who have had a previous pregnancy affected by congenital heart defects or not. The results will be compared with those of women without such a history. This may shed light on new insights into mechanisms that confer an increased risk of having pregnancies affected by congenital heart defects.

2. Subjects and methods

The work has been carried out on 50 mothers. They are divided into two groups who were attending or were following up in the Medical Genetics center and the Pediatric Cardiology Clinic, Faculty of Medicine, Ain Shams University, to establish a maternal risk profile for nonsyndromic congenital heart defects that would enhance current preventive strategies. Their age ranged from 20 to 39 years with the mean of 26.59± years. Mothers were divided into:

Group 1 *(Cases):* 25 mothers with a history of congenital heart defects in previous children.

Group 2 *(Controls):* 25 mothers whose previous children were unaffected by any birth defect including congenital heart defects.

For both groups Time of last pregnancy before participating in the study ranges between 11 and 18 month.

All study procedures were approved by the Ethics Committee of Ain Shams University. All included women were interviewed in person using structured questionnaire and informed orally about the procedures and the aim of the study and a written consent was taken to participate in the study. The work is carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

All included women were subjected to the following:

A. Full history taking laying stress on mother's age, full obstetric history, informations about the current use of multivitamin, cigarettes smoking, caffeine intake, dietary micronutrient intake including folate, vit B-12 and vit B6, history of chronic diseases, history of taking medications (e.g. antiepileptic drugs) and time between last pregnancy and participation in the study
B. Complete general examination.
C. Withdrawal of a fasting blood sample by routine venipuncture to measure plasma concentration of:
   - Vitamin B-12 and folic acid.
   - Homocysteine.
   - Methionine.
   - S-adenosylmethionine (SAM)
   - S-adenosylhomocysteine (SAH).

Sample preparation

Blood samples were collected into evacuated tubes containing EDTA, immediately chilled on ice, and centrifuged at 4000×*g* for 10 min at 4 °C. Aliquots of the plasma layer were transferred into cryostat tubes and stored at −20 °C until analysis.

Plasma vitamin B-12 and folic acid concentration

They were measured by using Radio immunoassay kit [Simul TRAC – SNB RadioAssay kit for Vitamin B-12 (^{67}Co) & Folate (^{126}I)] MP Biomedicals [16,17].

Plasma(Homocysteine – Methionine-SAH-SAM)

Was measured using High-Performance Liquid chromatography (HPLC) [18]. HPLC (Beckman) system Gold, dual pump, Module:125. Kanauer Injector, with a 20 µL loop. Module 166 variable UV detector. HPLC column "phenomenex" (Lichrosorb RP-18; 5 µm, 250 × 4.6 mm, USA).

Development of standard curves

Standard curves of (Homocystiene-Methionine-SAH-SAM) were plotted using four different concentrations (2, 10, 50,

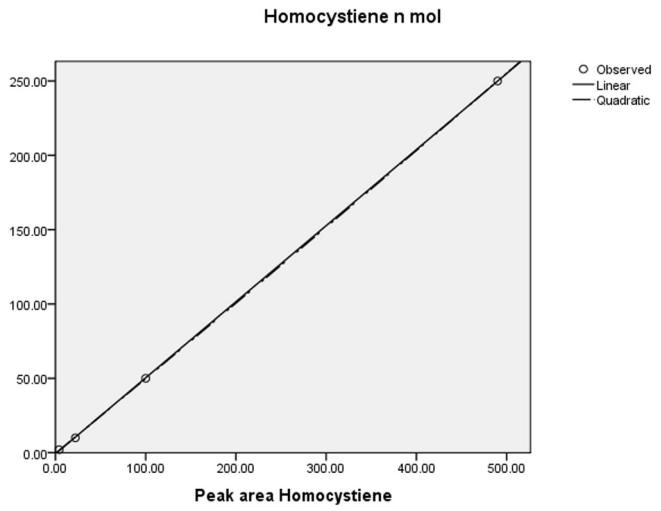

Fig. 1. Standard curve of Homocystiene.

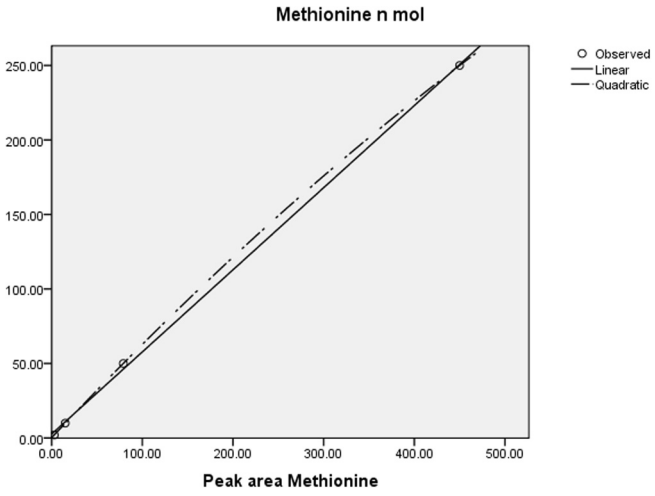

Fig. 2. Standard curve of Methionine.

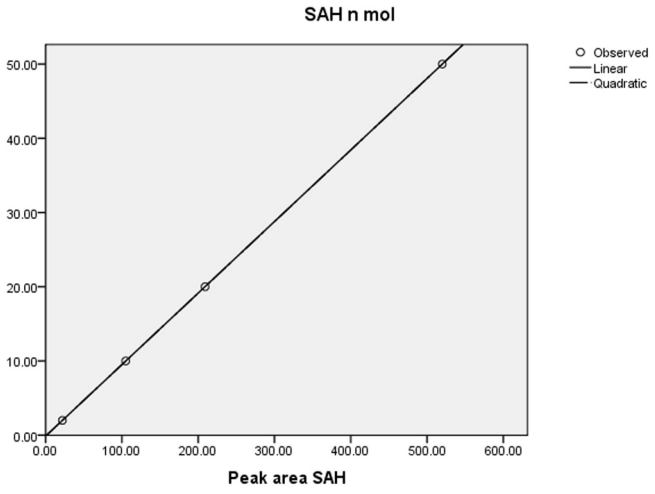

Fig. 3. standard curve of SAH.

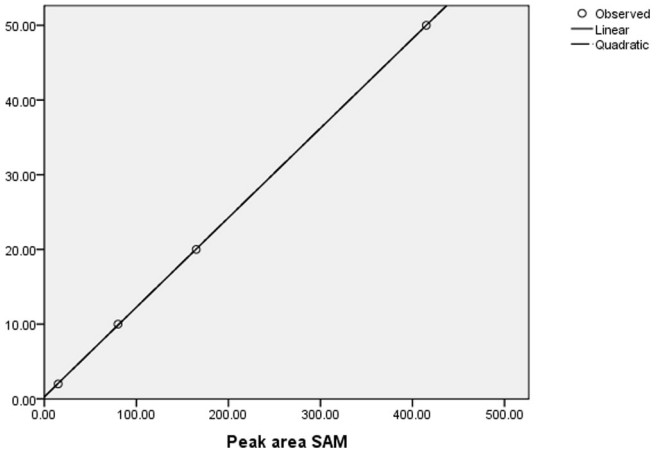

Fig. 4. standard curve of SAM.

250 nmol/20 μL). The ratio of peak area of each standard was determined and entered against concentration of the standard to plot the standard curves (Figs. 1–4).

Statistical method

IBM SPSS statistics (V.21.0,IBM Corp; USA,2012) was used for data analysis. Data were expressed as Mean – SD for quantitative parametric measures in addition to Median Percentiles for quantitative non-parametric measures and both number and percentage for categorized data.

3. Results

Mothers' ages in cases ranged between 20 and 39 years with mean of ±27.82 years and in controls ranged between 20 and 38 years with mean of ±25.36 years with no significant difference between them (P > 0.05) (Table 1). The educational level was less than high school in the majority of cases (52%), while in the controls the majority (40%) were college educate. However the educational level did not vary significantly between the two groups (P > 0.05). There was no significant difference between the two

Table 1
Demonstration of the mean age and SD of cases and controls at the time of study participation.

	Number	Range	Mean	S.D.	t	P
Cases	25	20–39	27.82	±5.4305	>0.05	−1.614
Controls	25	20–38	25.36	±5.3454		

P-Value: >0.05 Non significant.

Table 2
Time between last pregnancy and participation in the study.

	Number	Range	Mean	SD
Cases	25	11–28	16.44	13.070
Controls	25	12–16	12.4	6.6895
Z = −1.331	P = 0.183			

Table 3
Correlation between cases and control group as regards environmental & obstetric history.

Points of compare	Case		control		X2	p-value
	No	%	No	%		
Educational level						
Less than high school	13	52%	7	28%	4.371	0.112
Completed high school	8	32%	8	32%		
College education	4	16%	10	40%		
Smoking						
Negative	18	72%	22	88%	2.000	0.157
Positive	7	28%	3	12%		
Dietary caffeine intake					0.000	–
Low	17	68%	17	68%		
High	8	32%	8	32%		
Vitamin supplementation					5.195	0.023
Negative	18	72%	10	40%		
Positive	7	28%	5	60%		
History of Abortion					3.000	0.83
Negative	12	48%	18	72%		
Positive	13	52%	7	28%		
History of Still-births					3.191	0.74
Negative	22	88%	25	100		
Positive	3	12%	0	0		
History of Early neonatal deaths					5.357	0.021
Negative	18	72%	24	96		
Positive	7	28%	1	4		
Total number of cases	No					%
Case	25					100
Control	25					100

Table 4
Categories of congenital heart disease in previous pregnancies of cases.

Category		Number	Percent
Left to right shunt	VSD	8	32%
	ASD	5	20%
	PDA	2	8%
	Total	**15**	60%
Cyanotic heart disease	Tetralogy of fallot	3	12%
	TGA	2	8%
	DORV	1	4%
	Total	**6**	24%
Valvular lesions	P.S.	2	8%
	Coarc	1	4%
	Single ventricle	1	4%
	Total	**4**	16%

Abbreviations: VSD = Ventricular septal defect. ASD = Atrial septal defect. PDA = - patent ductus arteriosus. PS = Pulmonary stenosis. Coarc = Coarctation of aorta TGA = Transposition of great vessels. DORV = Double outlet right ventricle.

groups as regards history of smoking, exposure to environmental tobacco smoke or caffeine intake (P >0.05). There was no significant difference between group 1 and group 2 as regards the time of last pregnancy and date of blood withdrawal for study participation (P > 0.05) (Table 2). There is a significant difference between cases and controls as regards history of still births(12% in cases in contrast to 0% in controls). Also There is a significant difference between cases and controls as regards history of early neonatal deaths (28% in cases versus 4% in controls) (P < 0.05) (Table 3). The most frequent reported congenital cardiovascular malformations in our study is ventricular septal defect (VSD) (32%) and the next malformation in frequency is atrial septal defect (ASD) 20% Table 4. All biomarker concentrations, except for methionine, were significantly higher in cases than controls (P < 0.05) (Cases had higher mean homocysteine (48.028 nmol/L), SAH (21.578 n mol/L) and SAM (14.283 n mol/L) concentrations while controls had higher mean methionine (96.402 nmol/L) concentrations (Table 5, Fig. 5). The mean folic acid and vitamin B12 concentrations for controls were significantly higher than that for cases (10.645 ng/mL vesus 6.672 ng/mL) and (882.5 pg/mLversus 523.4 pg/mL) consecutively (Table 5, Fig. 6). Also there was a positive significant correlation between S-adenosylhomocyteine (SAH) and S-adenosylmethionine (SAM) [r = 0.495 p = 0.012], among cases. While there was a negative highly significant correlation between (SAM) and (Vit-B12) [r = −0.636, p < 0.001] (Table 6).

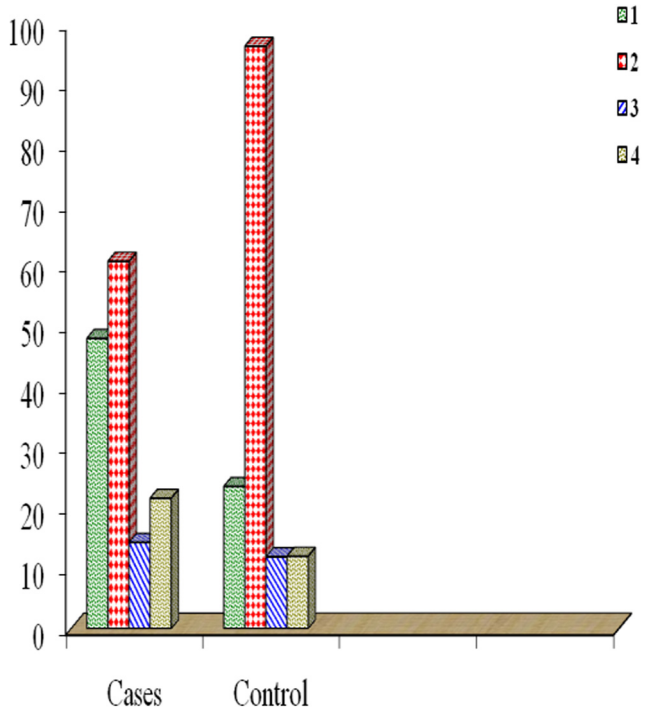

Fig. 5. Shows comparison between patients and controls as regards plasma concentrations of (1) Homocysteine-(2) Methionine (3) SAM-(4) SAH.

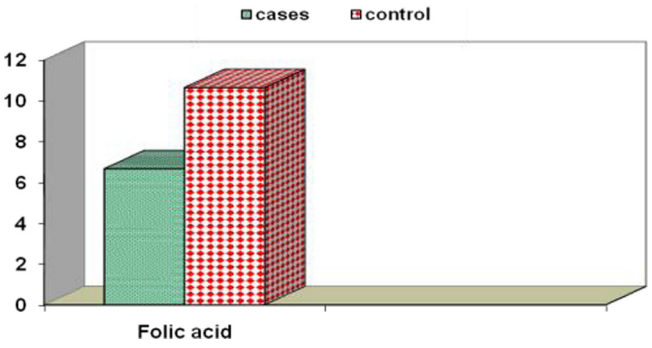

Fig. 6. Shows comparison between patients and controls as regards plasma concentrations of folic acid.

Table 5
Plasma biomarkers concentrations among cases and controls.

Biomarker	Cases			Controls			P
	n	Mean ± SD	Median (min,max)	n	Mean ± SD	Median (min, max)	
Homocysteine (nmol/L)	25	48.028 (19.469)	50.474 (16.38 93.61)	25	23.527 (16.852)	15.655 (3.98 67.5)	<0.001
Methionine (nmol/L)	25	60.795 (35.674)	61.585 (10 181.9)	25	96.402 (62.631)	80.549 (32 253.5)	0.059
SAM (nmol/L)	25	14.283 (4.611)	14.0340 (5.55 22.3)	25	11.906 (14.476)	9.3165 (4.3 80.8)	<0.001
SAH (nmol/L)	25	21.578 (4.34791)	(11.12 29.55)	25	11.982 (3.35680)	(6.03 18.7)	<0.001
Folic acid (ng/mL)	25	6.672 (5.601)	4.6 (1.523)	25	10.654 (6.115)	9 (132)	<0.001
Vitamin B-12 (pg/mL)	25	523.4 (304.996)	440 (90 1300)	25	882.5 (504.847)	680 (430 2200)	<0.001

SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine.

Table 6
Correlation between maternal biomarkers among cases and control group.

	Homocysteine		Methionine		Folic-acid		Vit-B12		SAM	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
SAH	r = 0.198	0.007	r = 0.285	0.18	r = −0.124	0.167	r = −0.273	0.077	r = 0.495	0.335
	p = 0.342	0.975	p = 0.167	0.39	p = 0.556	0.435	p = 0.186	0.72	p = 0.012	0.102
SAM	r = 0.028	0.243	r = 0.074	0.19	r = −0.161	0.034	r = −0.636	0.077		
	p = 0.893	0.242	P = 0.726	0.363	p = 0.442	0.875	p = 0.001	0.72		
Vit-B12	r = −0.155	0.325	r = −0.017	0.035	r = 0.267	0.177				
	p = 0.46	0.087	p = 0.937	0.87	p = 0.198	0.408				
Folic-acid	r = −0.145	0.032	r = −0.274	0.361						
	p = 0.488	0.883	p = 0.184	0.083						
Methionine	r = −0.378	0.69								
	p = 0.062	0.742								

4. Discussion

Risk factors for CHDs have to be identified in order to develop and implement effective primary prevention programs. Preconceptional screening of these risk factors and the development of (nutritional) interventions will contribute to future risk assessment and, thereby, prevention of CHDs. The maternal nutritional status has been shown to be involved in the pathogenesis of related birth defects [19].

In our study the majority of cases were low educated (52%), while 40% of controls were highly educated. These findings are consistent with Lu et al., [20] who reported that dietary vitamin B12 is only available from animal sources like fish and red meat. Therefore, low educated people may not have enough income for these more expensive foods or they are not correctly informed to buy and prepare healthy food.

In the current study history of abortion and early neonatal deaths were significantly higher among cases than controls [(52%) versus (28%), (28%) versus (4%)] consecutively. The increase in total plasma maternal homocysteine (tHcy) among our cases could be the cause of these adverse pregnancy outcomes as it is a strong and independent risk factor for vascular disease. Similar results were obtained by Burke et al., [21] who reported similar relations between total homocysteine (tHcy) concentration and congenital heart, premature delivery, recurrent abortions, preeclampsia and placental abruption.

In the current study history of taking vitamins supplementation before and during pregnancy was (60% in controls versus 28% in cases). This was in agreement with Lorenzo et al., [22] study who found that women using multivitamin supplements in the periconceptional period were at significantly lower risk of having babies with congenital heart defects than women not using multivitamins.

In our study, we found that VSD was the most common lesion reported (32%) followed by ASD (20%). This coincides with Zaher and Abd Elmohsen [23] who reported that VSD was the most common congenital heart defect (30.6%). However Lin et al., [24] reported that VSD represents 15.7% of their cases. Factors, such as spontaneous closure of the lesion, and the non recognition of minimum or small septal defects by the physician responsible for primary care, may have contributed to the difference between our figures and those in other studies. Therefore, the incidence of VSD will be much higher if all newborns are examined, lower if only those newborns with murmurs are examined and lower still if accession of these subjects is delayed until one year of age [25]. As regards cyanotic heart defects, tetralogy of fallot was the most frequent type recorded 12%, followed by transposition of great arteries 8%, and double outlet right ventricle 4%. Complex defects, which prevailed in other studies, manifesting in the first days of life, such as hypoplastic left ventricle, single ventricle and Ebstein's anomaly were less frequently found in our study. Because these patients die early, we may suppose that many of them didn't receive specialized care in the proper time or even die without a diagnosis [26]. The results of our study shows that plasma concentrations of homocysteine (48.028 nmol/L), SAH (21.578 nmol/L) and SAM (14.283 nmol/L) were significantly elevated among case subjects while plasma concentration of vitamin B12 (882.5 pg/mL) and folic acid (10.654 ng/mL) were significantly elevated among control subjects. There was no significant difference in plasma concentration of methionine between cases and controls. Possible causes for the altered biomarkers include deficiencies in folate or vitamin B-12 or both, genetic polymorphisms encoding enzymes in the methionine cycle, or exogenous factors [15]. The hyperhomocysteinaemia among the cases can largely be explained by the significantly lower folate and/ or vitamin B_{12} status. Therefore, malnutrition and polymorphisms in folate and vitamin B_{12} genes may play a role. Other causes of the maternal hyperhomocysteinaemia, however, have to be considered as well, such as low vitamin B_2 or B_6 levels, high body mass index (BMI) or glucose intolerance [27].

Folate and vitamin B_{12} are involved in the remethylation of homocysteine and donation of one-carbon groups to proteins, lipids and nucleotides, whereas vitamin B_6 is important in the transsulphuration of homocysteine [28]. The reduced plasma concentration of vitamin B12 and folic acid among cases suggested that folate is a key factor in cardiovascular development. Insufficient intake of B-vitamins results in biochemical derangements leading to hyperhomocysteinemia and DNA hypomethylation that may contribute to the development of CHDs [29].

Our findings of elevated homocysteine among case subjects are consistent with other studies comparing vitamin-dependent homocysteine metabolism among mothers of offspring with congenital heart defects [15], orofacial clefts [30] and neural tube defects (NTDs) [31] with those of mothers with normal formed offspring. Possible mechanisms by which homocysteine may have an embryotoxic effect include; elevated homocysteine may have an indirect embryotoxic effect by increasing oxidative stress through excessive production of reactive oxygen species and by decreasing the glutathione-dependent antioxidant-defense mechanisms [32]. Chronic oxidative stress is also associated with low concentrations of vitamin B-6, reduced glutathione (GSH), and cysteine and high concentrations of oxidized glutathione (GSSG) [33]. Furthermore, lifestyle factors such as alcohol intake and cigarette smoking are associated with increased oxidative stress, which can decrease the functional activity of methionine synthase through limiting the availability of reduced vitamin B-12 [34]. In our sample the number of smokers among case subjects was relatively higher than among control subjects, but the results were not significantly different. We examined our data to determine whether the concentrations of homocysteine and folate are correlated with the time interval between the end of pregnancy and the blood drawn in case

and control subjects. No discriminatory trend was found. Therefore, it is likely that, the metabolic patterns observed even more than a year after pregnancy reflect stable adult profiles.

To conclude, A high maternal homocysteine concentration is associated with an increased risk of having a child with CHD, in addition low maternal folate and Vitamin B-12 intakes detrimentally affect the embryonic cardiovascular development. Several methodologic limitations of our study should be considered, as venipuncture was done after the pregnancies had ended. Thus, our measurements may not represent the biomarker concentrations at the time of organogenesis.

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