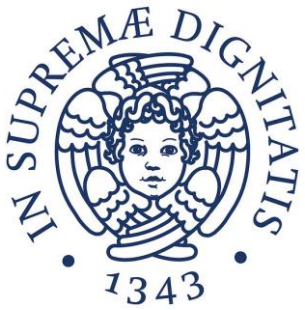

University of Pisa
Research Doctorate School in Biological and Molecular Sciences

Program: Biology

Congenital Heart Diseases: parental exposures and gene-environment interactions

Supervisor:
Dr. Maria Grazia Andreassi

PhD Student:
Dr. Monica Cresci

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Index

ABSTRACT

Congenital heart defects (CHDs) are the most prevalent of all birth defects, arising from the complex interplay of environmental exposures and genes. The molecular causes of most CHDs as well as the modifiable environmental risk factors, (especially for paternal exposure) remain largely unknown. Thus, there is an increasing interest in the study of gene-environment interaction in the pathogenesis of CHDs. The major aim of this project was to expand the knowledge of CHD etiology with specific attention at the identification of genetic and environmental risk factors. The effects of environmental factors might be modified by the genes responsible for the activation and detoxification of toxicant agents, contributing to an increased resistance (or sensitivity) to cardiac teratogenesis. Thus, the knowledge of genetic variants that can modify a person's risk of environmental exposure-induced disease may identify new potential therapeutic targets and appropriate preventive strategies.

In the first part of the study, we analyzed the association between different parental environmental exposures and CHD risk. Moreover, it has been investigated if the presence of specific polymorphisms in genes involved in toxicant metabolism, glutathione-S transferase: *GSTM1* and *GSTT1*, in the children might modulate the risk of CHD associated to toxicant exposure. In a case-control study , 360 parents of a child with CHD and 360 parents of a child without any congenital malformations, were compared in terms of lifestyle habits and toxicant exposures.

The results showed that parental smoking (≥15 cigarettes/day) was significantly associated with CHD risk (OR 2.1, 95% CI 1.3-3.5, p=0.002). Moreover, both maternal (OR 2.6, 95% CI 1.6-4.2, p<0.0001) and paternal (OR 2.5, 95% CI 1.6-3.8,

p<0.0001) occupational/environmental exposure to toxicants increased the risk of CHD. In addition, a significant additive risk (OR 4.5, 95% CI 2.5-8.3, p<0.0001) was found when both parents were exposed to toxicants. Regarding to genotype, *GSTM1* and *GSTT1* polymorphisms were investigated in 180 children with CHD. Both maternal (OR 3.6, 95% CI 1.1-11.2, p=0.03) and paternal (OR 3.3, 95% CI 1.0-10.8, p=0.03) exposure to toxicants increased the CHD risk in children who carried the combined null *GST* genotypes. The effect for the combined null genotypes was also stronger (OR 6.5, 95% CI 1.5-28.0, p=0.01) when both parents were exposed.

In the second part of the project, we analyzed the joint effect of the glutathione-S transferase P1 (*GSTP1)* genetic polymorphism (Ile105Val) and maternal environmental exposure, on CHD risk. The *GSTP1* gene is highly expressed early in fetal life and is the most abundant phase II xenobiotic metabolism enzyme in a human placenta. Fetal inherited *GSTP1* Ile105Val polymorphism may modify the metabolism and excretion of xenobiotics from fetal tissue and increase the risk of CHD. In a case-control study, 190 children with CHD and 190 healthy children were genotyped for the *GSTP1* Ile105Val polymorphism. All the mothers completed a structured questionnaire on the demographic as well as the preconceptional and lifestyle exposures.

No significant differences in Ile105Val genotype frequencies were observed between CHD and healthy children (p=0.9) as well as no evidence of significant interaction between the maternal exposure and *GSTP1* polymorphism was found.

In the last part of the project, we investigated whether the *ISL1* (rs1017) single-nucleotide polymorphism, in 3'-UTR region, conferred susceptibility to CHD. Indeed, the LIM homeodomain transcriptor factor *ISL1* is a known marker for undifferentiated cardiac progenitor cells that give rise to both the right ventricle and

the inflow and outflow tracts. To date, contradictory findings about the role of the *ISL1* rs1017 single-nucleotide polymorphism on increased risk of CHD have been reported.

In a case-control study, 309 patients with CHD and 500 healthy controls were genotyped for the *ISL1* rs1017 polymorphism. No significant difference in the genotype and variant allele distribution was found between patients and controls. In addition, the *ISL1* rs1017 polymorphism was not associated to the risk of CHD neither overall ($p=0.7$) nor stratifying the population by sex and CHD classification.

All these findings suggest that common genetic variants, not necessarily disease-causing, may contribute to increase the risk of CHD, especially interacting with environmental factors. Further studies are required to better define the role of genetic factors and their potential interaction with environmental factors on the risk of CHD.

INTRODUCTION

Congenital heart defects (CHDs) are due to an abnormal development of the heart during embryogenesis and fetal life, between the second and ninth week of gestation. CHDs are the most common occurring congenital malformations in newborn, and is the most frequent non-infectious cause of infant death (Hoffman and Kaplan, 2002; Garg, 2006). The incidence of moderate and severe forms of CHD is about 8/1,000 live births (19/1,000 when bicuspid aortic valve is included), and of all forms increases to 75/1,000 live births if tiny muscular ventricular septal defects, present at birth, and other trivial lesions are included (Pierpont et al., 2007; van der Linde, 2011).

About 20% of heart defects can be associated with extracardiac abnormalities, as part of a more complex syndrome, involving chromosomal anomalies, such as the trisomies (e.g. chromosomes 21, 18, and 13), or microdeletion 22q11, which is well-established chromosome cause of DiGeorge syndrome (Oyen et al., 2009; Richards and Garg, 2010).

Nevertheless, many types of CHDs are more frequently diagnosed as isolated and non-syndromic, and single gene mutations have been shown to contribute to the occurrence of malformations (Ware and Jefferies, 2012). To date, more of 30 genes have been linked to non-syndromic forms of CHD and the contribution of which is presumed to be relatively small (Blue et al, 2012).

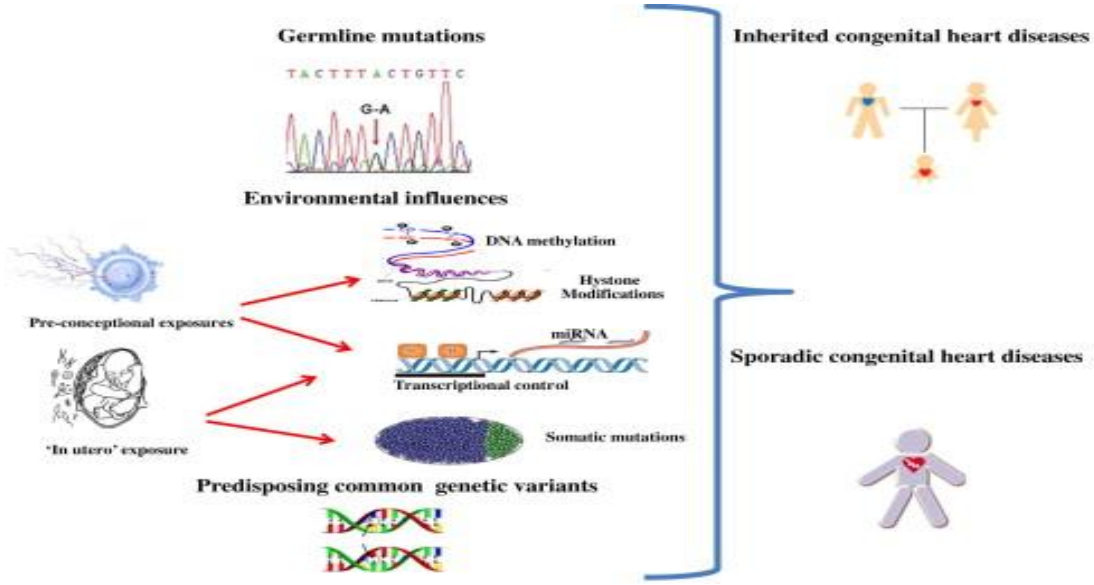
A family history has been described in both syndromic and isolated defects in 1-6% of the cases (Calcagni et al., 2007). Similarly, an increased risk of pediatric heart disease recurrence in family members of affected individuals has been shown (Garg, 2006; Ransom and Srivastava, 2007). Moreover, if more than one sibling is affected,

the recurrence risk can increase to 10% (Nora and Nora, 1988). Furthermore, the most non-syndromic CHDs occur sporadically, without a familial history of disease and a clear Mendelian inheritance. It is estimated that about 80% of CHDs with unknown aetiology has a multifactorial origin with an key interplay of genetic and environmental factors.

Controversy, it has suggested that multiple somatic mutations –mutations present in affected tissue but not in the germline one - may cause sporadic CHDs (Reamon-Buettner and Borlak, 2006). Indeed, the presence of several somatic mutations has been shown in cardiac transcription factors genes, such as *NKX2.5*, *GATA4* and *HAND1*, from the Leipzig (Germany) collection of malformed hearts 20-year stored in formalin (Reamon-Buettner et al., 2004; Reamon-Buettner and Borlak, 2004; Reamon-Buettner and Borlak, 2006; Reamon-Buettner et al., 2007). However, subsequent studies have not replicated this finding (Wang et al., 2001; Draus et al., 2009; Esposito et al., 2011; Salazar et al., 2011).

Recently, novel findings supported that epigenetic alterations, such as methylation status, dysregulation of small-non coding RNAs (microRNAs) and histone modification may contribute to understand the molecular basis of CHDs and that congenital heart diseases might be, in part, a consequence of a change in the control of the epigenome induced by the environment (Zhao et al., 2005; Montgomery et al., 2007; Zhao et al., 2007; Movassagh et al., 2010) (Figure 1).

Fig. 1: Schematic representation of the pathogenesis of congenital heart defects.



Cardiac transcription factor genes: Genetic Hypothesis

The knowledge about the genetic component of the congenital heart disease is very difficult, due to the complex interplay from different genes in different space and different times (Pierpont et al., 2007).

During the embryonic development, the heart is one of the first organs to form.

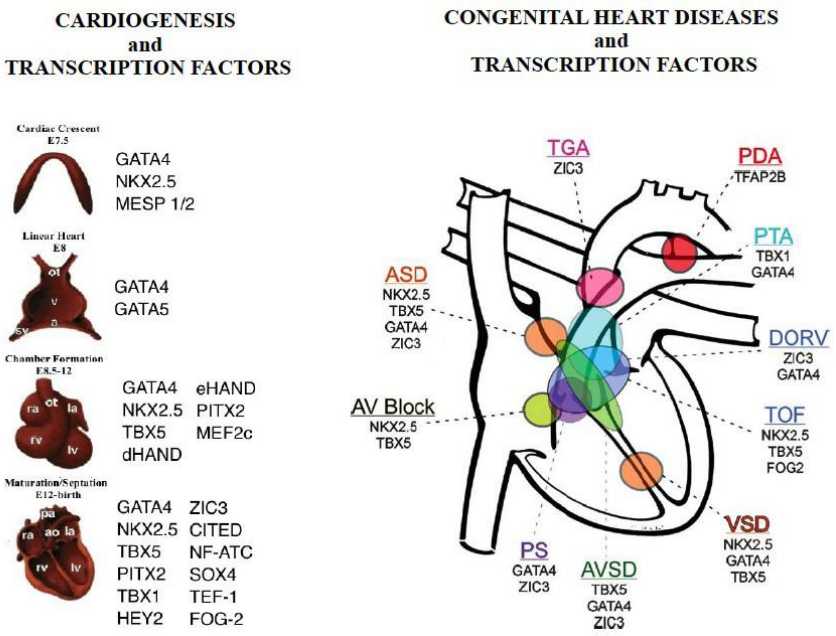
A high degree of conservation is observed during early-stage heart development in zebrafish, chick, frog, mouse, and human (Brand, 2003).

Because dysregulation of heart development is at root of CHD, a clear picture of how the heart forms is crucial for understanding the genesis of this disease.

In the last decade, it has been suggested that most of the inherited forms of CHD are a result of mutations in genes know to be essential for cardiac development, such as specific transcription factors genes (TFs). These genes, regulate specific events in

heart morphogenesis, through an intricate process, where TFs regulate each other's expression in order to stabilize and reinforce the cardiac gene program (Olson 2006; Nemer, 2008; Mc Bride et al., 2010; Ware and Jefferies, 2012) (Fig.2).

Fig. 2.: Transcription factors and congenital heart diseases.

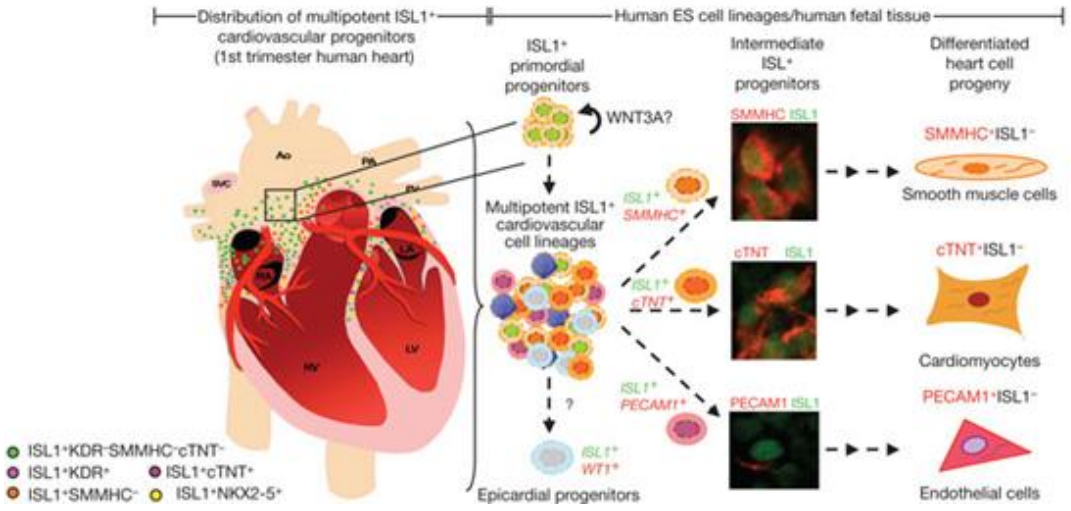


Because of their important role in the orchestration of cardiac development, mutations in these genes may induce a significant disruption/dysregulation of downstream gene expression and, thus, lead to cardiovascular malformations, as evidenced by the findings in transgenic mice (Biben et al., 2000; Bruneau et al., 2001; Zhang et al., 2013).

Moreover, the progenitor cells originating from the first heart field, second heart field and cardiac neural crest contribute to the cardiac morphogenesis (Buckingham et al., 2005; Shan et al., 2012).

ISL1 is a LIM homeodomain TF considered to be the most important marker of cardiac progenitor cell lineages in the secondary heart-field differentiation (Moretti et al., 2006; Kang et al., 2009; Stevens et al., 2010; Klaus et al., 2012) (Fig. 3).

Fig.3: *ISL1* functions (adapted from Bu et al., 2009)



ISL1 cardiovascular progenitors give rise to right ventricle and inflow and outflow tracts, which are affected by several cardiovascular malformations, e.g., transposition of the great vessels, tricuspid atresia and tetralogy of Fallot (Laugwitz et al., 2008). Animal experimental models have shown that homozygous mutants for *ISL1* developed a severe cardiac phenotype (Laugwitz et al., 2008), whereas mice totally knocked out for this gene were lacking in the outflow tract, in the right ventricle and in several part of the atria (Cai et al., 2003; Lin et al., 2006). Recently, common genetic variants and specific haplotypes in the *ISL1* gene have been found to contribute to the risk of CHDs in the white and black/African-American populations (Stevens et al., 2010). On the contrary, a more recent paper (Xue et al., 2012) showed that *ISL1* common variant rs1017 did not play a crucial role in conferring

susceptibility to sporadic CHD in Chinese population. Moreover, another recent paper, identified the presence of six known and five novel *ISL1* variants, investigating the potential contribution of *ISL1* in cardiomyopathies. The authors also describes a novel p.Asn252Ser *ISL1* gain-of-function variant, which could lead to greater activation of downstream targets, such as Mef2c, which are known to be involved in cardiac development, dilation, and hypertrophy (Friedrich et al., 2013).

Etiology of Congental Heart Disease: gene-environment interaction

In approximately 80% of CHD cases, the cause is multifactorial. Direct evidence regarding environmental exposures and the risk of CHD is very limited (Jenkins et al., 2007; Blue et al., 2012). Unfortunately, less is known about modifiable "non genetic" factors.

Several studies have suggested that some parental occupational and/or environmental exposures may be significantly associated with an increased prevalence of birth defects in offspring, especially for selected congenital heart defects (Dolk and Vrijheid, 2003; Chapin et al., 2009; Rankin et al., 2009; Strickland et al., 2009; Desrosiers et al., 2012). In particular, the maternal environmental risk factor known to influence the incidence of CHD are rubella, pre-gestational diabetes and exposure to teratogens, such as thalidomide, retinoic acid and indomethacin, and exposure to chemicals at work (Øyen et al., 2000).

Every human cell, including the spermatozoa and the oocytes, can suffer DNA mutations due to the exposure to environmental toxicants. Gametic DNA mutations preceding the conception can induce miscarriage, death or congenital defects.

Several studies have shown that maternal exposure is associated with a variety of adverse pregnancy outcomes including preterm birth, low birth weight, and birth defects (Brent, 2004; Kuehl and Loffredo, 2005; Jenkins et al., 2007; Patel et al., 2010). Conversely, the information available regarding environmental exposures of father as risk factor for birth defects, in particular for CHD, is very limited (Jenkins et al., 2007).

The toxicity of xenobiotics for embryonic tissues depends on the biotransformation process during which reactive products are formed (phase I) and detoxified (phase II). Several enzymes (and their gene families) are involved in this process. In particular, the impact of parental exposure on birth defects in the offspring might be affected by the presence of polymorphisms in genes responsible for the activation and detoxification of toxicant agents, contributing to an increased resistance or sensibility to cardiac teratogenesis (Loffredo, 2000; Kuehl and Loffredo, 2005; Patel et al., 2010).

Glutathione S-tranferase enzymes (*GSTM1, GSTT1, GSTP1)*

Genetic polymorphisms in the Glutathione S-Transferase (GST) enzymes, which provide critical defense against numerous toxins, might modulate the effect of toxic agents such as xenobiotics compounds. (Kuehl and Loffredo, 2005; Shi et al., 2008). Eight cytosolic GSTs are known to be expressed in humans: alpha (A), mu (M), pi (P), theta (T), kappa (K), omega (O), sigma (S) and zeta (Z). Each class consists of several distinct subclasses, with some overlap in tissue expression (Hayes and Strange, 2000). The *GSTM1* and *GSTT1* genes are located in chromosome 1 and 22

respectively. Their activity is the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione, which are water-soluble and can be excreted from the body. The gene coding for *GSTM1* and *GSTT1* exhibits a deletion polymorphism, which in case of homozygozity leads to absence of phenotypic enzyme activity.

The presence of these polymorphisms in association to maternal cigarette smoking has been associated to an increased risk for orofacial defects (Olshan et al., 2005). Moreover, the role of *GST* polymorphisms and their interaction with environmental pollutants on the risk of birth defects has also been examined (Garlantézec et al., 2012).

The *GSTP1* gene, highly expressed in early fetal life, is the most abundant phase II xenobiotic metabolism enzyme in the human placenta (Ahmad et al., 1990; Becket et al., 2000; Raijmakers et al. 2001).

An A to G transition at nucleotide 313 in exon 5 of the *GSTP1* gene, which replaces isoleucine (*Ile*) at codon 105 with valine (*Val*) within the active site of the enzyme, has been shown to result in altered enzyme activity (Zimniak et al. 1994). Therefore, fetal inherited *GSTP1* Ile105Val polymorphism might modify the metabolism and excretion of xenobiotics capable of crossing the placental barrier from fetal tissue.

AIM

Most of the known CHDs occur through a heterogeneous and complex process in which predisposing genetic factors interact with environmental factors. The environmental effects may be modified by genes involved in the activation or detoxification of toxicant agents, contributing to an increased resistance (or sensitivity) to cardiac teratogenic substances. Accordingly, recent studies have shown the fundamental role of single-nucleotide polymorphisms (SNPs) and/or mutations in genes critical for detoxification pathway, in the pathogenesis of CHDs.
In this framework, the first hypothesis of this project, was that environmental factors interact with genetic predisposing factors in the pathogenesis of CHDs. In order to reach this aim, the specific objectives were:

- to examine the association between the environmental exposure of both parents and CHD risk.
- to explore the modification effects of metabolizing gene polymorphisms (*GSTM1, GSTT1, GSTP1)* in association with parental exposure to toxicants.

In the last part of the project, since the importance of TFs genes in the control of heart development, it has been hypothesized that "common" sequence variations in these genes might be one of causative mechanism of CHD. In order to reach this aim, the specific objective was:

- to evaluate if the presence of a common variant of TF gene , *ISL1* conferred an increased susceptibility to CHD.

MATERIALS AND METHODS

Study population

The study population consisted of 309 patients, who were diagnosed with isolated, non-syndromic CHD (197 males [21.3 ±25.2 years], including 200 pediatric [4 ± 5.6 years] and 109 adult patients [52.5 ± 17.2 years] with bicuspid aortic valve, BAV); a control group of 500 healthy subjects [272 males; 15.7 ± 21.3 years] comprising 300 newborn and 200 adult subjects (39.8 ± 13.7 years). Moreover, we enrolled 360 parents of a child with CHD and 360 parents of a child without any congenital malformations. Both case and control parents completed a structured questionnaire on the demographic, preconceptional, and lifestyle exposures. We collected environmental and occupational exposure data from specific questions on potential teratogens/mutagens that have been linked to human reproductive impairment, including ionizing radiation, solvents, pesticides, asbestos and heavy metals. For the parents, the exclusion criteria were mothers who reported inconsistent use of B vitamin and folate supplements in the periconception period; the inability to obtain complete information about the occupational, demographic and lifestyle data from both parents. A sample of blood was obtained from 309 patients with CHD and 500 healthy subjects. The questionnaire model used is shown below.

MATERNAL EXPOSURE before and during pregnancy

1. Age at conception: _____

2. Employment: _____ From how many years? _____

3. Genetic disease: [N] [Y] Type: _____

   Chronic disease: [N] [Y] Type: _____

4. Have anyone in your family had genetic disease or cancer?

   Sibling: [N] [Y] Explain: _____

   Mother: [N] [Y] Explain: _____

   Father: [N] [Y] Explain: _____

   Mother family: [N] [Y] Explain: _____

   Father family: [N] [Y] Explain: _____

5. Have you had any medical exam? [N] [Y] If YES, explain:

	BEFORE THE PREGNANCY		FIRST 3 MONTHS OF PREGNANCY	
	NUMBER	LOCATION	NUMBER	LOCATION
Rx				
TAC				
Scintigraphy				
Angiography				
Mammography				
Radiotherapy				
Ultrasound				
Magnetic Resonance				
Other				

6. Smoking habits:
   - [0] No smoker
   - [1] Ex-smoker
   - [2] Smoker

   If ex-smoker:

   Age of beginning _____

   Age of end _____ N°cigarettes/die _____

   If smoker:

   Age of beginning _____ N°cigarettes/die _____

Smoking during first 3 months: [N] [Y] Cigarettes/days _____

7. Environmental tobacco smoke:

   Smoker cohabitants? [N] [Y]

   If Yes, how many? [1] [2] [>2]

   Smoker at work? [N] [Y]

   If Yes, how many? [1] [2] [>2]

7. Did you drink before the pregnancy? N Y Wine (glasses/days) _____
Beer _____
Alcohol _____

Did you drink during first 3 months? N Y

8. Did you take any prescription drugs before the pregnancy? N Y
If "yes", please indicate type: _____
_____

Did you take any prescription drugs during first 3 months? N Y
If "yes", please indicate type: _____

9. Have you been exposed to potentially toxic substances at home or elsewhere before the pregnancy?

Ionizing radiation:	N Y	Type:	_____
Pesticides:	N Y	Type:	_____
Chemical substances:	N Y	Type:	_____
Heavy metals:	N Y	Type:	_____
Anesthetic gases:	N Y	Type:	_____

Have you been exposed to potentially toxic substances during first 3 months?

Ionizing radiation:	N Y	Type:	_____
Pesticides:	N Y	Type:	_____
Chemical substances:	N Y	Type:	_____
Heavy metals:	N Y	Type:	_____
Anesthetic gases:	N Y	Type:	_____

10. Have you had any disease during the pregnancy? N Y
If "yes", please indicate type: _____

11. Where did you live?

City centre ☐ Suburbs ☐ Industrial zone ☐ Rural area ☐

On which floor?

Ground floor ☐ 1° floor ☐ 2° floor ☐ 3° floor or over ☐

CHILDREN

12. Date of birth: _____ Gender: M F

Weight at birth (g): _____ Week of gestation: _____

Twin birth: N Y

Other pregnancies

13. Number of previous pregnancies: ☐

Have the children birth defect, handicaps or genetic disease? N Y

Explain:

_____

_____

Other pathological conditions:

_____

_____

14. Other events:

☐ Stillbirth
☐ Miscarriages
☐ Therapeutic abortion
☐ Children who died other than in accidents

Date of event: _____

Cause or diagnosis, if known:

_____

_____

_____

_____

_____

FATHER EXPOSURE at the time of conception

1. Age at conception: _____

2. Employment: _____ From how many years? _____

3. Genetic disease: [N] [Y] Type: _____
   Chronic disease: [N] [Y] Type: _____

4. Have anyone in your family had genetic disease or cancer?
   - Sibling: [N] [Y] Explain: _____
   - Mother: [N] [Y] Explain: _____
   - Father: [N] [Y] Explain: _____
   - Mother family: [N] [Y] Explain: _____
   - Father family: [N] [Y] Explain: _____

5. Have you had any medical exam? [N] [Y] If YES, explain:

	NUMBER	LOCATION	DATA
Rx			
TAC			
Scintigraphy			
Angiography			
Radiotherapy			
Magnetic Resonance			
Ultrasound			
Other			

6. Smoking habits:
   - [0] No smoker
   - [1] Ex-smoker
   - [2] Smoker

If ex-smoker:

Age of beginning _____
Age of end: _____ N°cigarettes/die _____

If smoker:

Age of beginning _____ N°cigarettes/die _____

7. Environmental tobacco smoke:

Smoker cohabitants? [N] [Y]

If Yes, how many? [1] [2] [>2]

Smoker at work? [N] [Y]

If Yes, how many? [1] [2] [>2]

8. Did you drink? N Y

Wine (glasses/days)_____
Beer _____
Alcohol_____

9. Did you take any prescription drugs? N Y If "yes", please indicate type:

_____

_____

10. Have you been exposed to potentially toxic substances at home or elsewhere?

Ionizing radiation:	N	Y	Type: _____
Pesticides:	N	Y	Type: _____
Chemical substances:	N	Y	Type: _____
Heavy metals:	N	Y	Type: _____
Anesthetic gases:	N	Y	Type: _____

11. Where did you live?

City centre ☐ Suburbs ☐ Industrial zone ☐ Rural area ☐

On which floor?

Ground floor ☐ 1° floor ☐ 2° floor ☐ 3° floor or over ☐

DNA extraction

Genomic DNA was extracted from whole-blood samples through Biorobot EZ1 (Qiagen) that allows to extract DNA, automatically, from both blood and tissue, from 6 together samples in a single step:

- sample lysis,
- DNA binding to magnetic particles,
- washing and elution of the DNA.

DNA quantitative assessment

We have prepared DNA elutions 1:250 on sterilized water for spectrophotometric quantification at 260 nm and 280 nm. The spectrophotometric analysis determines the quantitative concentration of the DNA and qualitative information. In fact, the ratio of the absorbance at 260 nm and 280 nm represent a good index of the sample purity. For the DNA, the well index is 1.7-1.9. The formula for the final concentration of the sample was:

FINAL CONCENTRATION=λ260 x dilution factor.

Genotyping analysis

Glutathione S-Transferase M1 and T1 (*GSTM1* and *GSTT1*)

The *GSTM1* and *GSTT1* genotypes were determined using a co-amplification polymerase chain reaction approach, with the *GSTM4* gene, which is never deleted, as the internal control to distinguish the null genotypes from aborted polymerase chain reaction. The primers sequences and PCR conditions were:

- for the *GSTM1* amplification:

forward 5'-CGC CAT CTT GTG CTA CAT TGC CCG-3'

reverse 5'-TTC TGG ATT GTA GCA GAT CA-3'

First cycle

denaturing	94°	5 min

35 cycles

denaturing	94°	1 min
annealing	52°	1 min
elongation	72°	1 min

Last cycle

elongation	72°	10 min

- for the *GSTT1* amplification:

forward 5’- TTC CTT ACT GGT CCT CAC ATC TC -3’

reverse 5’- TCA CCG GAT CAT GGC CAG CA -3’

First cycle

denaturing	94°	5 min

35 cycles

denaturing	94°	1 min
annealing	56°	1 min
elongation	72°	1 min

Last cycle

elongation	72°	10 min

- for the *GSTM4* amplification:

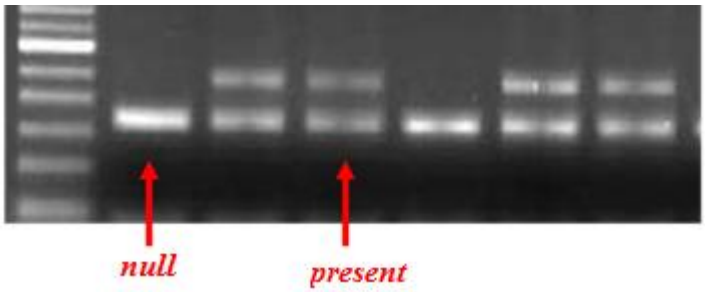
forward 5’-CGC CAT CTT GTG CTA CAT TGC CCG-3’

reverse 5’-ATC TTC TCC TCT TCT GTC TC-3’

Agarose gel electrophoresis

PCR reaction was appears in the 1.5% agarose gel, stained with ethidium bromide 10 mg/ml at final concentration of 0.3%. We have loaded the wells of the gel with 10 µl of PCR product with 3 µl of "*loading*" buffer (L.B.: 0.25% bromphenol blue, 0.25% cyanol xylene, 15% glycerol) and a DNA marker of 100 bp (*PRIME*). The electrophoresis occurred at 100 V in TBE 1X buffer (Tris-base 4 mM, 0.9 M boric acid, 50 mM EDTA, pH 8). The internal standard fragments amplified from the *GSTM4* gene was 157 bp. A 230-bp fragment was amplified for the *GSTM1* gene, and a 480-bp fragment was obtained for the *GSTT1* gene. The absence of amplified products was consistent with the null genotypes (Fig. 4).

Fig. 4: *GST* electrophoresis.



Glutathione S-Transferase P1 (*GSTP1*) Ile105Val polymorphism

A PCR-restriction fragment length polymorphism method was used to determine the allele distribution of the *GSTP1* Ile105Val polymorphism. The primers sequences were:

forward 5'-ACC CCA GGG CTC TAT GGG AA -3'

reverse 5'-TGA GGG CAC AAG AAG CCC CT-3'

The PCR condition involved:

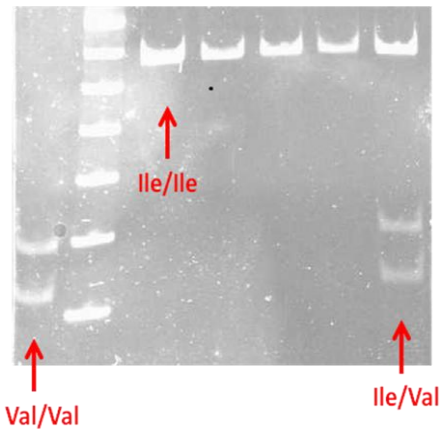
First cycle		
denaturing	94°	5 min
32 cycles		
denaturing	94°	30 s
annealing	64°	50 s
elongation	72°	50 s
Last cycle		
elongation	72°	10 min.

The 176-bp amplified *GSTP1* gene fragment was subjected to restriction digestion in a 15-µl reaction volume containing 7.5 µl of PCR product, 100 mmol/l of NaCl, 50 mmol/l of Tris-HCl, pH 7.9, 10 mmol/l of $MgCl_2$, 1 mmol/l dithiothreitol, and 5 U of BsmAI enzyme at 55° overnight.

Polyacrylamide gel electrophoresis

The digested PCR products were separated by electrophoresis, occurred at 200 V, using polyacrylamide gel (size: 10.1 cm x 9.7 cm, 1.0 mm thickness) at 8% (acrylamide stock solution 20%; 19:1 acrylamide:bisacrilamide), TBE 1X (Tris-base 4 mM, 0.9 M boric acid, 50 mM EDTA, pH 8), TEMED 0.13% and APS 0.1%. We have loaded the wells of the gel with 15 µl of digestion product with 4 µl of "loading" buffer (L.B.: 0.25% bromphenol blue, 0.25% cyanol xylene, 15% glycerol) and a DNA marker of 20 bp (PRIME). The polyacrylamide gel was stained, though immersion, with a solution composed with 40 µl of ethidium bromide 10 mg/ml and 100 ml TBE 1X, for 30 minutes at final concentration of 2.1%. The genotype was determined by analysis of the bands on the gel as follow: homozygous wild-type for isoleucine (II), one band (176 bp); homozygous mutated for valine (VV), two bands (91 and 85 bp); and heterozygous (IV), three bands (176, 91, and 85 bp) as shown in Fig. 5.

Fig.5: Electrophoresis gel for *GSTP1* gene



ISL1 rs1017 polymorphism *(ISL1)*

A PCR-restriction fragment length polymorphism method was used to determine the allele distribution of the rs1017 polymorphism. The primer sequences, were designed using the Primer3 program (http://primer3.sourceforge.net/):

forward 5'-CCT TCA GGA AGG TGG AGC TG-3'

reverse 5'-CGC TTG TGG CAA AAT AGA GG-3'.

PCR conditions were as follow:

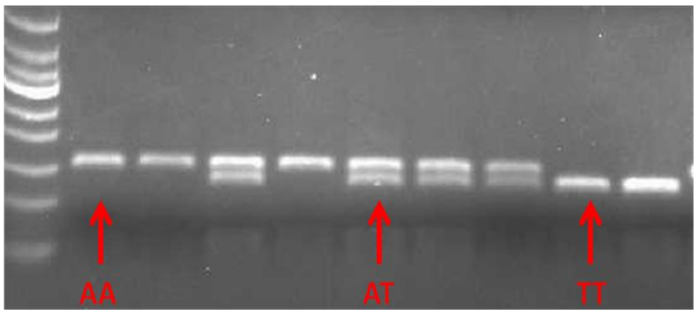
First cycle		
denaturing	94°	5 min
35 cycles		
denaturing	94°	30 s
annealing	56°	30 s
elongation	72°	30 s
Last cycle		
elongation	72°	7 min.

The 248-pb amplified *ISL1* gene fragment was subjected to restriction digestion in a 9-µl reaction volume containing 4 µl of PCR product, 100 mmol/l of NaCl, 50 mmol/l of Tris-HCl (pH 7.9), 10 mmol/l of MgCl2, 1 mmol/l dithiothreitol, and 2.5 U of *DraI* enzyme at 37° overnight.

Agarose gel electrophoresis

The digested PCR products were separated by electrophoresis using 1.5% agarose gel, stained with ethidium bromide 10 mg/ml at final concentration of 0.3%. We have loaded the wells of the gel with 5 µl of PCR product with 2 µl of "*loading*" buffer (L.B.: 0.25% bromphenol blue, 0.25% cyanol xylene, 15% glycerol) and a DNA marker of 100 bp (*PRIME*). The electrophoresis occurred at 100 V in TBE 1X buffer (Tris-base 4 mM, 0.9 M boric acid, 50 mM EDTA, pH 8). A representative gel is shown in Fig. 6. The genotype was determined by analysis of the bands on the gel: homozygous wild-type for adenine (AA), one band (156 bp); homozygous mutated for thymine (TT), one band (134 bp); and heterozygous (AT), two bands (156 and 134 bp).

Fig. 6:Electrophoresis gel for *ISL1* gene.



Statistical analysis

Statistical analyses of the data were conducted using the StatView statistical package, version 5.0.1 (Abacus Concepts, Berkeley, California). Data are expressed as the mean ± SD. For statistical analysis, all determinants were dichotomized except for the age variable. Smokers were classified as individuals who smoked at least three cigarettes for day at the time of the conception, ex-smokers as those who stopped smoking at least 6 months before inclusion in the study, and nonsmokers as person who never smoked. Smokers also were divided into medium smokers (3-14 cigarettes a day) and heavy smokers (≥ 15 cigarettes a day). For consumption frequency of alcoholic drinks per day (beer, wine, liquor) and nondrinkers as those who drank less than three drinks for day. Given the relatively small number of exposed mothers in any particular environmental or occupational category, the statistical analysis for toxicant exposure included both environmental and occupational exposure to X-rays, chemicals, anesthetics, industrial cleaning agents and solvents, exhaust and welding fumes, paint/varnish/thinner, asbestos, heavy metals and pesticides. The differences between the mean values of two continuous variables were evaluated using the unpaired Student *t* test. The differences in non continuous variables and genotype distribution were tested using chi-square analysis. Unconditional logistic regression analysis was used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) for the association between CHD and parental exposure. The ORs were also adjusted for potential confounding factors. A 2-tailed p value <0.05 was chosen as the level of significance.

RESULTS

Parental exposures analysis

We used a prospective and paired case-control study design (1:1). Data obtained from questionnaires showed that the mothers of patients with CHD had a significantly lower age than the controls (p=0.01) but no significant differences between the two groups were found for paternal characteristics.

The CHD cases had a significantly lower birth weight (p=0.01) compared to the control children and the children with CHD were also more often conceived with artificial fertilization (p=0.05). In table 1 are summarized the specific occupational and professional risk factors for CHD.

Table 1: Occupational/environmental exposure among CHD and control parents

Variable	CHD parents	Control parents	p value
Maternal occupational exposures			**0.004**
Risk factor (n)	28 (15.5%)	11 (6.1%)	
Paint/ varnish/thinner/solvents	9	0	
X-rays (hospital workers)	4	2	
Cleaning agents and solvents	4	1	
Industrial chemicals and solvents	4	1	
Hair dyes	2	2	
Textile dye	2	0	
Chemical laboratory exposure	1	1	
Asbestos	1	0	
Pesticides	1	0	
Anesthetic gas	0	4	
Paternal occupationale exposures	60 (33.3)	41 (22.8)	**0.03**
Risk factor (n)			
Paint/ varnish/thinner/solvents (shoe factory)	14	4	
Cement dust	15	8	
Industrial chemicals and solvents	11	6	
Hair dyes	1	1	
Cleaning agents and solvents	1	1	
X-rays (hospital workers)	1	4	
Textile dye	1	0	
Exhaust and welding fumes	2	3	
Chemical laboratory exposure	1	-0	
Asbestos	6	1	
Heavy metals	4	5	
Pesticides	3	1	
Anesthetic gas	0	7	
Maternal environmental exposures	45 (25.0)	25(13.9)	**0.001**
Risk factor (n)			
Waste sites	9	0	
Thermal power plant	8	1	
Industrial pollution	9	5	
Electromagnetic field	4	0	
Environmental pollution	3	3	
X-rays (radiotherapy; Chernobyl)	2	0	
Asbestos	1	1	
Pesticide sites	5	0	
Drugs	4	15	
Paternal environmental exposures	40 (22.2)	12 (6.7)	**<0.0001**
Risk factor, n			
Waste sites	9	0	
Thermal power plant	9	0	
Industrial pollution	8	5	
Electromagnetic field	3	0	
Environmental pollution	3	0	
Asbestos	0	1	
Pesticide sites	5	0	
Drugs	3	6	

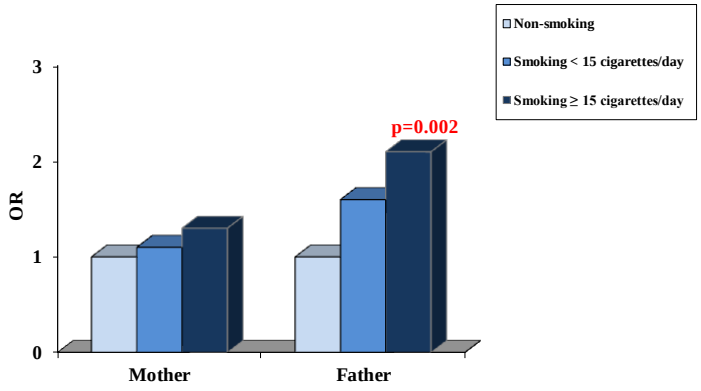
The parental exposure to environmental factors and CHD risk is listed in Table 2.

Table 2: Parental exposure to environmental risk factors and CHD risk

Variable	CHD parents (n=360)	Control parents (n=360)	OR (C.I. 95%)	p
Active smoking (positive)				
Mother	50 (27.8)	45 (25.0)	1.2 (0.7-1.8)	0.6
Father	81 (45.0)	65 (36.1)	**1.7** (1.1-2.6)	0.02
Diagnostic x-ray exposure (positive)				
Mother	49 (27.2)	38 (21.1)	1.3 (0.8-2.2)	0.2
Father	45 (25.0)	37 (20.6)	1.3 (0.8-2.1)	0.3
Heavy drinking (positive)				
Mother	1 (0.6)	1(0.6)	1.0 (0.1-16)	0.99
Father	7 (3.9)	7 (3.9)	1.0 (0.3-2.9)	0.99
Occupational/environmental exposure (positive)				
Mother	70 (38.9)	34 (18.9)	**2.6** (1.6-4.2)	<0.0001
Father	88 (48.9)	49 (27.2)	**2.5** (1.6-3.8)	<0.0001

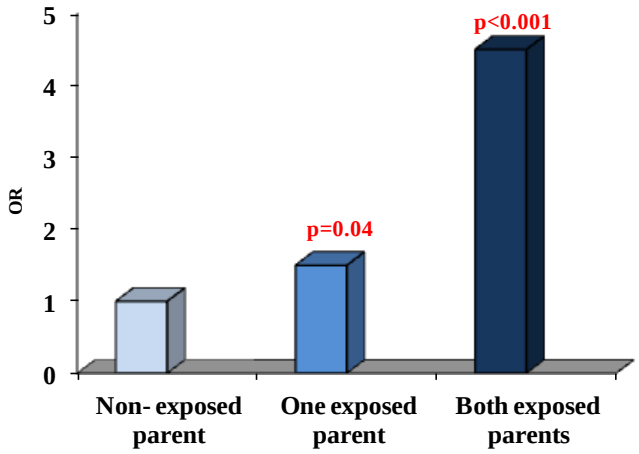
Maternal smoking was not significantly associated with an increased risk of CHD (OR 1.2, 95% CI 0.7 to 1.8) but the fathers who were smoking showed an increased risk of CHD in their offspring (OR 1.7, 95% CI 1.1 to 2.6, p=0.02), especially fathers who were heavy smokers (≥ 15 cigarettes/day) had an high risk of having children with CHD (Figure 7). Conversely, parental alcohol use, the existence of a chronic disease or a disease in the first trimester of pregnancy and the exposure to diagnostic radiographs did not show to influence CHD risk.

Figure 7: Parental smoking and CHD risk.



Both maternal (OR 2.6, 95% CI 1.6 to 4.2, p<0.0001) and paternal (OR 2.5, 95% CI 1.6 to 3.8, p<0.0001) occupational/environmental exposure to toxicants increased the risk of CHD. Therefore, the exposure of both parents to toxicants is associated to significant additive risk (OR 4.5, 95% CI 2.5 to 8.3, p<0.0001) of CHD in the offspring (Fig.8).

Fig.8: Interactive effect between maternal and paternal exposures on CHD risk.



Moreover, on multivariate analysis, a father's heavy smoking (OR 1.9, 95% CI 1.1 to 3.3) and maternal exposure (OR 2.1, 95% CI 1.2 to 3.6) and paternal exposure (OR 1.8, 95% CI 1.1 to 3.0) to toxicants were the main determinants of CHD risk.

Gene-environment interactions analysis

In a case-only approach, we genotyped 180 children with CHD for *GST* polymorphisms, considering their association to parental exposure. A case-only design was chosen because it is considered the most powerful method to study gene-gene and gene-environment interaction in disease etiology (Gauderman, 2002)

The frequency of *GSTM1* null genotype, in our population, was 57.5%, and the frequency of the *GSTT1* null genotype was 16.7%. We did not detect an interactive effect of *GSTM1* or *GSTT1* null genotypes and maternal and paternal exposures (Table 3).

Table 3: Gene-environment interactions for combination of glutathione S-transferase (GST) genes and parental exposure.

Positive parental exposure	GSTM1 Present	GSTM1 Absent	OR (CI 95%)	p Value
Mother				
Active Smoking				0.3
Negative	54	61	1.0	
Positive	19	31	1.4 (0.7-2.9)	
Diagnostic x-ray exposure				0.1
Negative	61	70	1.0	
Positive	17	32	1.6 (0.8-3.2)	
Other toxicant exposures				0.2
Negative	52	58	1.0	
Positive	26	44	1.5 (0.8-2.9)	
Father				
Active Smoking				0.8
Negative	34	45	1.0	
Positive	36	45	1.0 (0.5-1.8)	
Diagnostic x-ray exposure				0.6
Negative	60	75	1.0	
Positive	18	27	1.2 (0.6-2.4)	
Other toxicant exposures				0.1
Negative	45	47	1.0	
Positive	33	55	1.6 (0.9-2.9)	

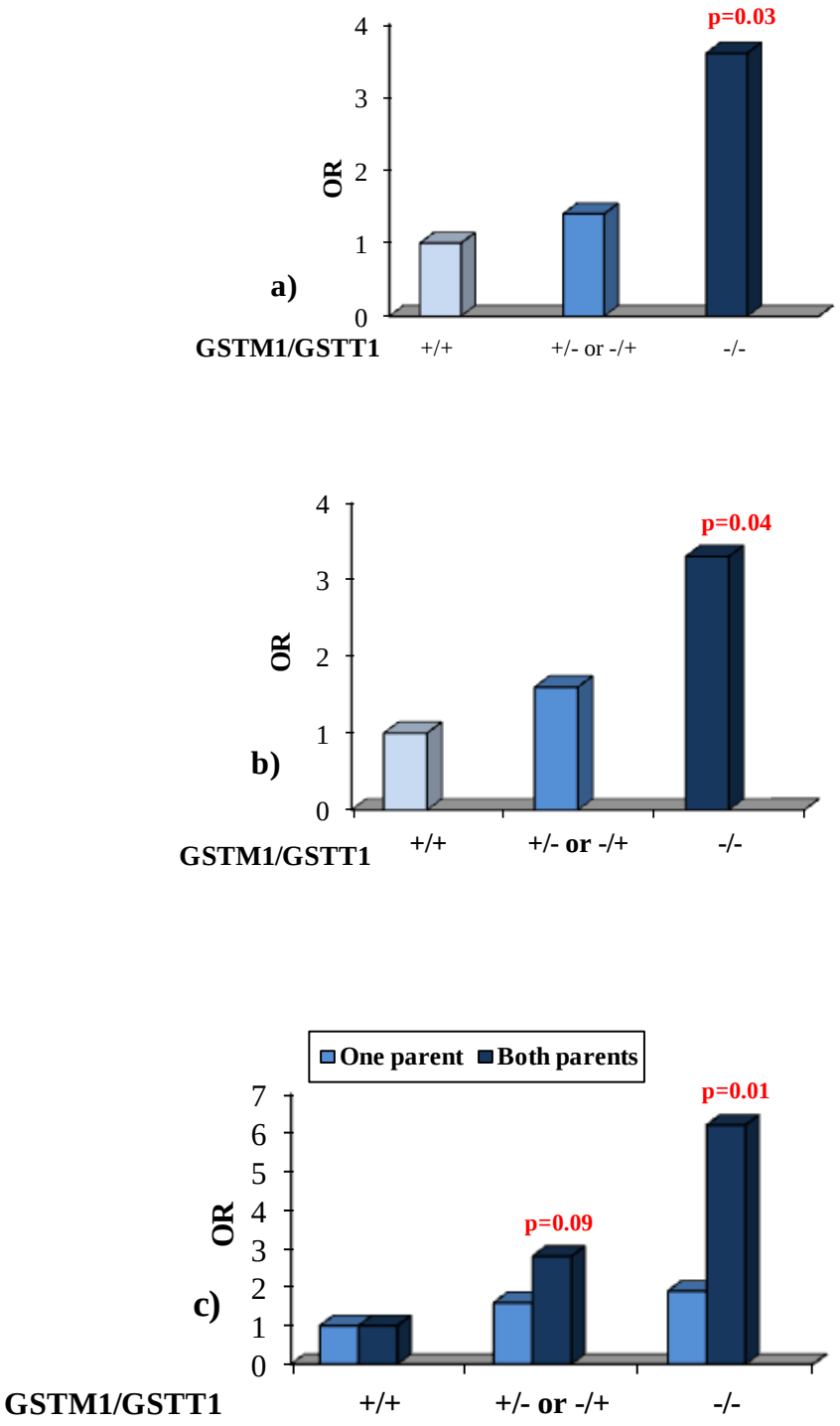
Positive parental exposure	GSTT1 Present	GSTT1 Absent	OR (CI 95%)	p Value
Mother				
Active Smoking				0.1
Negative	93	22	1.0	
Positive	45	5	0.5 (0.2-1.3)	
Diagnostic x-ray exposure				0.9
Negative	109	22	1.0	
Positive	41	8	1.0 (0.4-2.3)	
Other toxicant exposures				0.08
Negative	96	14	1.0	
Positive	54	16	2.0 (0.9-4.5)	
Father				
Active Smoking				0.4
Negative	63	16	1.0	
Positive	69	12	0.7 (0.3-1.4)	
Diagnostic x-ray exposure				0.1
Negative	109	26	1.0	
Positive	26	4	0.4 (0.1-1.2)	
Other toxicant exposures				0.09
Negative	81	11	1.0	
Positive	69	19	2.0 (0.9-4.5)	

However, the risk of CHD tended to be greater in children carrying the *GSTM1* and *GSTT1* null genotypes who had parents exposed to toxicants.

A significant interaction was found for the combined null GSTs and both maternal (OR 3.6, 95% CI 1.1 to 11.2, p=0.03) and paternal (OR 3.3, 95% CI 1.0 to 10.8, p=0.03) exposure to toxicants.

Finally, children with the combined null *GST* genotype had a greater risk than children carrying wild-type *GST* genes when both parents were exposed (Figure 9).

Fig.9: ORs stratified by children's GST genes and (A) maternal, (B) paternal, and (C) parental occupational/environmental exposure to toxicants.



Glutathione S-Transferase P1 (*GSTP1*)

Gene-environment interaction analysis

In order to investigate the role of *GSTP1* Ile105Val and CHD risk, we used a paired case-control study design (1:1). The frequency of *GSTP1* Ile105Val in both CHD cases and control subjects satisfied the Hardy-Weinberg equilibrium and was comparable with that described in the Caucasian population.

The genotype distribution was not significantly different between the patients and the control subjects (p=0.9), as shown in table 4.

Tab. 4: Genotype distribution of *GSTP1* polymorphism.

***GSTP1* polymorphism**	**Case Population n(%)**	**Control Population n (%)**	**p value**
Ile/Ile Ile/Val Val/Val	109(57) 66(35) 15(8)	110(58) 65(34) 15(8)	0.9

Moreover, we found no association between *GSTP1* Ile105Val polymorphism in children and maternal exposure. Indeed, no significant gene-environment interactions were observed (Table 5). Logistic regression did not show an increased risk for CHD in the presence of positive maternal exposure and *GSTP1* polymorphism.

Table 5: Gene-environment interactions for *GSTP1* genes and maternal exposure.

Maternal exposures	**GSTP1 controls (n)**			**p**	**GSTP1 cases (n)**			**p**
	Ile/Ile	**Ile/Val**	**Val/Val**		**Ile/Ile**	**Ile/Val**	**Val/Val**	
Active Smoking								
No	69	38	8	0.7	68	40	11	0.6
Yes	41	27	7		40	26	5	
Occupational exposures								
No	100	58	13	0.8	89	56	13	0.8
Yes	10	7	2		43	25	5	
Environmental exposures								
No	96	50	13	0.1	83	50	12	0.9
Yes	14	15	2		26	16	3	

ISL1 rs1017 polymorphism *(ISL1)*

Genetic analysis

A case-control study design was also used to investigate the association between *ISL1* and CHD. The observed genotype frequency was in agreement with Hardy-Weinberg equilibrium.

No significant difference between the case and control groups was found in term of genotype and allele distribution, as shown in table 6.

At logistic analysis, the rs1017 AT genotype and the mutated TT genotype were not associated with the risk of CHD (OR 1.0, 95% CI 0.7 to 1.3, p=0.8; OR 1.1, 95% CI 0.7 to 1.7, p=0.6 respectively). Moreover there was no appreciable difference between CHD risk and the presence of T allele (OR 1.0, 95% CI 0.7 to 1.5, p=0.7).

Tab.6: Main effects of ISL1 rs1017 on CHD risk in the case-control study.

Genotypes	No. (%) cases (n=309)	No. (%) controls (n=500)	OR (CI%95)	p-Value
rs1017				
AA	142 (46)	229 (46)	1.0	
AT	130 (42)	204 (41)	1.0 (0.7-1.3)	0.8
TT	37 (12)	67 (13)	1.1 (0.7-1.7)	0.6
AT + TT	167 (54)	271 (54)	1.1(0.7-1.5)	0.7
Allele				
A	414 (67)	662 (66)	1.0	
T	204 (33)	338 (34)	1.2 (0.7-1.7)	0.6

Furthermore, when we compared the *ISL1* rs1017 genotype distribution among the different groups of patients and controls, no significant difference was also observed (Table 7).

Table 7: *ISL1* rs1017 genotype distribution in different groups of patients and controls.

Genotypes	**No. (%) CHDs (n=200)**	**No. (%) BAV (n=109)**	**No. (%) pediatric controls (n=300)**	**No. (%) Adult controls (n=200)**
rs1017				
No. (%) genotypes				
AA	93 (46)	49 (45)	144 (48)	85 (43)
AT	82 (41)	48 (44)	114 (38)	90 (45)
TT	25 (13)	12 (11)	42 (14)	25 (13)
T allele frequency	0.33	0.33	0.33	0.35

Finally, we evaluated the influence of polymorphism on CHD risk also stratifying the population for sex and CHD classification, but no differences in the various subgroups were found (table 8).

Table 8: Stratified analysis and *ISL1* rs1017 polymorphism.

Variables	OR (95% CI)	p-Value
Overall	1.1 (0.7-1.5)	0.7
Sex		
Male	0.8 (0.5-1.3)	0.4
Female	0.9 (0.7-1.3)	0.8
CHD classification		
Bicuspid Aortic Valve	1.0 (0.7-1.6)	0.9
Complex CHD	0.9 (0.6-1.4)	0.6
Tetralogy of Fallot	1.0 (0.6-1.8)	0.9
Septation defects	1.3 (0.7-2.4)	0.5
Patent ductus arteriosus	0.9 (0.3-2.7)	0.9
Aortic coarctation	0.7 (0.3-2.4)	0.7
Other CHDs	1.5 (0.4-6.0)	0.6

DISCUSSION

Our research project shows the importance of cumulative effect of genetic and environmental risk factors in the etiology of CHD. For the first time, we have demonstrated that *GSTM1* and *GSTT1* polymorphisms mediate the risk of CHD in the presence of a positive history of parental exposure to toxicants. Conversely, *GSTP1* Ile105Val polymorphism showed no association with the maternal toxicant exposure and the risk to develop a CHD. Moreover, *ISL1* rs1017 polymorphism was not associated with an increased risk to develop CHDs. Our data underline the fundamental role of gene-environment interactions in the pathogenesis of congenital heart disease.

Parental exposures analysis

Environmental factors potentially harmful in the aetiology of CHDs are unknown. The best available information comes from the Baltimore-Washington Infant Study (BWIS), conducted in the Baltimore-Washington area between 1981 and 1989 and the Finnish study conducted by the National Public Health Institute (in Helsinki) in cases and controls born during 1982 to 1984 (Källén, 1999; Malik et al., 2008). Several other studies investigated the association between occupational hazards, including the exposure of chemicals, and specific phenotypes of congenital malformation (Thulstrup and Bonde 2006; Snijder et al., 2012). Some of these studies found indication for effects of chemical on fetal development but the evidence remains equivocal.

The maternal environmental risk factor known to influence the incidence of CHD are rubella, pre-gestational diabetes and exposure to teratogens, such as thalidomide, retinoic acid and indomethacin, and exposure to chemicals at work (Øyen et al., 2000).

In particular, maternal occupation exposures to solvent-containing products, mineral oil products, dyes, lacquers, paints and pesticides have been associated with an increased risk for CHD (Loffredo 2000; Gilboa et al., 2012).

A number of studies have also investigated maternal cigarettes smoking and congenital heart disease, providing controversial results, probably owing to variations in the method, disease categorization, sample size, or confounding bias (Ferencz et al, 1997; Källén et al., 1999; Woods et al., 2001; Kuciene and Dulskiene, 2008; Patel et al., 2012). An analysis of the BWIS data identified a possible dose-response relationship between the number of cigarettes smoked and non-syndromic atrio-ventricular septal defects (Ferencz et al, 1997; Alverson et al. 2011).

In a population-based case-control study of 3,067 infants with non-syndromic CHD and 3,947 infants without birth defects, the association was stronger for mothers who reported heavier tobacco consumption (≥25 cigarettes/day) during pregnancy (Malik et al., 2008). This dose-effect relation was confirmed in another recent studies of children with CHD and maternal smoking exposure (Krapels et al. 2006; Patel et al., 2012).

Few studies have evaluated the effect of paternal exposure to environmental factors in the preconception period. Specifically, paternal exposures to lifestyle substances (Marijuana), paint stripping and ionizing radiation exposure have been associated with certain malformations in the BWIS (Correa-Villasenor et al, 1993; Ewing et al., 1997). An association between parental smoking, particularly when both parents

were smokers, and the risk of conotruncal heart defects, including D-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle and truncus arteriosus has also been reported (Wasserman et al. 1996). A very recent study found that several occupations were associated with an increased prevalence of various birth defects categories (Desrosiers et al., 2012). Moreover, another study found a more than fivefold increase in risk mortality and deaths due to congenital malformation, especially for cardiac defects, for paternal occupational exposures, possibly to organic solvents during preconception period (Sung et al., 2009). In addition, recent studies have revealed that paternal smoking and occupational exposure, are risk factors for congenital defects, such as orofacial, anorectal malformations and CHDs (Krapels et al., 2006; van Rooij et al.,2010; Snijder et al., 2012) supporting the existing data on the great vulnerability of the male reproductive system to environmental exposures. At present, societal concern is growing about the particular sensitivity of the male germ line to genetic transmissible effects, (Cordier, 2008; Gianicolo et al., 2010) that might play a crucial role in the origin of congenital defects in general, and in particular CHD (Kuehl and Loffredo, 2005). It is has been suggested that male exposure may exert a teratogenic effect through toxicant compounds adsorbed to sperm and transmitted to a woman in the ejaculate (Chapin et al., 2004). The contaminant is absorbed by the woman, where it might reach and adversely affect a current pregnancy and, perhaps, remain in the woman's body to influence future pregnancies (Gianicolo et al., 2010). In contrast, toxicant compounds may act in human seminal fluid as endocrine disrupting agents causing direct germ line DNA damage or epigenetic changes (Cordier, 2008; Gianicolo et al., 2010). Teratogenic, carcinogenic, and endocrine disrupting agents, such as pesticide residues, heavy metals organic solvents (benzene, toluene, and xylene), nicotine,

aromatic hydrocarbons and precursors of mutagenic nitrosamines, have all been detected in human seminal fluid (Hales and Robair, 2001; Cordier, 2008; Gianicolo et al., 2010). Recent studies also showed that these environmental toxicants can induce oxidative DNA damage, mutations and chromosomal aberrations, such as DNA strand breaks and aneuploidy, in human seminal fluid (Hales and Robair, 2001; Cordier, 2008; Gianicolo et al., 2010). Therefore, it is plausible that exposure to toxicant agents of both parents during the preconceptional period and 'in utero' during pregnancy, might have a pivotal role in the pathogenesis of CHDs affecting genome and the so-called 'epigenome' (Gianicolo et al., 2010).

Gene-environmental interactions

It is increasing the evidence that people have different susceptibility to develop a disease induced by toxic agents (Kuehl and Loffredo, 2005; Shi et al., 2008). Specifically, the inheritance of particular genotypes for metabolizing systems and DNA repair pathways, might modulate the effect, leading to varying susceptibility to the congenital malformations of toxicants (Kuehl and Loffredo, 2005; Shi et al., 2008). The toxicity of xenobiotics for embryonic tissues depends on the biotransformation process during which reactive products are formed (phase I) and detoxified (phase II). Several enzymes (and their gene families) are involved in this process including glutathione transferases. In particular, the GSTs are the polymorphic super-gene family of detoxification enzymes, that are involved in metabolism of numerous toxins and provide critical defense against xenobiotics (Bolt and Their, 2006).

GSTM1 and *GSTT1* are the most extensively studied genes in the GST gene superfamily. There has been some, albeit contradictory, evidence of their enzymatic expression during the early stages of embryonic and fetal development in most tissues (Raijmakers et al., 2001; Shi et al., 2007). For example, *GSTM1,* appears to be expressed in the fetus early in gestation (Raijmakers et al., 2001), while the *GSTT1* enzyme has been shown to be expressed only in craniofacial structures during fetal life (Shi et al., 2007). The polymorphism in *GSTM1* and *GSTT1* gene loci is caused by a deletion which results in the absence of enzyme, especially in individuals with null genotypes. Deletion of the *GSTM1* and *GSTT1* genes, resulting in loss of functional activity, has been reported in approximately 50 and 20% of the white population, respectively, which might predispose to greater health effects from toxic xenobiotics (Bolt and Their, 2007). Previous studies reported that deletion in the *GSTM1* and *GSTT1* genes might contribute to the development of congenital malformations, such as oral cleft defects, hypospadias and cardiac congenital defects (Shi et al., 2008; van der Zanden et al., 2012). In particular, an elevated relative risk of cleft palate in infants with the *GSTT1* null genotype has been detected and whose mothers were exposed to certain occupational chemicals (Shi et al., 2007).

Glutathione S-Transferase P (*GSTP1*)

In this research project, the genotype distribution of the *GSTP1* Ile105Val polymorphism was not significantly different between the patients and control subjects. Moreover, no adverse effect of *GSTP1* Ile105Val polymorphism in the presence of maternal exposure was observed.

Studies show that *GSTP1* is responsible for the detoxification of benzo(a)pyrene diol epoxide (Robertson et al., 1986). The presence of Ile105Val variant diminishes its enzyme activity (Ali-Osman et al., 1997; Watson et al., 1998) impairing the excretion of toxicants. Hayes and Strange have reported a significant association of valine allele with susceptibility to the development of certain tumors, including bladder, breast, lung, and multiple myeloma (Hayes and Strange, 2000). Cancer research has also found suggestive interaction between the presence of this polymorphism and risk of oral and bladder cancer, especially among smokers (Soya et al., 2007) but the results are contradictory (López-Cima et al., 2012). In addition, the Val105 allele also is reported to increase the risk of asthma and susceptibility to the effects of ozone on breathing difficulties among children with asthma (Romieu et al., 2006). These findings suggest that variable *GSTP1* expression may be an important determinant of susceptibility to environmental diseases.

GSTP1 gene is highly expressed early in fetal life and appears to be the predominant GST present in the human placenta (Ahmad et al., 1990; Beckett et al., 1990; Raijmakers et al., 2001). A recent study showed a significant association between *GSTP1* Ile105Val polymorphism and Hirschsprung disease, a common congenital intestinal defects (Gao et al., 2011). Conversely, studies on esophageal atresia, orofacial clefts and congenital heart defects have found no association between the Ile105Val variant and the disease (Ramirez et al., 2007; Filonzi et al., 2010). Recently, it has been suggested that the loss of expression of the gene *GSTP1* is caused by promoter hypermethylation in several types of cancer, such as prostate and breast cancer (Millar et al., 2000Zhang et al., 2005). The loss of *GSTP1* leads to elevated levels of electrophilic intermediates, resulting in increased DNA damage and mutations (Lee et al., 1994).

ISL1 rs1017 polymorphism *(ISL1)*

The formation of the three major cell types of the heart-cardiomyocytes, smooth muscle, and endothelial cell lineages-has been largely ascribed to a set of non-overlapping embryonic precursor derived from distinct origin. Several signaling molecules, included bone morphogenetic proteins and fibroblast growth factors, are required to initiate the cardiomyogenic program. Subsequently, a unique combinatorial subset of transcriptional factors such as GATA, HAND, and TBX interact to generate different cardiac cells types (Kelly and Buckingham, 2002). Several studies revealed that single gene mutations in different cardiac TFs could be responsible for inherited and sporadic forms of CHDs (Hatcher et al., 2003; Pulignani et al., 2011). The LIM homeodomain transcription factor *ISL1* is critically involved in embryonic cardiogenesis and is a specific marker for a distinct population of undifferentiated cardiac progenitor cells that give rise to the cardiac segments in secondary heart fielding.

ISL1 function is required for these progenitors contributing to the proliferation, survival, and migration of cardiac progenitors into the forming heart (Bu et al., 2009; Kang et al., 2009). Its absence is associated with ablation of the entire second heart field, and ISL1+ progenitor cells have been shown to form essential components of the heart, such as the atria, ventricles, coronary arteries, and the conduction system (Cai et al., 2003). Animal experimental studies have shown that both the deficiency and the mis-expression of *ISL1* might cause deep developmental defects, growth retardation and death during embryogenesis (at approximately embryonic day [ED] 10.5), thus supporting the importance of a correct regulation of *ISL1* gene expression during the fetal life (Ahlgren et al., 1997; Brade et al., 2007; Kappen and Salbaum,

2009; Golzio et al., 2012). Histological analysis of mutant hearts from murine fetuses (ED 9.0-9.5) showed that homozygous *ISL1* mutants had serious cardiac phenotypes characterized by a severe decrease in tissue mass characterized by loss of some segments (Cai et al., 2003). In addition, *ISL1*-deficient hearts fail to undergo a correct looping morphogenesis and show a common atrium and an uni-ventricular chamber (Cai et al., 2003). A very recent study describes a new gain-of-function of p.Asn252Ser variant in the human *ISL1* gene, which could potentially lead to greater activation of downstream targets involved in cardiac development, dilation, and hypertrophy (Friedrich et al., 2013). Stevens et al recently showed that two different *ISL1* haplotypes contributed to the risk of CHD in the white and black/African-American populations (Stevens et al., 2010). In particular, two specific polymorphisms, rs1017 and rs3762977, were associated with cardiac congenital defects. The rs1017 SNP (located in 3'UTR) increased the risk of CHD in the United States white population but not in black/African American populations, whereas the rs3762977 SNP (located in 5'UTR) contributed to the risk of CHDs in black/African-American population but not in the white population. Conversely, a more recent article showed that *ISL1* common variant rs1017 did not play a crucial role in conferring susceptibility to sporadic CHD in the Chinese population.

Our results are in line with Xue et al., underling the importance of additional studies to better define the association between the genetic variants in *ISL1* gene and CHD risk.

CONCLUSIONS

Our result showed that paternal smoking and exposure to toxicants for both parents, affects the risk of children with CHD and the polymorphisms in *GSTM* and *GSTT1* genes can modify a person's risk of toxicant exposure-induced disease. On the contrary, we did not find any association between the presence of polymorphism in *GSTP1* gene and the effect of positive maternal exposure to toxicants, on the risk for CHD. Similarly, we found no association between *ISL1* rs1017 polymorphism and increased risk to develop CHD.

In conclusion, our findings support the notion that cardiac development, regulated by a complex mechanism that involves the role of many different genes, is largely influenced by environmental factors.

Furthermore, understanding the biologic impact of gene-environmental interactions may provide a key insight into the prevention of these congenital malformations in future generations. Understanding the genetic basis and the molecular mechanisms of CHD may allow the identification of family members at risk as well as to identify new possible therapeutic targets and appropriate preventive strategies because environmental factors can be modified in contrast to genetic factors.

Further research based on so-called "omics" technologies, such as transcriptomic, proteomic, metabolomic, exposomic) are fundamental to improve the knowledge of the genetic defects involved in the development of CHD, and to better understand the complex interactions between genes and environment.

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Smoking and Congenital Heart Disease: The Epidemiological and Biological Link

Emilio Antonio Luca Gianicolo[1], Monica Cresci[2], Lamia Ait-Ali[2], Ilenia Foffa[2] and Maria Grazia Andreassi[2,*]

[1]*CNR, Institute of Clinical Physiology, Lecce, Italy,* [2]*CNR, Institute of Clinical Physiology, Massa and G. Monasterio Foundation, G. Pasquinucci Hospital, Massa, Italy*

Abstract: Cigarette smoking is a powerful human germ cell mutagen and teratogen. Congenital heart defects (CHD) are the most prevalent of all birth defects and leading cause of death in the first year of life. The purpose of this article is to review the epidemiology of the impact of cigarette smoking on CHD risk as well as to discuss the potential biological mechanisms of smoking–mediated abnormal cardiac development. Although epidemiological studies of association between parental smoking and CHD are limited, biological evidence supports the concept that cigarette smoking may substantially contribute to the aetiology of CHD through induction of either male and female germ-cell mutation or interference with epigenetic pathways. Further research is needed to better define the relationship between parental smoking and the risk of heart defects as well as to assess parental–fetal gene-smoking interactions.

Keywords: Congenital heart defects, parental smoking, pathogenesis.

INTRODUCTION

Congenital heart defects (CHDs), defined as "a gross structural abnormality of the hearth or intrathoracic great vessels that is actually or potentially of functional significance" [1], are the most prevalent of all birth defects and a leading cause of death in the first year of life [2]. The incidence -not the "true incidence" that would need to include occurrences among spontaneous abortions [3]- of moderate and severe forms of CHD is about 6/1,000 live births, 19/1,000 live births if the potentially serious bicuspid aortic valve is included. Moreover, the incidence of all forms increases to 75/1,000 live births if tiny muscular ventricular septal defects present at birth and other trivial lesions are included [4].

Over the past decade, experiments in several model systems have led to the identification of numerous genes and molecular events required for normal heart development [5-8]. Heart formation is a complex process that is highly conserved among vertebrates and is controlled by a network of evolutionarily conserved cardiac transcription factors (TFs) that regulate each other's expression in order to stabilize and reinforce the cardiac gene program [7,8]. CHDs are due to an abnormal development of the heart during the embryogenesis and the fetal life, between the second and the ninth week of gestation. Generally, CHDs occur on a sporadic and did not have a familial history of the disease [9].

The proportion of cases of CHD that are potentially attributable to environment is currently unknown. Attributable risk calculation suggests that the fraction of cases due to identifiable and potentially modifiable factors may be 30% for some types of defects [10].

Currently, it is believed that environmental risk factors for CHD could dominate in the aetiology of CHD, but knowledge of such factors is limited [9,11].

Definitive environmental risk factors include maternal rubella infection, pregestational diabetes and exposure to teratogens, such as thalidomide, retinoic acid and indomethacin [11].

On the contrary, information available regarding paternal environmental exposure as risk factor for CHD is very limited [11]. However, there is ample evidence of male-mediated developmental toxicity in experimental models, and some evidence of transgenerational effects, showing a particular sensitivity of the male germ line to these transmissible effects [12]. Cigarette smoking is clearly the human germ cell mutagen and teratogen with the greatest overall adverse impact on fetal development [13-15]. Tobacco smoke is known to be toxic to humans, and it is generally considered as the most extreme example of a human systemic mutagen [13]. It contains over 4,000 chemicals, many of which are genotoxic and possible carcinogens, including a complex mixture of over 4000 compounds such as nicotine, carbon monoxide and polycyclic aromatic hydrocarbons [16].

The aim of this paper is to review the epidemiology of the impact of cigarette smoking on CHD risk, as well as to discuss the potential biological mechanisms of smoking–mediated abnormal cardiac development.

SMOKING AND CHD: EPIDEMIOLOGICAL STUDIES

As already pointed out by other authors [3], the epidemiology of CHD is best known from *case-control* studies in spite of their retrospective, non-randomized nature which limits the conclusions that can be extracted from them.

Several studies reported associations between parental cigarette smoking and CHD, but there is little consensus on the real risk. In Wasserman *et al*. [17], for all conotruncal heart defects (including D-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle, truncus arteriosus) relatively elevated risks were observed for both maternal and paternal smoking when evaluated separately (Table **1**). Both parents smoking increases the risk up to 1.9 (95% CI: 1.2-3.1) and 2.5 (95% CI: 1.3-4.8), respectively. Furthermore, risk of tetralogy of Fallot was observed by the authors for involuntary maternal smoke exposures among women who did not smoke in the period from one month before through three months after conception. Paternal smoking showed associations in the absence of maternal smoking for both limb reduction defects (OR=2.1; 95%CI: 1.3-3.6) and amniotic band (OR=3.0; 95% CI: 1.3-6.9).

In the Baltimore-Washington Infant Study (BWIS), cigarette smoking in early pregnancy was associated in a dose-dependent manner with transposition of the great arteries, with ventricular septal defects (VSDs) and with pulmonic valve stenosis [18]. The effects were observed in possibly susceptible subgroups, such as older mothers and those with a history of spontaneous abortion.

Among infants with Down syndrome, Torfs and Christianson have reported associations of maternal smoking during the first trimester of pregnancy with a cardiac defect (OR=2.0; 95% CI: 1.2-3.2). [19]. An analogous relative increase in risk was observed for atrioventricular (AV) canal. For atrial septal defect/patent foramen

***Address correspondence to this author at the CNR Institute of Clinical Physiology, G. Pasquinucci Hospital, Via Aurelia Sud-Montepepe, 54100 Massa, Italy; Tel: +39 0585 493646; Fax: +39 0585 493601;
E-mail: andreas@ifc.cnr.it

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Table 1. Reported Exposures with a Risk for Specific Cardiovascular Malformations

Study, Year	Kind of Cardiovascular Malformations	Mother/Father Smoker	OR (CI 95%)
	Conotruncal heart defects (a)	Both smokers	1.9 (1.2-3.1)
	d-transposition of the great arteries	Both smokers	2.5 (1.3-4.8)
Wasserman *et al*., 1996	Tetralogy of Fallot	Maternal involuntary smoke exposure at work (b)	1.9 (1.3-6.6)
	Limb reduction defects	Father smoker	2.1 (1.3-6.6)
	Amniotic band	Father smoker	3.0 (1.3-6.9)
Ewing *et al*., 1997	Isolated membranous ventricular septal defects (VSD)	Father – marijuana use(c)	1.4 (1.1-1.8)
	Transposition of the great arteries	Mother smoker	1.3 (1.0-1.7)
	All truncus anomalies	Mother smoker	1.2 (1.0-1.5)
	Atrial septal defects	Mother smoker	1.6 (1.0-2.6)
Källen, 1999	Registered in MBR only	Mother smoker	1.3 (1.0-1.6)
	All PDA (full term)	Mother smoker	1.3 (1.1-1.6)
	All heart conditions (PDA included)	Mother smoker	1.1 (1.0-1.2)
	Grouped cardiac defects (d)	Mother smoking	2.0 (1.2-3.2)
	Atrioventricular canal	Mother smoking	2.3 (1.2-4.5)
Torfs and Christianson, 1999	Tetralogy of Fallot	Mother smoking	4.6 (1.2-17.0)
	Atrial septal defects without ventricular septal defect	Mother smoking	2.2 (1.1-4.3)
Woods S and Raju, 2001	Cardiovascular system congenital anomalies	Mother smoking	1.6 (1.1–2.2)
	Right ventricular outflow tract obstructions	Mother heavy smoker (≥25 cigarettes per day)	2.4 (1.2-4.5)
	Pulmonary valve stenosis	Mother heavy smoker (≥25 cigarettes per day)	2.3 (1.1-4.8)
		Mother light smoker (≤14 cigarettes per day)	1.4 (1.2-1.8)
	Septal defects	Mother medium smoker (15-24 cigarettes per day)	1.5 (1.1-2.0)
Malik *et al*., 2008		Mother heavy smoker (≥25 cigarettes per day)	2.1 (1.2-3.5)
	Ventricular septal defects	Mother light smoker (≤14 cigarettes per day)	1.3 (1.0-1.7)
		Mother light smoker (≤14 cigarettes per day)	2.0 (1.5-2.8)
	Atrial septal defects	Mother medium smoker (15-24 cigarettes per day)	1.8 (1.1-3.0)
	Atrioventricular septal defects	Mother medium smoker (15-24 cigarettes per day)	2.2 (1.0-4.6)

(a) Includes d-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle, truncus arteriosus
(b) Includes only women who did not smoke cigarettes in the period from 1 month before through 3 months after conception
(c) Authors recommend extreme caution in the interpretation of these results.
(d) Among infants with Down Syndrome

ovale (ASD/PFO), the OR was lower; for tetralogy of Fallot it was higher.

Källen [20] did not find any association for the total of heart malformations (OR= 1.07; 95% CI: 0.98-1.17), but mixed results in specific groups or phenotypes analysis (Table **1**).

Woods and Raju in a retrospective large cohort (N=18,016) of live births showed that maternal smoking during pregnancy was associated with cardiovascular system abnormalities [21].

Recently, Malik *et al*. reported, that maternal smoking during pregnancy man associated with septal right sided obstructive defects in a population based case-control study of 3,067 infant with nonsyndromic CHD and 3,947 infancts without birth defects [22].

Further research is, however, needed to determine whether there is a relationship between maternal smoking and risk of heart defects based on large population-based studies using more standardized case ascertainment and classification methods [11].

At the present time, there is growing concern that paternal factors may play a role in the origin of congenital defects in general and of CHD [11]. However, few studies have been conducted to evaluate the role of paternal exposures in the origin of CHD.

Paternal exposures to lifestyle substances (Marijuana) were also associated with certain malformations in the BWIS, particularly, membranous VSD [23].

Savitz *et al*. identified paternal cigarette smoking, alcohol intake, and older age on the risk of congenital cardiac anomalies, using data from 1959 to 1966 of Kaiser Health Plan members who participated in the Child Health and Development Study [24].

Although epidemiological studies of association between parental smoking and CHD are limited, biological evidence supports the concept that cigarette smoking may substantially contribute to the aetiology of CHD.

MOLECULAR MECHANISMS IN CHD AND SMOKING–MEDIATED BIOLOGICAL EFFECTS

Over the past years, human genetic studies have identified that several syndromic and familial cases of CHD are caused by single-gene mutations in cardiac TFs that regulate specific events in heart development, such as ventricular septation or outflow tract morphogenesis [7,8].

Although germline mutations have also been shown to be involved in cases of sporadic CHD, the frequency is low ranging from 0 to about 3% [6].

Therefore, the fundamental etiology of more common "sporadic" form of CHD remains unknown [25]. Recently, some studies have also provided evidence that somatic mutations and mosaicism in cardiac TF genes were present in the diseased heart tissues of patients, but absent in unaffected heart tissue of the same patients [26,27]. Somatic mosaicism refers to the condition in which a mutation arises after fertilization such that only a subset of cells or tissues harbors the defects, underlines the potential importance of environmental factors in determining genetic damage. Furthermore, accumulating evidence than also strongly suggested that alterations of the epigenetic programming may play an equally important role in generating differences in gene expression in the absence of DNA sequence variation, affecting cardiovascular development, especially in response to environmental toxicant exposure [28-31].

The most likely scenario is that the exposure to environmental toxicants, such as smoking, of both parents during the preconceptional period and 'in utero' during the pregnancy may cause CHD affecting genome and the so-called 'epigenome' (Fig. **1**).

It is plausible that smoking may exert a teratogenic effect through tobacco exposure of the mother or *via* direct toxic exposure of the seminal fluid, and spermatogenesis, thereby inducing genotoxic effects. There are many constituents of tobacco and tobacco smoke that are carcinogens, teratogenic or fetotoxic agents in laboratory animals.

Indeed, both spermatozoa and the oocytes can be vulnerable to DNA damage from the exposure to tobacco smoke [13,32]. The proportion of diploid oocytes in the ovary increases with the number of cigarettes smoked per day [33], and smoking in pregnant women is associated with an increased risk of trisomy 21 offspring [34].

Possible mechanisms of compromised oocyte quality include the presence of toxins derived from tobacco smoke in follicular fluid. The follicular fluid concentrations of the heavy metal cadmium [13], a known ovarian toxin, are higher in smokers than in nonsmokers. Women exposed to passive smoke in the home had also detectable levels of cotinine, a major metabolite of nicotine, in follicular fluid [13]. Moreover, the period of rapid embryonic and

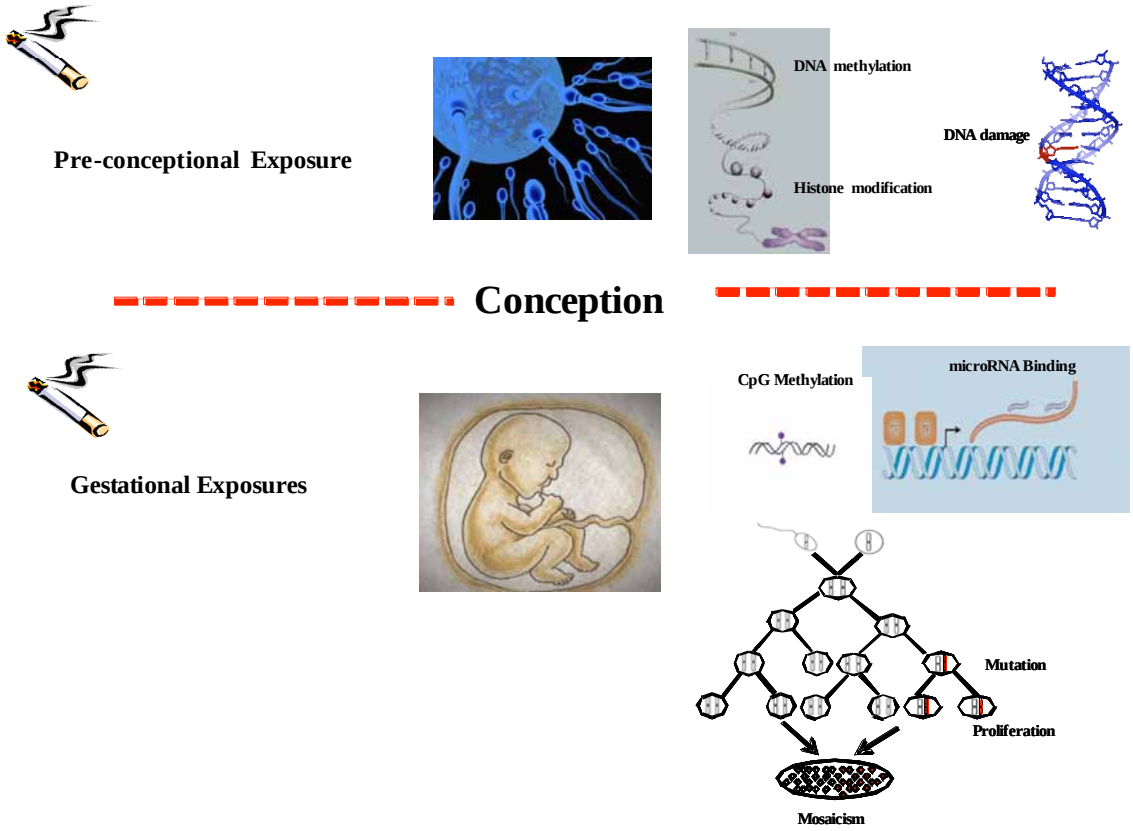

Fig. (1). Potential smoking–mediated biological effects from pre-conceptional and teratogenic exposures.

fetal development during pregnancy is associated with increased sensitivity to environmental factors, including cigarette smoking.

During this period, complex and rapid change is normal, from the molecular level through all the biochemical and physical processes that determine the course of development. Cell division, migration, differentiation, and apoptosis all must occur in the correct sequence and spatial orientation, coordinated through a large number of control and signaling systems.

Nicotine and carbon monoxide both rapidly crosses the placenta, with chronic exposure, levels of either or both in the fetal compartment exceed those in the maternal compartment [15].

A significant higher level of micronuclei (a result of chromosomal damage) has been reported in the cord blood from newborns of smoking mothers compared to non smokers [35].

Maternal smoking of 10 or more cigarettes per day for 10 or more years, including during pregnancy, is associated with increased chromosomal instability in amniocytes, expressed as an increase of structural chromosomal abnormalities and chromosomal lesions, which is not influenced by maternal age [36].

Exposure to cigarette smoke may cause epigenetic changes without changing the DNA sequence, leading to subtle changes in gene expression, resulting in an elevated predisposition to congenital anomalies [37].

Paternal exposures to tobacco smoke that cause germline DNA or chromatin damage and may affect consequently offspring health are well documented in animal model [38].

Seminal fluid of smokers contains nicotine, its metabolites, aromatic hydrocarbons, and precursors of mutagenic nitrosamines [39-42].

It has been suggested that these toxicant compounds can be adsorbed to sperm and be transmitted to a woman in the ejaculation (Fig. **2**). The contaminant is absorbed by the female, where it may reach and adversely affect a current pregnancy and, perhaps, remain in the woman's body to influence future pregnancies [43]. On the other hand, several evidences suggest that cigarette smoke constituents and their DNA-reactive metabolites gene react directly with spermatozoa [44,45].

In mice, an increased incidence of mutations was documented in spermatogonial cells of animals exposed to tobacco smoke [38]. Data also suggested that mutations accumulate with extended exposure [38].

Increased levels of major form of oxidative DNA damage, 8-hydroxydexyguanosine (8-OH-dG), are also present in spermatozoa of smokers of 20 cigarettes a day, compared with non smokers [45,46], and in dose-related association with the concentrations of cotinine in seminal plasma [47].

Importantly, paternal smoking habit has also been associated with an increased rate of DNA adducts in embryos from smokers compared to nonsmokers, indicating transmission of modified DNA originating from parental smoking [48].

However, it is now clear that people have different susceptibility to the effects of toxic agents exposure, and some individuals are more susceptible to the adverse effects of tobacco exposure than others [49]. Inheritance of particular genotypes for metabolizing systems and DNA repair pathways may modulate the effect leading to varying susceptibility to the congenital malformations of cigarette smoke [50].

Studies of gene-smoking interaction may be a promising avenue for future research on risk factors for CHD, as has been the case in other malformations, such as oral cleft defects [50].

Therefore, these evidences support the concept that cigarette smoking may substantially contribute to the etiology of CHD through induction of germ-cell mutation or interference with epige-

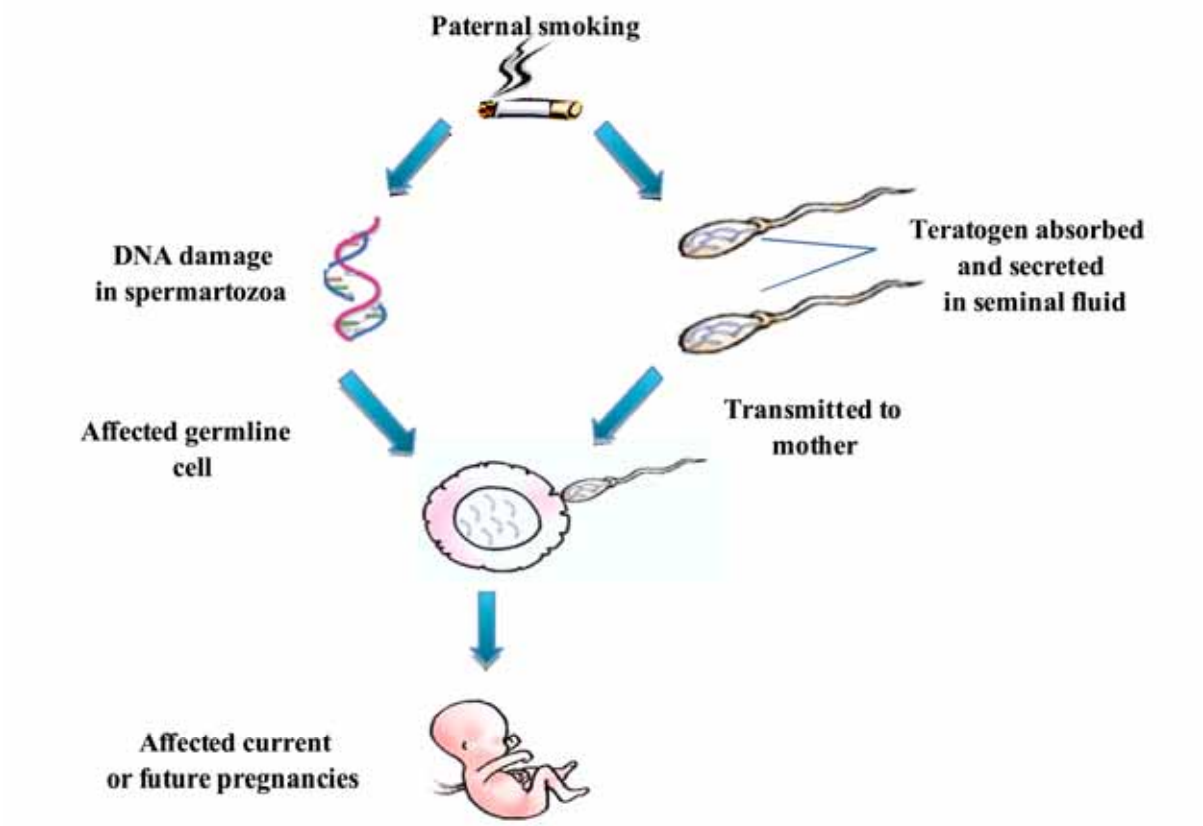

Fig. (2). Schematic pathways of sperm-mediated effects on congenital risk.

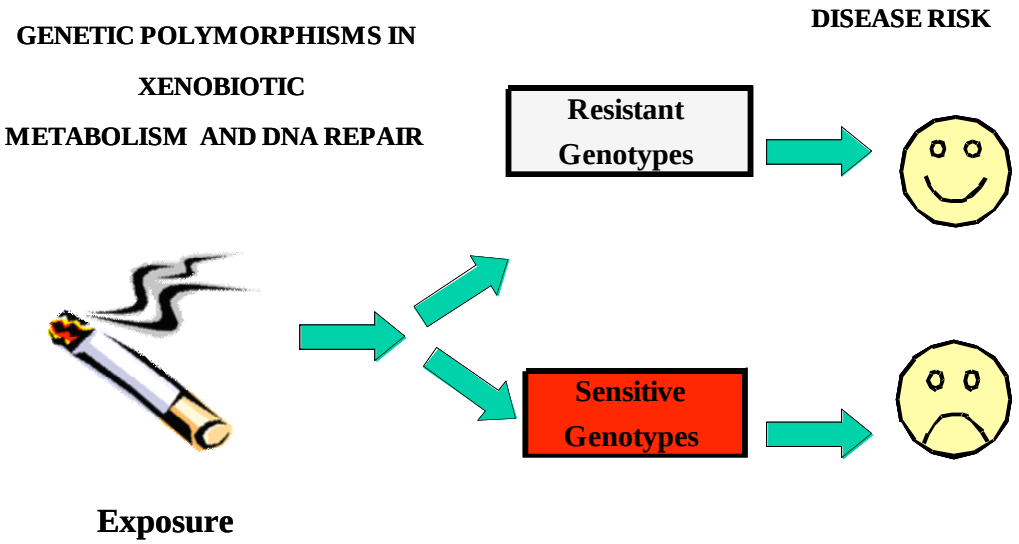

Fig. (3). The smoking exposure–disease model: parental and fetal polymorphisms can modify the individual risk for disease.

netic pathways. In this scenario, parental–fetal polymorphisms in environmental response genes can modify a person's risk for smoking exposure-disease (Fig. **3**).

CONCLUSION

Despite the presence of known mutagens and toxicant in cigarette smoke, there is currently no clear evidence to show that exposure to cigarette smoke, can result in heritable genetic mutation and increase the risk of CHD.

Further research is needed to better understand the epidemiological impact of tobacco-related on cardiac development, as well as to obtain advance knowledge in disease-causing mechanisms related to smoking exposure. Exploring CHD-smoking effect may also provide an excellent model in order to better understand the role of environmental factors to CHD, especially aimed at studying the role of paternal exposures on CHD risk.

A better understanding of the molecular basis of CHD as well as the examination of the influence of gene-environment interactions is expected to facilitate the prevention of CHD and as to improve genetic counselling and care of affected individuals and their families.

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Maternal and Paternal Environmental Risk Factors, Metabolizing GSTM1 and GSTT1 Polymorphisms, and Congenital Heart Disease

Monica Cresci, MSc[a], Ilenia Foffa, MSc, PhD[a], Lamia Ait-Ali, MD, PhD[a], Silvia Pulignani, MSc[a], Emilio Antonio Luca Gianicolo, MSc, PhD[a], Nicoletta Botto, MSc, PhD[b], Eugenio Picano, MD, PhD[a], and Maria Grazia Andreassi, MSc, PhD[a,*]

Congenital heart defects (CHDs) are the most prevalent of all birth malformations arising from the complex interplay of environmental exposures and genes. Modifiable environmental risk factors are still largely unknown, especially for paternal exposure. The aim of the present study was to examine the association between the environmental exposures of both parents and CHD risk and to explore the modification effect of metabolizing gene polymorphisms in children who lack the genetic capacity to produce the glutathione S-transferase (GST) GSTM1 and GSTT1 enzymes. A total of 330 parents of a child with CHD and 330 parents of a child without any congenital malformations were compared in terms of lifestyle habits and toxicant exposure. GST gene polymorphisms were investigated in 180 patients with CHD (104 males, age 4.9 ± 5.8 years). Paternal smoking (≥15 cigarettes/day) was significantly associated with CHD risk (odds ratio [OR] 2.1, 95% confidence interval [CI] 1.3 to 3.5, p = 0.002). Both maternal (OR 2.6, 95% CI 1.6 to 4.2, p <0.0001) and paternal (OR 2.5, 95% CI 1.6 to 3.8, p <0.0001) occupational/environmental exposures increased the risk of CHD. Also, a significant additive risk (OR 4.5, 95% CI 2.5 to 8.3, p <0.0001) was found when both parents were exposed to toxicants. Both maternal (OR 3.6, 95% CI 1.1 to 11.2, p = 0.03) and paternal (OR 3.3, 95% CI 1.0 to 10.8, p = 0.03) exposure to toxicants increased the CHD risk in children who carried the combined null GST genotypes. The effect for the combined null GST genotypes was also stronger (OR 6.5, 95% CI 1.5 to 28.0) when both parents were exposed. In conclusion, paternal smoking and exposure to toxicants for both parents affect the risk of children with CHD. Polymorphisms in GST genes can modify a person's risk of toxicant exposure-induced disease. **(Am J Cardiol 2011;108: 1625–1631)**

Congenital heart defects (CHDs) are due to an abnormal development of the heart during embryogenesis and fetal life, between the second and ninth week of gestation.[1,2] Generally, CHDs occur on a sporadic basis and do not have a familial history.[3,4] Most CHDs are thought to have a multifactorial origin, with an interplay of genetic and environmental effect. It has recently been suggested that environmental risk factors could dominate in the etiology of CHD[3]; however, knowledge of such factors is very limited, especially for paternal exposure.[4]

Furthermore, the effect of environmental factors might be modified by the genes responsible for the activation and detoxification of toxicant agents, contributing to an increased resistance (or sensitivity) to cardiac teratogenesis.[2,5] In particular, metabolizing genetic polymorphisms of the glutathione S-transferase (GST) enzymes, which provide critical defense against numerous toxins, might modulate the effects of the toxic agent.[5,6]

In the present study, we used a nested case-control design to examine the association between the environmental risk factors of both parents and CHD risk and to explore the modification effect of genetic susceptibility in children who lacked the genetic capacity to produce the GSTM1 and GSTT1 enzymes.

Methods

A prospective and paired case-control study (1:1) was conducted from October 2008 to April 2010 by inviting 360 parents of a child with isolated CHD and 360 parents of a child without any congenital malformations to participate. The study subjects were the parents of pediatric patients who had been admitted to our pediatric cardiac center and the parents of a healthy child who had been recruited in collaboration with the maternity and pediatric units. The matching criteria for the controls were the absence of a congenital malformation and age compatible with the observed age range of the patients. The exclusion criteria were mothers who reported inconsistent use of B vitamin and folate supplements in the periconception period; and an inability to obtain complete information about the occupational, demographic, and lifestyle data for both parents. The

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*Corresponding author: Tel: (+39) 0585-493-646; fax: (+39) 0585-493-601.

E-mail address: andreas@ifc.cnr.it (M.G. Andreassi).

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Table 1
Baseline characteristics of the study population

Variable	Cases	Controls	p Value
Mother			
Age (years)	30 ± 5.4	31 ± 4.8	0.01
History of chronic illness	21 (12%)	26 (14%)	0.4
Spontaneous miscarriage	34 (19%)	40 (22%)	0.4
Father			
Age (years)	33 ± 6.0	34 ± 5.1	0.3
History of chronic illness	29 (16%)	33 (18%)	0.6
Offspring			
Male gender	104 (58%)	105 (58%)	0.9
Birth weight (g)	3,178 ± 630	3,340 ± 550	0.01
Artificial fertilization	6 (3.3%)	1 (0.6%)	0.05

Table 2
Parental exposure to environmental risk factors and CHD risk

Variable	CHD Parents (n = 360)	Control Parents (n = 360)	OR (95% CI)	p Value
Active smoking (positive)				
Mother	50 (27.8%)	45 (25.0%)	1.2 (0.7–1.8)	0.6
Father	81 (45.0%)	65 (36.1%)	1.7 (1.1–2.6)	0.02
Diagnostic x-ray exposure (positive)				
Mother	49 (27.2%)	38 (21.1%)	1.3 (0.8–2.2)	0.2
Father	45 (25.0%)	37 (20.6%)	1.3 (0.8–2.1)	0.3
Heavy drinking (positive)				
Mother	1 (0.6%)	1 (0.6%)	1.0 (0.1–16)	0.99
Father	7 (3.9%)	7 (3.9%)	1.0 (0.3–2.9)	0.99
Occupational/ environmental exposure (positive)				
Mother	70 (38.9%)	34 (18.9%)	2.6 (1.6–4.2)	<0.0001
Father	88 (48.9%)	49 (27.2%)	2.5 (1.6–3.8)	<0.0001

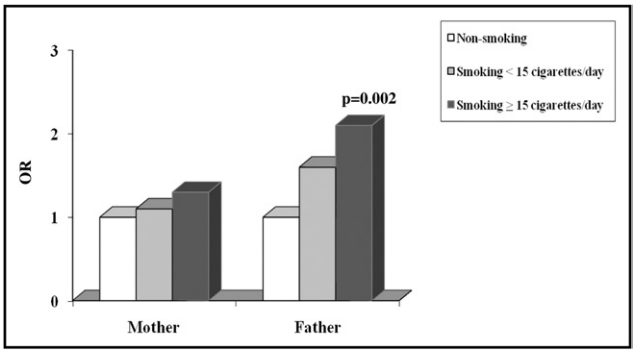


Figure 1. Parental smoking and CHD risk.

present study was conducted with informed consent of every participant, and the local ethical research committee approved it.

Both case and control parents completed a structured questionnaire on the demographic, preconceptional, and lifestyle exposures. We derived environmental and occupational exposure data from specific questions on potential

Table 3
Occupational/environmental exposure among congenital heart defect (CHD) and control parents

Variable	CHD Parents	Control Parents	p Value
Maternal occupational exposure			0.004
Risk factor (n)	28 (15.5%)	11 (6.1%)	
Paint/varnish/thinner/solvents	9	0	
X-rays (hospital workers)	4	2	
Cleaning agents and solvents	4	1	
Industrial chemicals and solvents	4	1	
Hair dye	2	2	
Textile dye	2	0	
Chemical laboratory exposure	1	1	
Asbestos	1	0	
Pesticides	1	0	
Anesthetic gas	0	4	
Paternal occupational exposure			0.003
Risk factor (n)	60 (33.3%)	41 (22.8%)	
Paint/varnish/thinner/solvents (shoe factory)	14	4	
Cement dust	15	8	
Industrial chemicals and solvents	11	6	
Hair dyes	1	1	
Cleaning agents and solvents	1	1	
X-rays (hospital workers)	1	4	
Textile dye	1	0	
Exhaust and welding fumes	2	3	
Chemical laboratory exposure	1	0	
Asbestos	6	1	
Heavy metals	4	5	
Pesticides	3	1	
Anesthetic gas	0	7	
Maternal environmental exposures			0.01
Risk factor (n)	45 (25.0%)	25 (13.9%)	
Waste sites	9	0	
Thermal power plant	8	1	
Industrial pollution	9	5	
Electromagnetic field	4	0	
Environmental pollution	3	3	
X-rays (radiotherapy; Chernobyl)	2	0	
Asbestos	1	1	
Pesticide sites	5	0	
Drugs	4	15	
Paternal environmental exposure			<0.0001
Risk factor (n)	40 (22.2%)	12 (6.7%)	
Waste sites	9	0	
Thermal power plant	9	0	
Industrial pollution	8	5	
Electromagnetic field	3	0	
Environmental pollution	3	0	
Asbestos	0	1	
Pesticide sites	5	0	
Drugs	3	6	

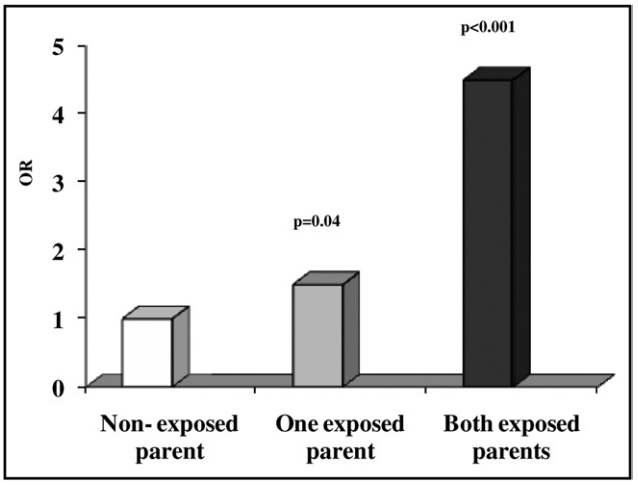


Figure 2. Interactive effect between maternal and paternal exposures on CHD risk.

teratogens/mutagens that have been linked to human reproductive impairment, including ionizing radiation, solvents, pesticides, asbestos, and heavy metals.

The paternal questionnaire carefully evaluated the 3-month period before conception, according to the duration of spermatogenesis (about 70 days). The maternal questionnaire covered the period before conception to the end of the first trimester of pregnancy. The first trimester of pregnancy was chosen because that it is most critical period for embryogenesis associated with increased sensitivity to environmental factors. The maternal questionnaire gathered information on personal characteristics and lifestyle habits, health, pregnancy aspects, medication during pregnancy, occupational, and other potential harmful exposures (e.g., diagnostic radiation exposure). The paternal questionnaire collected data on personal characteristics, health, occupational history, and other potential harmful exposures (e.g., diagnostic radiation exposure). Parental illness data included diseases of the thyroid, kidney, liver, gut, stomach, pancreas, gastrointestinal, respiratory, and urinary disorders.

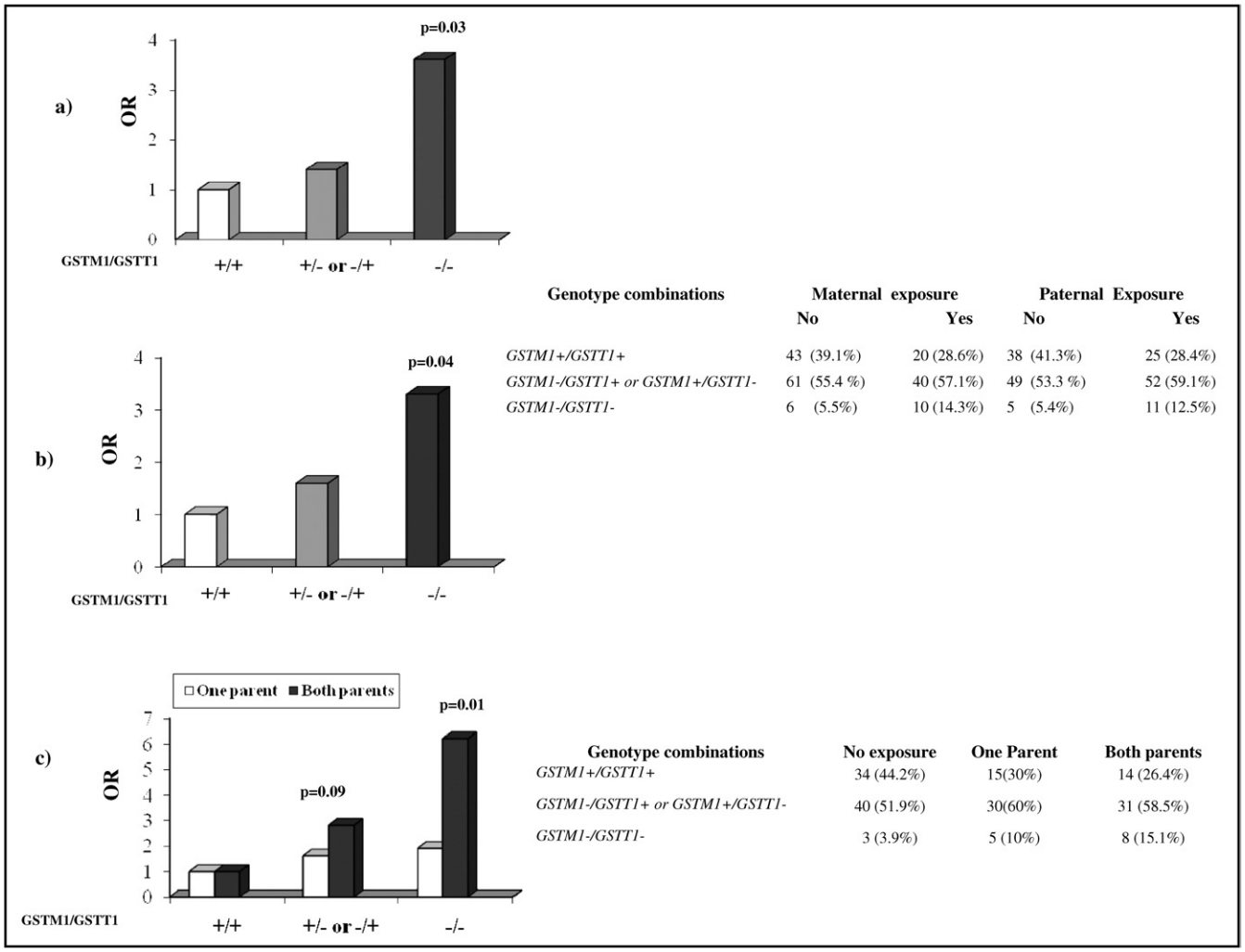
Using a case-only design, DNA was obtained from 180 patients with CHD (104 males, age 4.9 ± 5.8 years). Genotyping analysis was performed by our laboratory staff, who were unaware of the clinical data. Genomic DNA was extracted from peripheral blood leukocytes. The GSTM1 and GSTT1 genotypes were determined using a co-amplification polymerase chain reaction approach with the GSTM4 gene, which is never deleted, as the internal control to distinguish the null genotypes from aborted polymerase chain reaction. Primer sequences, annealing temperatures, and digest conditions were performed according to previously published protocols.[7] The internal standard fragment amplified from the GSTM4 gene was 157 bp. A 230-bp fragment was amplified for the GSTM1 gene, and a 480-bp fragment was obtained for the GSTT1 gene. The absence of amplified products was consistent with the null genotypes.

For statistical analysis, all determinants were dichotomized, except for the age variables. We considered as smokers those who smoked ≥3 cigarettes/day at conception; exsmokers were those who had stopped smoking ≥6 months before study inclusion; and nonsmokers were those who had never smoked. The smokers were also divided into groups according to smoking level: medium smokers (3 to 14 cigarettes/day) and heavy smokers (≥15cigarettes/day). The consumption frequency of alcoholic beverages (beer, wine, liquor) was classified in drinkers who drank ≥3 alcoholic drinks/day (beer, wine, liquor) and nondrinkers, who drank <3 drinks/day.

Given the relatively small number of exposed parents in any particular environmental and occupational category, statistical analysis for toxicant exposure was performed, including both environmental and occupational exposure to x-rays, chemicals, anesthetics, industrial cleaning agents and solvents, exhaust and welding fumes, paint/varnish/thinner, asbestos, heavy metals, and pesticides.

Table 4
Gene–environment interactions for combination of glutathione S-transferase (GST) genes and parental exposure

Positive Parental Exposure	GSTM1		OR (CI 95%)	p Value
	Present	Absent		
Mother				
Active smoking				0.3
Negative	54	61	1.0	
Positive	19	31	1.4 (0.7–2.9)	
Diagnostic x-ray exposure				0.1
Negative	61	70	1.0	
Positive	17	32	1.6 (0.8–3.2)	
Other toxicant exposures				0.2
Negative	52	58	1.0	
Positive	26	44	1.5 (0.8–2.9)	
Father				
Active smoking				0.8
Negative	34	45	1.0	
Positive	36	45	1.0 (0.5–1.8)	
Diagnostic x-ray exposure				0.6
Negative	60	75	1.0	
Positive	18	27	1.2 (0.6–2.4)	
Other toxicant exposures				0.1
Negative	45	47	1.0	
Positive	33	55	1.6 (0.9–2.9)	
	GSTT1			
Mother				
Active smoking				0.1
Negative	93	22	1.0	
Positive	45	5	0.5 (0.2–1.3)	
Diagnostic x-ray exposure				0.9
Negative	109	22	1.0	
Positive	41	8	1.0 (0.4–2.3)	
Other toxicant exposures				0.08
Negative	96	14	1.0	
Positive	54	16	2.0 (0.9–4.5)	
Father				
Active smoking				0.4
Negative	63	16	1.0	
Positive	69	12	0.7 (0.3–1.4)	
Diagnostic x-ray exposure				0.1
Negative	109	26	1.0	
Positive	26	4	0.4 (0.1–1.2)	
Other toxicant exposures				0.09
Negative	81	11	1.0	
Positive	69	19	2.0 (0.9–4.5)	



Genotype combinations	Maternal exposure		Paternal Exposure	
	No	Yes	No	Yes
GSTM1+/GSTT1+	43 (39.1%)	20 (28.6%)	38 (41.3%)	25 (28.4%)
GSTM1-/GSTT1+ or GSTM1+/GSTT1-	61 (55.4 %)	40 (57.1%)	49 (53.3 %)	52 (59.1%)
GSTM1-/GSTT1-	6 (5.5%)	10 (14.3%)	5 (5.4%)	11 (12.5%)

Genotype combinations	No exposure	One Parent	Both parents
GSTM1+/GSTT1+	34 (44.2%)	15(30%)	14 (26.4%)
GSTM1-/GSTT1+ or GSTM1+/GSTT1-	40 (51.9%)	30(60%)	31 (58.5%)
GSTM1-/GSTT1-	3 (3.9%)	5 (10%)	8 (15.1%)

Figure 3. ORs stratified by children's GST genes and *(A)* maternal, *(B)* paternal, and *(C)* parental occupational/environmental exposure to toxicants.

Statistical analyses of the data were conducted using the StatView statistical package, version 5.0.1 (Abacus Concepts, Berkeley, California). Data are expressed as the mean ± SD. Differences between the mean values of 2 continuous variables were evaluated using the unpaired Student *t* test. Differences in noncontinuous variables and genotype distribution were tested using chi-square analysis. Unconditional logistic regression analysis was used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) for the association between CHD and parental exposure. The ORs were also adjusted for potential confounding factors. A 2-tailed p value <0.05 was chosen as the level of significance. In the genetic study, we used a case-only study design because it is considered the most powerful method to study gene–gene and gene–environment interaction in disease etiology, achieving greater statistical power than a case-control study of the same size.[8] With independence assumed between the exposure and the genotype, case-only ORs for the relevant interactions and 95% CIs were estimated by using the presence or absence of the GST variant as the dependent dichotomous variable in the logistic regression models that tested for an association with parental exposure. The study was powered to allow an interaction of magnitude of 2.3 with ≥80% power and an error rate of 5% under a sample size of 170 only-cases.[8]

Results

The demographic characteristics of the study population are listed in Table 1. The mothers of the patients with CHD had a significantly lower age than the controls ($p = 0.01$). No significant differences between the 2 groups were found for paternal characteristics. The CHD cases had a significantly lower birth weight ($p = 0.01$) compared to the control children. The children with CHD were also more often conceived with artificial fertilization ($p = 0.05$).

The parental exposure to environmental factors and CHD risk is listed in Table 2. Maternal smoking was not significantly associated with an increased risk of CHD (OR 1.2, 95% CI 0.7 to 1.8). Fathers who were smokers showed an increased risk of CHD in their offspring (OR 1.7, 95% CI 1.1 to 2.6, $p = 0.02$), especially fathers who were heavy smokers (≥15 cigarettes/day) had a high risk of having children with CHD (Figure 1). Parental alcohol use did not influence the risk of CHD, and neither did the existence of a chronic disease or disease in the first trimester of preg-

nancy. Exposure to diagnostic radiographs was more frequently seen among the case-parents. Both maternal (OR 2.6, 95% CI 1.6 to 4.2, p <0.0001) and paternal (OR 2.5, 95% CI 1.6 to 3.8, p <0.0001) occupational/environmental exposure to toxicants increased the risk of CHD. The specific occupational and professional risk factors for CHD are listed in Table 3. In addition, the exposure of both parents to toxicants showed a significant additive risk (OR 4.5, 95% CI 2.5 to 8.3, p <0.0001) of CHD in the offspring (Figure 2). On multivariate analysis, a father's heavy smoking (OR 1.9, 95% CI 1.1 to 3.3) and maternal exposure (OR 2.1, 95% CI 1.2 to 3.6) and paternal exposure (OR 1.8, 95% CI 1.1 to 3.0) to toxicants were the main determinants of CHD risk. In the case-only study, we considered the association between GST polymorphisms in children and parental exposure. The frequency of GSTM1 null genotype was 57.7%, and the frequency of the GSTT1 null genotype was 16.7%. The genotype distribution was within the range previously reported for GSTM1 and GSTT1 null genotypes in other white populations.[8–10] We did not detect an interactive effect of GSTM1 or GSTT1 null genotypes and active maternal and paternal smoking (Table 4). The risk of CHD tended to be greater in children carrying the GSTM1 and GSTT1 null genotypes who had parents exposed to toxicants. A significant interaction was found for the combined null GSTs and both maternal (OR 3.6, 95% CI 1.1 to 11.2, p = 0.03) and paternal (OR 3.3, 95% CI 1.0 to 10.8, p = 0.03) exposure to toxicants (Figure 3). Finally, children with the combined null GST genotypes had a greater risk (OR 6.5; 95% CI 1.5 to 28.0) than children carrying wild-type GST genes when both parents were exposed (Figure 3).

Discussion

Our study showed that paternal smoking and exposure to toxicants for both parents affect the risk of children with CHD, supporting the hypothesis of the pivotal influence of the environmental risk factors for congenital malformations.[9] In addition, our gene-environment analyses suggested that null GSTs genes can modify a person's risk of toxicant exposure-induced disease. Few epidemiologic studies have investigated the association between parental exposure and CHD risk. The best available information comes from the Baltimore-Washington Infant Study (BWIS), conducted in the Baltimore-Washington area from 1981 to 1989, and the study conducted in Finland by the National Public Health Institute in Helsinki of cases and controls born from 1982 to 1984.[10,11] The maternal environmental risk factors known to influence the incidence of CHD include rubella, pregestational diabetes, exposure to teratogens, such as thalidomide, retinoic acid, and indomethacin, and exposure to chemicals at work.[3] In particular, maternal occupational exposure to solvent-containing products, mineral oil products, dyes, lacquers, paints, and pesticides has been associated with an increased risk of CHD.[2] A number of studies have also investigated maternal cigarette smoking and congenital heart disease, providing controversial results, probably owing to variations in the method, disease categorization, sample size, or confounding bias.[10,12–14] In the BWIS, cigarette smoking in early pregnancy was associated in a dose-dependent manner with transposition of the great arteries, ventricular septal defects, and pulmonary valve stenosis.[10] In a population-based case-control study of 3,067 infants with nonsyndromic CHD and 3,947 infants without birth defects, the association was stronger for mothers who reported heavier tobacco consumption (≥25 cigarettes/day) during pregnancy.[15] This dose–effect relation was confirmed in a recent case–control study of 157 neonates diagnosed with CHD at the University of Patras Medical School and 208 normal neonates.[16] Few studies have evaluated the effect of paternal exposure to environmental factors in the preconception period. Specifically, paternal exposures to lifestyle substances (marijuana), paint stripping, and ionizing radiation exposure have been associated with certain malformations in the BWIS.[17,18]

An association between parental smoking, particularly when both parents were smokers, and the risk of conotruncal heart defects, including D-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle, and truncus arteriosus, has also been reported.[19] A very recent study found a more than fivefold increase in risk mortality and deaths due to congenital malformation, especially for cardiac defects, for paternal occupational exposures, possibly to organic solvents during preconception.[20]

In addition, recent studies have revealed that paternal smoking and occupational exposure are risk factors for congenital defects, such as orofacial and anorectal malformations,[21,22] supporting the existing data on the great vulnerability of the male reproductive system to environmental exposures.

At present, societal concern is growing about the particular sensitivity of the male germ line to genetic transmissible effects[9,23] that might play a crucial role in the origin of congenital defects in general and CHD in particular.[5] It is has been suggested that male exposure might exert a teratogenic effect through toxicant compounds adsorbed to the sperm and transmitted to a woman in the ejaculate. The contaminant is absorbed by the women, where it might reach and adversely affect a current pregnancy and, perhaps, remain in the woman's body to influence future pregnancies.[9] In contrast, toxicant compounds might act in human seminal fluid as endocrine disrupting agents causing direct germ line DNA damage or epigenetic changes.[9,23] Teratogenic, carcinogenic, and endocrine disrupting agents, such as pesticide residues, heavy metal organic solvents (benzene, toluene, and xylene), nicotine, aromatic hydrocarbons, and precursors of mutagenic nitrosamines, have all been detected in human seminal fluid.[9,23,24]

Recent studies also showed that these environmental toxicants can induce oxidative DNA damage, mutations, and chromosomal aberrations, such as DNA strand breaks and aneuploidy, in human seminal fluid.[9,23,24] Therefore, it is plausible that exposure to toxicant agents of both parents during the preconceptional period and "in utero" during pregnancy might have a pivotal role in the pathogenesis of CHD affecting genome and the so-called epigenome.[9] With regard to malfunctions in the epigenetic mechanisms, our findings also support recent evidence that have demonstrated an increased risk of cardiovascular birth defects associated with in vitro fertilization resulting from epige-

netic modifications in the early stage of the development of the embryos produced by in vitro fertilization.[25]

Actually, evidence is increasing that people have different susceptibility to the effects of toxic agents, with some more susceptible to the adverse effects of environmental toxicants than others.[5,6]

Specifically, the inheritance of particular genotypes for metabolizing systems and DNA repair pathways might modulate the effect, leading to varying susceptibility to the congenital malformations of toxicants.[5,6]

In particular, the GSTs are a polymorph supergene family of detoxification enzymes that are involved in the metabolism of numerous toxins and provide critical defense against drugs and industrial chemicals.[26] GSTM1 and GSTT1 are the most extensively studied genes in the GST gene superfamily. There has been some, albeit contradictory, evidence of their enzymatic expression during the early stages of embryonic and fetal development in most tissues, including the heart.[27,28] Deletion of the GSTM1 and GSTT1 genes, resulting in loss of functional activity, has been reported in approximately 50% and 20% of the white population, respectively, which might predispose to greater health effects from toxic xenobiotics.[26] Previous studies reported that deletion in the GSTM1 and GSTT1 genes might contribute to the development of congenital malformations, such as oral cleft defects.[6] In particular, an elevated relative risk of cleft palate in infants with the GSTT1 null genotype has been detected and whose mothers were exposed to certain occupational chemicals.[28] This is the first study to shown that GST polymorphism might mediate the risks of parental exposure to toxicants for CHD, supporting the need for future research on the gene–environment interactions and CHD risk.[2]

Some important limitations must be considered when interpreting the results of our study. First, all the risk factors studied were self-reported; thus, random misclassification of exposure and recall bias could not be excluded. Some variables might be prone to over- and underestimation, thereby affecting the risk estimates. Regardless, although recall bias can never be excluded, evidence is emerging that its role is considered rather small and merely nondifferential in case-control studies focused on congenital malformations. Second, no exposure dose or biomarker of exposure was available. In addition, our study discriminated only between carriers (GST presence) and noncarriers (GST absence) and did not investigate a gene dosage effect determined by copy number variation (from 0 to 2 gene copies per allele per gene) on the risk of CHD. Finally, an undoubted limitation of our study was that the modest size of our population might have limited some associations between parental exposure and genetic polymorphisms CHD risk. It is possible that unknown confounders might have contributed to the risks we observed. However, some of our results, especially for paternal exposure, confirm well-documented effects in animal models,[29] and they are supported by the strong biologic plausibility.

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ORIGINAL ARTICLE

Maternal Environmental Exposure, Infant *GSTP1* Polymorphism, and Risk of Isolated Congenital Heart Disease

Monica Cresci · Ilenia Foffa · Lamia Ait-Ali · Silvia Pulignani · Alessandra Kemeny · Emilio Antonio Luca Gianicolo · Maria Grazia Andreassi

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Abstract The *GSTP1* gene, highly expressed early in fetal life, is the most abundant phase 2 xenobiotic metabolism enzyme in a human placenta. Fetal inherited *GSTP1* Ile105Val polymorphism may modify the metabolism and excretion of xenobiotics from fetal tissue and increase the risk of congenital heart disease (CHD). This study aimed to analyze the joint effects of *GSTP1* genetic polymorphism (Ile105Val) and maternal environmental exposure on CHD risk. Within a case-control design, a total of 190 children with CHD (104 boys age 4 ± 5.6 years) and 190 healthy children (114 newborn boys) were genotyped for the *GSTP1* Ile105Val polymorphism. Mothers completed a structured questionnaire on the demographics as well as the preconceptional and lifestyle exposures. A higher frequency of mothers of children with CHD (38 %) reported a positive history of exposure to toxicants (occupational and environmental) than mothers of healthy children (23 %) ($p = 0.0013$). Logistic regression analysis showed that maternal occupational and environmental exposures increased the risk of CHD (odds ratio, 2.6; 95 % confidence interval, 1.6–4.2; $p < 0.0001$). No significant differences in Ile105Val genotype frequencies were observed between the children with CHD and the healthy children ($p = 0.9$). Furthermore, case-control analysis showed no evidence of significant interaction between the maternal exposures and *GSTP1* polymorphism. Maternal exposure to toxicants increased the risk of children with CHD. However, fetal *GSTP1* Ile105Val polymorphism did not increase the risk of CHD.

Keywords Congenital heart disease · *GSTP1* polymorphism · Maternal exposure · Xenobiotic metabolism enzymes

Monica Cresci and Ilenia Foffa contributed equally to the study.

M. Cresci · I. Foffa (✉) · L. Ait-Ali · S. Pulignani · M. G. Andreassi
Unità di Ricerca Genetica, G. Pasquinucci Hospital, CNR Istituto di Fisiologia Clinica, Via Aurelia Sud-Montepepe, 54100 Massa, Italy
e-mail: ilenia.foffa@ifc.cnr.it

A. Kemeny
U.O. Ostetricia e Ginecologia, Dipartimento Materno Infantile, Az. USL1 Carrara, Via Aurelia Sud, 54100 Massa, Italy

E. A. L. Gianicolo
CNR Istituto di Fisiologia Clinica, c/o Campus Universitario Ecotekne, Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy

Human embryonic development is determined by both maternal environmental exposure and fetal genetic properties, which in turn depend on maternal genetic attributes and behaviors [23]. In fact, fetuses are more sensitive than adults to the toxicity of many environmental toxicants [26]. In particular, the placenta has many physiologic functions in addition to the metabolism of xenobiotics [18].

Several studies have shown that maternal exposure is associated with a variety of adverse pregnancy outcomes including preterm birth, low birth weight, and birth defects [4, 6, 13, 14, 23]. Among the birth defects, congenital heart disease (CHD) is the most common type and the leading noninfectious cause of death in the first year of life [9, 12].

The impact of maternal exposure on childhood birth defects risk may be modulated by the presence of polymorphisms in genes involved in the detoxification pathways. Especially metabolizing genetic polymorphisms of the glutathione S-transferase (GST) enzymes, which provide a critical defense against numerous toxins, may

modulate the effect of the toxic agent [14, 23]. Accordingly, our recent study showed an interactive effect of *GSTM1* or *GSTT1* null genotypes on children with CHD and maternal and paternal exposures [6].

Eight cytosolic GSTs are known to be expressed in humans: alpha (A), mu (M), pi (P), theta (T), kappa (K), omega (O), sigma (S), and zeta (Z). Each class consists of several distinct subclasses, with some overlap in tissue expression [11].

The *GSTP1* gene, highly expressed early in fetal life, is the most abundant phase 2 xenobiotic metabolism enzyme in a human placenta [1, 3, 19]. An A to G transition at nucleotide 313 in exon 5 of the *GSTP1* gene, which replaces isoleucine (*Ile*) at codon 105 with valine (*Val*) within the active site of the enzyme, has been shown to result in altered enzyme activity [29]. Therefore, fetal inherited *GSTP1* Ile105Val polymorphism may modify the metabolism and excretion of xenobiotics capable of crossing the placental barrier from fetal tissue.

This study aimed to determine the prevalence of *GSTP1* Ile105Val polymorphism in young patients with CHD and in a group of healthy children. In addition, we applied a case-only design to analyze the joint effects of the *GSTP1* Ile105Val polymorphism and maternal environmental exposure on CHD risk.

Materials and Methods

Subjects and Epidemiologic Information

This case-control study included 190 patients (104 boys and 86 girls) with a diagnosis of isolated and nonsyndromic CHD admitted at our pediatric cardiac center and a control group of 190 newborn healthy children (114 boys and 76 girls) recruited in collaboration with the maternity and pediatric units.

All the mothers completed a structured questionnaire on demographics as well as preconceptional and lifestyle exposures. We derived environmental and occupational exposure from specific questions on potential teratogens/mutagens that have been linked to human reproductive impairment such as ionizing radiation, solvents, pesticides, asbestos, and heavy metals.

The questionnaire gathered information on personal characteristics, lifestyle habits, health, pregnancy aspects, medication during pregnancy, occupational history, and other potential harmful exposures (e.g., radiation diagnostic exposure). Maternal illness included diseases of the thyroid, kidney, liver, gut, stomach, pancreas, and heart as well as gastrointestinal, respiratory, and urinary disorders.

This study was conducted with the informed consent of every participant and approved by the local Ethical Research Committee.

GSTP1 Genotyping

We obtained DNA from the CHD patients and the control children. Genotyping analysis was performed by our laboratory staff, who were unaware of the clinical data. Genomic DNA was extracted from whole-blood samples, and a polymerase chain reaction (PCR)-restriction fragment length polymorphism method was used to determine the allele distribution for *GSTP1*.

The PCR mixture (50 µl) was prepared containing 50 ng of DNA, 5 µl of buffer (500 mmol/l KCl/100 mmol/l Tris-HCl, pH 8.3/15 mmol/l $MgCl_2$), 1 µl of 2.5 mmol/l deoxynucleotide triphosphates, and 2.5 µl each of the forward primer 5′-ACC CCA GGG CTC TAT GGG AA-3′ and the reverse primer 5′-TGA GGG CAC AAG AAG CCC CT-3′ and 1 U of *Taq* polymerase.

The PCR condition involved denaturing at 94° for 5 min, followed by 32 cycles of denaturing at 94° for 30 s, annealing at 64° for 50 s, elongation at 72° for 50 s, and finally incubation at 72° for 10 min to allow a complete extension of all PCR fragments. The 176-bp amplified *GSTP1* gene fragment was subjected to restriction digestion in a 15-µl reaction volume containing 7.5 µl of PCR product, 100 mmol/l of NaCl, 50 mmol/l of Tris-HCl, pH 7.9, 10 mmol/l of $MgCl_2$, 1 mmol/l of dithiothreitol, and 5 U of BsmAI at 55° overnight.

The digested PCR products were separated by electrophoresis using 8 % polyacrylamide gel and stained with ethidium bromide (10 mg/ml). The genotype was determined by analysis of the bands on the gel as follows: homozygous wild-type for isoleucine (II), one band (176 bp); homozygous mutated for valine (VV), two bands (91 and 85 bp); and heterozygous (IV), three bands (176, 91, and 85 bp).

Statistical Analyses

Statistical analyses of the data were conducted with the Statview statistical package, version 5.0.1 (Abacus Concepts, Berkeley, CA, USA). Data are expressed as mean ± standard deviation.

For statistical analyses, all determinants were dichotomized except for the age variable. Smokers were classified as individuals who smoked at least three cigarettes per day at the time of the conception, ex-smokers as those who stopped smoking at least 6 months before inclusion in the study, and nonsmokers as persons who never smoked. Smokers also were divided into medium smokers (3–14 cigarettes a day) and heavy smokers (≥15 cigarettes a day).

For consumption frequency of alcoholic beverages (beer, wine, liquor), drinkers were classified as those who drank three or more alcoholic drinks per day (beer, wine, liquor) and nondrinkers as those who drank less than three drinks per day.

Given the relatively small number of exposed mothers in any particular environmental or occupational category, the statistical analysis for toxicant exposure included both environmental and occupational exposure to x-rays, chemicals, anesthetics, industrial cleaning agents and solvents, exhaust and welding fumes, paint/varnish/thinner, asbestos, heavy metals, and pesticides.

The differences between the mean values of two continuous variables were evaluated by the unpaired Student's *t* test. The differences in noncontinuous variables and genotype distribution were tested by chi-square analysis. Unconditional logistic regression analysis was used to estimate odds ratios (ORs) and 95 % confidence intervals (CIs) for the association between CHD and maternal exposure.

The ORs also were adjusted for potential confounding factors. We considered that a sample size of at least 150 subjects per group was sufficient to detect an association between *GSTP1* Ile105Val polymorphism and CHD with a statistical power (β) of 80 % and a significance value of 0.05 (α) for an OR of 2.0 or higher given an allele frequency of 0.3. In addition, the study was powered to allow for an interaction with a magnitude of 2.3 having at least 80 % power and an error rate of 5 % under a sample size of 170 case-only [6]. A two-tailed *p* value lower than 0.05 was chosen as the level of significance.

Results

The demographic details of the study population are summarized in Table 1. The mothers of the patients with CHD had a significantly lower age at conception than the control mothers ($p = 0.009$). The CHD cases had a significantly lower birth weight ($p = 0.01$) than the control children. The maternal exposures to environmental factors are listed in Table 2.

Table 1 Baseline characteristics of the study population

	Case children ($n = 190$) *n* (%)	Control children ($n = 190$) *n* (%)	*p* Value
Offspring			
Male gender	104 (55)	114 (60)	0.2
Age (years ± SD)	4 ± 5.6	At birth	<0.0001
Birth weight (g)	3,168	3,317	0.01
Artificial fertilization	5 (2)	2 (1)	0.2
Mother			
Age (years ± SD)	30 ± 5.3	32 ± 5.4	0.009
History of chronic illnesses	21 (11)	20 (10)	0.8
Spontaneous miscarriages	32 (17)	48 (25)	0.04

Table 2 Maternal exposure to environmental risk factors

	CHD parents	Control parents	*p* Value
Occupational/environmental exposure (yes): *n* (%)	73 (38)	44 (23)	0.0013
Paint/varnish/thinner/solvents	8	4	
X-rays	7	4	
Asbestos	2	6	
Chemical laboratory exposure	2	3	
Cleaning agents and solvents	4	5	
Hair dye	2	2	
Industrial chemicals	5	1	
Pesticides	6	1	
Textile dye	2	0	
Drugs	4	12	
Electromagnetic field	4	0	
Environmental pollution	4	3	
Waste site	8	0	
Industrial pollution	8	3	
Thermal power plant	7	0	
Active smoking (yes): *n* (%)	52 (27)	45 (24)	0.6

CHD congenital heart disease

The analysis of maternal habits showed that 73 mothers of the children affected by CHD (38 %) had a positive history for exposure to toxicants (occupational and environmental) compared with 44 (23 %) mothers of the control children ($p = 0.0013$). No significant differences was found for maternal smoking habits in the two groups ($p = 0.62$). Multivariate logistic regression analysis showed that the risk for CHD was increased in presence of occupational/environmental maternal exposure to toxicants (OR, 2.6; 95 % CI, 1.6–4.2; $p < 0.0001$).

The frequency of *GSTP1* Ile105Val in both the CHD cases and the control subjects satisfied the Hardy-Weinberg equilibrium and was comparable with that previously observed in Caucasian subjects. The genotypes and variant allele distributions were not significantly different between the patients and the control subjects (Table 3). In the case-control study, we found no association between *GSTP1* Ile105Val polymorphism in children and maternal exposure.

No significant gene–environment interactions were observed (Table 4). Logistic regression did not show an increased risk for CHD in the presence of positive maternal exposure and *GSTP1* polymorphism.

Discussion

Our study clearly shows that maternal exposure to toxicants affects the risk of children with CHD, supporting the hypothesis positing a pivotal influence of environmental

Table 3 Genotype distribution, *GSTP1* polymorphism, and risk of congenital heart disease (CHD)

GSTP1 polymorphism	Case population *n* (%)	Control population *n* (%)	*p* Value
Ile/Ile	109 (57)	110 (58)	0.9
Ile/Val	66 (35)	65 (34)	
Val/Val	15 (8)	15 (8)	

risk factors on congenital malformations. In contrast, the genotype distribution of the *GSTP1* Ile105Val polymorphism was not significantly different between the patients and the control subjects. Nor were any adverse effects of *GSTP1* Ile105Val polymorphism in the presence of maternal exposure observed. However, our findings underscore the fundamental role of maternal exposure in the pathogenesis of CHD, consistent with previous observations [6].

Indeed, we also recently demonstrated that the presence of polymorphisms in *GSTM1* and *GSTT1* genes modified an individual's risk of toxicant exposure-induced CHD. To our knowledge, no studies in the literature have examined the effects of fetal *GSTP1* Ile105Val polymorphism in combination with maternal exposures.

Studies show that *GSTP1* is responsible for the detoxification of benzo(a)pyrene diol epoxide [21], and the presence of Ile105Val variant diminishes its enzyme activity [2, 27], which in turn may impair the excretion of toxicants. Several studies have reported a significant association of valine allele with susceptibility to the development of certain tumors including bladder, breast, lung, and multiple myeloma [11]. Cancer researchers also have found suggestive interaction between the presence of this polymorphism and the risk of oral and bladder cancer, especially among smokers [24]. In addition, the Val105 allele also is reported to increase the risk of asthma and susceptibility to the effects of ozone on breathing difficulties among children with asthma [22].

These findings suggest that variable *GSTP1* expression may be an important determinant of susceptibility to environmental diseases.

Although the *GSTP1* gene is highly expressed early in fetal life and appears to be the predominant GST present in the human placenta [1, 3, 19], data on maternal exposure and the role of *GSTP1* polymorphisms in the pathogenesis of congenital malformation [5, 10, 16, 24, 25] are insufficient.

A recent study showed a significant association between *GSTP1* Ile105Val polymorphism and Hirschsprung disease, a common congenital intestinal defect [8]. Conversely, studies on esophageal atresia, a life-threatening congenital condition, and orofacial clefts have found no association between the Ile105Val variant and the disease [7, 20].

The current study is one of the first to examine the role of *GSTP1* polymorphism in the etiology of CHD related to maternal exposure. We found no association between the presence of this polymorphism and the effect of positive maternal exposure to toxicants on the risk for congenital heart disease.

Recently, it has been suggested that the loss of expression of the gene *GSTP1* is caused by promoter hypermethylation in several types of cancer such as prostate and breast cancer [17, 28]. The loss of *GSTP1* leads to elevated levels of electrophilic intermediates, resulting in increased DNA damage and mutations [15].

Some important limitations must be considered when the results of this study are interpreted. First, all risk factors studied were self-reported, so random misclassification of exposure and recall bias cannot be excluded. Some variables may have been prone to over- and underestimation, thereby affecting the risk estimates. However, although recall bias can never be excluded, evidence is emerging that its role is rather small, and almost no differential in

Table 4 Gene–environment interactions for *GSTP1* genes and maternal exposure

Maternal exposures	GSTP1 controls (*n*)			*p* Value	GSTP1 cases (*n*)			*p* Value
	Ile/Ile	Ile/Val	Val/Val		Ile/Ile	Ile/Val	Val/Val	
Active smoking								
No	69	38	8	0.7	68	40	11	0.6
Yes	41	27	7		40	26	5	
Occupational exposures								
No	100	58	13	0.8	89	56	13	0.8
Yes	10	7	2		43	25	5	
Environmental exposures								
No	96	50	13	0.1	83	50	12	0.9
Yes	14	15	2		26	16	3	

case-control studies have focused on congenital malformations [6]. Second, no exposure dose or biomarkers of exposure were available. Finally, an undoubted limitation of our study was the modest size of our population when we analyzed genotype groups.

Further research is needed to understand better the epidemiologic impact of toxicant agents on cardiac development and to investigate gene–environmental interaction effects. A better understanding of both genetic and environmental factors is expected to facilitate the prevention of CHD and to reduce the occurrence of these congenital malformations in future generations.

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ORIGINAL ARTICLE

Lack of Association of the 3′-UTR Polymorphism (rs1017) in the *ISL1* Gene and Risk of Congenital Heart Disease in the White Population

Monica Cresci · Cecilia Vecoli · Ilenia Foffa · Silvia Pulignani · Lamia Ait-Ali · Maria Grazia Andreassi

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Abstract Congenital heart defects (CHDs) are the most prevalent of all birth defects and the leading cause of death in the first year of life. The molecular causes of most CHDs remain largely unknown. The LIM homeodomain transcriptor factor *ISL1* is a marker for undifferentiated cardiac progenitor cells that give rise to both the right ventricle and the inflow and outflow tracts, which are affected by several cardiovascular malformations. Contradictory findings about the role of the *ISL1* rs1017 single-nucleotide polymorphism in increasing the risk of CHD have been reported. In this study, we aimed to investigate whether the *ISL1* rs1017 genetic polymorphism conferred susceptibility to CHD in the white population. In a case–control study design, 309 patients with CHD (197 men [age 21.3 ± 25.2]) and 500 healthy controls (272 men [age 15.7 ± 21.3]) were genotyped for the *ISL1* rs1017 polymorphism. No significant difference in the genotype and variant allele distributions was found between patients and controls. In addition, the *ISL1* rs1017 polymorphism was not associated with the risk of CHD neither overall ($p = 0.7$) nor stratifying the population by sex and CHD classification. In conclusion, *ISL1* common variant rs1017 is not associated with increased genetic risk of CHD in the white population.

Keywords Congenital heart disease · *ISL1* polymorphism · Common genetic variants · Cardiac transcriptor factors

Introduction

Cardiac development is a complex process controlled by an evolutionarily conserved network of transcription factors (TFs). Many nucleotide variants in cardiac TF genes, such as in *GATA4* and in *NKX2.5*, have been identified in patients with congenital heart disease (CHD) [15, 16]. Nevertheless, because of the very low frequency of these mutations in both familial and sporadic cases of CHD [3], the molecular causes of different forms of CHDs remain largely unknown.

ISL1 is a LIM homeodomain TF considered the most important marker of cardiac progenitor cell lineage in the secondary heart-field differentiation [9, 14, 17]. *ISL1* cardiovascular progenitors give rise to the right ventricle and inflow and outflow tracts, which are affected by several cardiovascular malformations, *e.g.*, transposition of the great vessels, tricuspid atresia, and tetralogy of Fallot [12]. The involvement of *ISL1* in the pathogenesis of different forms of CHD appears evident [4]. In fact, animal experimental models have shown that homozygous mutants for *ISL1* developed a severe cardiac phenotype [12] whereas mice totally knocked out for this gene were lacking in the outflow tract, in the right ventricle and in several part of the atria [5, 13]. Recently, common genetic variants and specific haplotypes in the *ISL1* gene have been found to contribute to the risk of CHD in the white and black/African-American populations, thus supporting the common variant–common disease hypothesis [17]. The aim of this study was to analyse whether the rs1017 polymorphism influences the susceptibility of isolated, nonsyndromic CHD in the white population.

Monica Cresci and Cecilia Vecoli contributed equally to this study.

M. Cresci · I. Foffa · S. Pulignani · L. Ait-Ali
National Research Council Institute of Clinical Physiology, via Aurelia Sud, 54100 Massa, Italy

C. Vecoli (✉) · M. G. Andreassi
National Research Council Institute of Clinical Physiology, via Moruzzi 1, 56124 Pisa, Italy
e-mail: vecoli@ifc.cnr.it

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Methods

Subjects and Data Collection

This study included a total of 309 patients who were diagnosed with isolated, nonsyndromic CHD (197 males [21.3 ± 25.2 years], including 200 pediatric [4 ± 5.6 years] and 109 adult patients [52.5 ± 17.2 years] with bicuspid aortic valve, BAV) plus a control group of 500 healthy subjects [272 males; 15.7 ± 21.3 years) comprising 300 newborn and 200 adult subjects (39.8 ± 13.7 years). A sample of venous blood was collected from controls, whereas a cord blood sample was obtained from healthy newborns. This study was conducted with informed consent of every participant subject/parent and was approved by the local Ethical Research Committee.

ISL1 Genotyping

Genomic DNA was extracted from whole-blood samples, and a polymerase chain reaction (PCR)–restriction fragment length polymorphism method was used to determine the allele distribution of the rs1017 polymorphism. The PCR mixture (50 µl) was prepared containing 50 ng DNA, 5 µl buffer (500 mM KCl/100 mM Tris–HCl [pH 8.3]/15 mM $MgCl_2$), 1 µl 2.5 mM dNTPs, 2.5 µl each of the forward primer (5′-CTT TCA GGA AGG TGG AGC TG -3′) and reverse primer (5′-CGC TTG TGG CAA AAT AGA GG -3′), and 1 U Taq polymerase. Primers were designed using the Primer3 program (http://primer3.sourceforge.net/). PCR conditions were as follows: denaturing at 94° for 5 min, then 35 cycles of denaturing at 94° for 30 s, annealing at 56° for 30 s and elongation at 72° for 30 s, and incubation for 7 min at 72° to allow a complete extension of all PCR fragments. The 248-bp amplified *ISL1* gene fragment was subjected to restriction digestion in a 9-µl reaction volume containing 4 µl of PCR product, 100 mM NaCl, 50 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$,1 mM dithiothreitol, and 2.5 U *DraI* at 37° overnight. The digested PCR products were separated by electrophoresis using 1.5 % agarose gel. A representative gel is shown in Fig. 1. The genotype was determined by analysis of the bands on the gel: homozygous wild-type for adenine (AA), one band (156 bp); homozygous mutated for thymine (TT), one band (134 bp); and heterozygous (AT), two bands (156 and 134 bp).

Statistical Analyses

Statistical analyses of the data were conducted with the Statview statistical package version 5.0.1 (Abacus Concepts, Berkeley, CA). Data are expressed as mean ± SD. Differences between the mean value of two continuous variables were evaluated by unpaired Student *t* test.

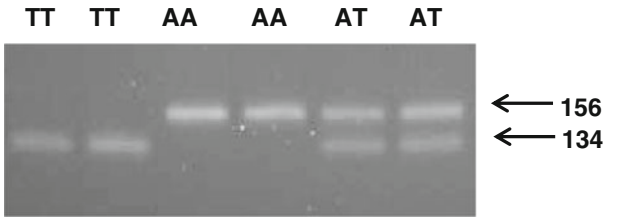

Fig. 1 Representative 1.5 % agarose gel used to separate by electrophoresis the digested PCR products. Homozygous wild-type for *AA*, one band (156 bp); homozygous mutated for *TT*, one band (134 bp); and heterozygous (*AT*), two bands (156 and 134 bp)

Differences in noncontinuous variables and genotype distribution were tested by Chi-square analysis. Unconditional logistic regression analysis was used to estimate odds ratios (ORs) and 95 % confidence intervals (CIs) for the association between CHD and the presence of polymorphism. ORs were also adjusted for potential confounding factors. We estimated that our sample size was able to detect a significant association between *ISL1* rs1017 polymorphism and CHD with a power >80 %, for an OR ≥2.0 given an allele frequency of 0.35. A two-tailed p value < 0.05 was chosen as the level of significance.

Results

Study Population

Table 1 lists no detectable statistically significant differences between cases and controls in terms of sex. Of the 309 CHD cases, 109 (35 %) had BAV, 89 (28 %) had complex CHD, 44 (14 %) had tetralogy of Fallot, 37 (12 %) had septation defect, 14 (5 %) had patent ductus arteriosus, 9 (3 %) had aortic coarctation, and 7 (3 %) had other CHD abnormalities.

Effect of the *ISL1* rs1017 Variant on CHD

The observed genotype frequency was in agreement with that expected under the Hardy–Weinberg equilibrium. No significant difference between the case and control groups was found in term of genotype and allele distribution (Table 2). At logistic analysis, the rs1017 AT genotype and the mutated TT genotype were not associated with the risk of CHD (OR 1.0, 95 % CI 0.7–1.3 and $p = 0.8$; OR 1.1, 95 % CI 0.7–1.7, $p = 0.6$ respectively). There was no appreciable difference between CHD risk and the presence of T allele (OR 1.0, 95 % CI 0.7–1.5, $p = 0.7$). When we compared the *ISL1* rs1017 genotype distribution among the different groups of patients and controls, no significant difference was also observed (Table 3). Finally, we

Table 1 Baseline characteristics of the study population

Characteristics	Cases ($n = 309$)	Controls ($n = 500$)
No. male sex (%)	197 (63)	272 (55)
Age (years)	21.3 ± 25.5	15.7 ± 21.3
CHD classification		
Bicuspid aortic valve	109 (5)	
Complex CHD	89 (28)	
Tetralogy of Fallot	44 (14)	
Septation defects	37 (12)	
Patent ductus arteriosus	14 (5)	
Aortic coarctation	9 (3)	
Other CHD	7 (3)	

Table 2 Main effects of *ISL1* rs1017 on CHD risk in the case–control study

Genotypes	No. (%) cases ($n = 309$)	No. (%) controls ($n = 500$)	OR (CI %95)	*P*
rs1017				
AA	142 (46)	22 (46)	1.0	
AT	130 (42)	204 (41)	1.0 (0.7–1.3)	0.8
TT	37 (12)	67 (13)	1.1 (0.7–1.7)	0.6
AT + TT	167 (54)	271 (54)	1.1 (0.7–1.5)	0.7
Allele				
A	414 (67)	662 (66)	1.0	
T	204 (33)	338 (34)	1.2 (0.7–1.7)	0.6

Table 3 *ISL1* rs1017 genotype distribution in different groups of patients and controls

Genotypes	No. (%) CHD ($n = 200$)	No. (%) BAV ($n = 109$)	No. (%) pediatric controls ($n = 300$)	No. (%) adult controls ($n = 200$)
rs1017				
No. (%) genotypes				
AA	93 (46)	49 (45)	144 (48)	85 (42)
AT	82 (41)	48 (44)	114 (38)	90 (45)
TT	25 (13)	12 (11)	42 (14)	25 (13)
T allele frequency	0.33	0.33	0.33	0.35

Table 4 Stratified analysis and *ISL1* rs1017 polymorphism

Variables	OR (95 % CI)	*P*
Overall	1.1 (0.7–1.5)	0.7
Sex		
Male	0.8 (0.5–1.3)	0.4
Female	0.9 (0.7–1.3)	0.8
CHD classification		
Bicuspid aortic valve	1.0 (0.7–1.6)	0.9
Complex CHD	0.9 (0.6–1.4)	0.6
Tetralogy of Fallot	1.0 (0.6–1.8)	0.9
Septation defect	1.3 (0.7–2.4)	0.5
Patent ductus arteriosus	0.9 (0.3–2.7)	0.9
Aortic coarctation	0.7 (0.3–2.4)	0.7
Other CHD	1.5 (0.4–6.0)	0.6

evaluated the influence of polymorphism on CHD risk also stratifying the population for sex and CHD classification (as listed in Table 1), but no differences in the various subgroups were found (Table 4).

Discussion

The formation of the three major cell types of the heart—cardiomyocytes, smooth muscle, and endothelial cell lineages—has been largely ascribed to a set of nonoverlapping embryonic precursor derived from distinct origin. Several signaling molecules, including bone morphogenetic proteins and fibroblast growth factors, are required to initiate the cardiomyogenic program. Subsequently, a unique combinatorial subset of transcriptional factors, such as GATA, HAND, and TBX, interact to generate different cardiac cells types [11]. The LIM homeodomain TF *ISL1* is a specific marker for a distinct population of undifferentiated cardiac progenitor cells that give rise to the cardiac segments in secondary heart fielding. *ISL1* function is required for these progenitors contributing to the proliferation, survival, and migration of cardiac progenitors in the forming heart [4, 9]. Animal experimental studies have shown that both the deficiency and the misexpression of *ISL1* might cause deep developmental defects, growth retardation, and death during embryogenesis (at approximately embryonic day [ED] 10.5), thus supporting the importance of correct regulation of *ISL1* gene expression during fetal life [1, 2, 7, 10]. Histological analysis of mutant hearts from murine fetuses (ED 9.0–9.5) showed that homozygous *ISL1* mutants had serious cardiac phenotypes characterized by a severe decrease in tissue mass characterized by loss of some segments [5]. In addition, *ISL1*-deficient hearts fail to undergo a correct looping morphogenesis and show a common atrium and an univentricular chamber [5].

To date, no *ISL1* coding mutations have been identified in humans, likely because of an embryonic lethal phenotype caused by a complete inactivation of this TF [5]. Stevens et al. [17] recently showed that two different *ISL1* haplotypes contributed to the risk of CHD in the white and black/African-American populations. In particular, two specific polymorphisms, rs1017 and rs3762977, were associated with cardiac congenital defects. The rs1017 SNP (located in 3′UTR) increased the risk of CHD in the United

States white population but not in the black/African-American population, whereas the rs3762977 SNP (located in 5′UTR) contributed to the risk of CHDs in the black/African-American population but not in the white population. Conversely, a more recent article [18] showed that *ISL1* common variant rs1017 did not play a crucial role in conferring susceptibility to sporadic CHD in the Chinese population. The investigators ascribed these different results to multiple factors, including etiologic heterogeneity between populations, study design, type of heart defects, and lack of information on potential effect modifiers in Stevens' article [17].

In accord with these last findings, we found that this intronic variant, rs1017, was not associated with CHD risk in the white population. An undoubted limitation of our study could be the modest size of the study population, which made difficult a correct stratification of analysis according to genotype, sex, and CHD etiology difficult. However, our results are in agreement with those of Xue et al., obtained in a large sample, suggesting that the identification of common variants associated with CHD risk in a specific, confined population could not to be due to a common genetic cause increasing the disease susceptibility. In fact, cardiac development is regulated by a complex mechanism that involves the expression of many different genes in different times, spaces, and orders and that is largely influenced by environmental factors [6]. Thus, great attention is necessary when common variants are ascribed as the cause conferring genetic susceptibility to CHD. For instance, a major limitation of Stevens' article could be that the investigators did not report or consider precise exclusion and inclusion criteria that would be necessary when a specific genetic association is found. Indeed, the potential influences of contaminant factors, such as the environmental effect, the use of some drugs (*i.e.*, teratogen elements) by the mother, the presence of parental disease (*i.e.*, diabetes), which are largely known to affect cardiac development [8], have not been defined.

In conclusion, we did not find any association between *ISL1* rs1017 and increased risk to develop CHD in the white population. Further studies, carefully designed to include the evaluation of more factors involved in the complex mechanism of cardiogenesis, are needed. Indeed, only a multifactorial analysis may allow us to better understand the molecular mechanism causing the cardiac congenital malformations.

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LETTER TO THE EDITOR

Genetics of congenital heart defects: is it not all in the DNA?

We read with great interest the paper by Wu et al[1] on the genetic analysis of the promoter region of the GATA4 gene in patients with ventricular septal defect (VSD).

The authors provide new important ideas about the potential role of genetic variants within regulatory region rather than the codifying sequences that may contribute to the etiology of VSD, the most common type of congenital heart defect (CHD). In this study, 5 heterozygous sequence variants were found within the promoter region of GATA4 gene in VSD patients but in none of the healthy controls. Although these variants do not interrupt the regulatory promoter regions, they seem to significantly alter the transcriptional activity of GATA4 gene promoter, which may contribute to the VSD.

In another study published by the same authors,[2] functional analysis showed that sequence variants within promoter regions significantly enhance the transcriptional activities of the NKX2-5 gene, which may lead to upregulated NKX2-5 gene expression, contributing to the VSD etiology.[2]

We believe that these observations underlie that the causative factors and the molecular mechanisms involved in the CHD etiology still remain largely elusive.

In recent years, several lines of evidence have highlighted the importance of GATA4, in association with a variety of binding partners like NKX2-5, in a specific transcriptional complex that confer tissue-specific gene expression during cardiogenesis and that can be altered during the development of CHD.[3] Indeed, mutations leading to gene haploinsufficiency in key cardiac transcription factors (TFs) are responsible for inherited and sporadic CHDs.[3]

Nevertheless, the study of genetic basis of CHDs is complicated by the fact that a given structural defect can be caused by more than one gene because TFs work in a synergic manner. In addition, the frequency of mutations in TF genes have been shown to be less than 3%, especially in the sporadic forms of CHD.[4,5] These observations suggest that the predisposition to the disease involves multiple factors, notably for isolated non-family CHDs, including complex gene interactions, several signaling pathways, and environmental influences as well as a combination of these factors.

Furthermore, the hypothesis of somatic mutations has been also suggested as novel genetic mechanism for CHDs.[6] This theory still remains a controversial scientific matter that needs further investigations.[7-9]

It is also important to remember that, during cardiogenesis, there is a cooperative relationship between tissue-specific TFs and epigenetic variations to specify cell fate and promote terminal differentiation.

Cytosine methylation at 5'-CpG-3 dinucleotide is the most common base modification in the eukaryotic genome that influence gene expression. Generally, CpG islands in the gene promoter are protected from DNA methylation, whereas CpG sites in gene-coding or non-coding region are commonly methylated.[10] However, the methylation pattern could be disrupted in diseases, and methylated CpG islands can be silenced or downregulated.[10] Consequently, DNA methylation may be proposed as a potential mechanism involved in CHDs.

Interestingly, histone deacetylases 1 and 2, key regulatory enzymes involved in the regulation of gene expression during development and throughout life, have mostly been characterized as having a role in cardiac morphogenesis, growth, and contractility.[11] Furthermore, the hearts of mutant mice displayed unusual morphologic abnormalities of the right ventricular chamber.[11]

Another potential mechanism on the basis of CHD is the microRNA (miRNA) post-transcriptional regulation that control key genetic programs in cardiovascular biology.[12] One of the best examples of miRNA regulation in the heart involves the basic helix-loop-helix TF, Hand2, and its repression by miR-1 and miR-133a, leading to VSD.[13] Indeed, miRNAs can inhibit translation and/or promote mRNA degradation depending on its degree of complementarity with the target.[12]

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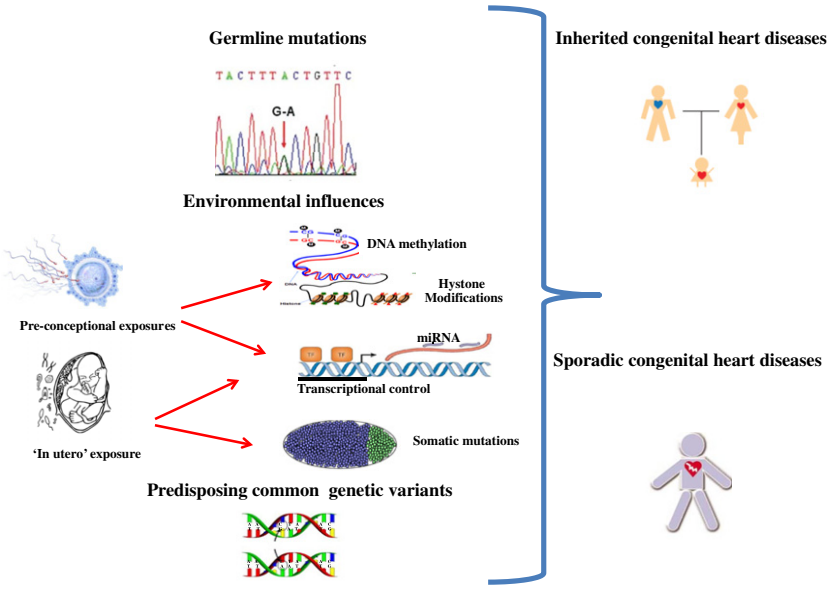

Fig 1. Pathogenesis of congenital heart defects. The most likely scenario is that the etiology of disease involves multiple factors, including different genetic loci, environmental influences, epigenetic factors (eg, DNA methylation or histone modifications) and miRNA dysfunction and/or a combination of these factors. (Color version of figure is available online.)

Finally, there is a recent evidence supporting the idea that common genetic variants, not necessarily disease-causing, may contribute to risk of CHD, especially interacting with environmental factors.

Indeed, we recently showed that exposure to toxicants from both parents affect the risk of children with CHD, supporting a pivotal influence of the environmental risk factors in the pathogenesis of congenital malformations.[14,15] In addition, our gene-environment analyses suggested that specific and common genetic variants in genes involved in detoxification pathways can modify a persons risk of toxicant exposure-induced disease.[14,15]

In conclusion, many questions still remain open about the disease. What are the genetic and epigenetic bases of different forms of CHD? What is the recurrence risk for parents of a child with CHD? Which is the risk of disease transmission in grown-up CHD patients? Future studies and more research in this area are greatly needed to provide insight into the molecular basis CHD as well as to answer these questions (Fig 1).

In the near future, it is expected that the power of next generation sequencing technologies may allow a more comprehensive analysis of genetic and epigenetic contributions to the pathogenesis of CHD.

Furthermore, understanding the biologic impact of gene-environmental interactions may provide a key insight into the prevention of these congenital malformations in future generations. Understanding the genetic basis and the molecular mechanisms of CHD may allow the identification of family members at risk as well as to identify new possible therapeutic targets and appropriate preventive strategies because environmental factors can be modified in contrast to genetic factors.

Silvia Pulignani
Monica Cresci
Maria Grazia Andreassi
Institute of Clinical Physiology, CNR
Massa, Italy

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