

Environmental risk factors for congenital heart disease

Environmental risk factors for CHD

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Abstract

Congenital Heart Disease (CHD) has many forms and a wide range of causes. Clinically it is important to understand the causes. This allows estimation of recurrence rate, guides treatment options and may also be used to formulate public health advice to reduce the population prevalence of CHD. The recent advent of sophisticated genetic and genomic methods has led to the identification of more than 100 genes associated with CHD. However, despite these great strides, to date only one-third of CHD cases have been shown to have a simple genetic cause. This is because CHD can also be caused by oligogenic factors, environmental factors and/or gene-environment interaction. Although solid evidence for environmental causes of CHD have been available for almost 80 years, it is only very recently that the molecular mechanisms for these risk factors have begun to be investigated. In this chapter we describe the most important environmental CHD risk factors, and what is known about how they cause CHD.

Introduction

The reasons for the frequent occurrence of birth defects has invited speculation since ancient times. Although the ancient Greeks recognised that some birth defects might be inherited, they also speculated that they might occur by bad influences during pregnancy. In the following millennia, the latter hypothesis was predominant, right up until the rediscovery of Mendel's work on genetics in the early twentieth century. For example, the early cardiac anatomist Thomas Peacock wrote in 1858: "The occurrence of accidents and strong impressions upon the mind of the mother are also supposed to conduce to the irregular development of the offspring" (Peacock 1858). He also noted that other cases appeared to have "an hereditary predisposition to defective development of the heart". 50 years later, the pioneering cardiac pathologist Maud Abbott wrote that CHD could occur through "baneful influences acting on the mother during the early weeks of pregnancy" (Abbott 1908). She also conceded that heredity might play some role, although not as strongly as in other birth defects such as polydactyly. The environmental hypothesis received its first solid evidence with the discovery in 1941 that maternal Rubella infection could cause a suite of birth defects, including CHD (Gregg 1941). Despite this, by late 1940s the mood was shifting in favour of genetics as the major cause of CHD (Taussig 1947; Campbell 1949) and by 1959 there was the first clear evidence for this with the identification of the chromosomal abnormalities underlying Down syndrome (Lejeune et al. 1959) and Turner syndrome (Ford et al. 1959). Despite the identification two years later of exposure to the morning sickness drug thalidomide as another environmental cause of CHD (Lenz 1961; McBride 1961; Pfeiffer and Kosenow 1961), by the 1960s the majority view was that genetics was the most important cause of CHD, and environmental factors were relegated to probably only having a supporting role in gene-environment interactions (Nora 1968). From the 1970s onwards, chromosomal deletions and genetic mapping studies identified approximate genome locations for a dozen or so inherited forms of CHD. This process accelerated with the advent of molecular biology in the 1980s, and by 1991 the first gene mutation associated with CHD was identified: *FIBRILLIN* in Marfan syndrome (Dietz et al. 1991). The rapid advances in genetic diagnoses captured the imagination of scientists and the general public alike, and research into possible environmental causes of CHD became unfashionable. In the 20 years since the sequencing of the human genome, intensive effort has been focused on understanding the genetic causes of CHD. This has been coupled with great advances in uncovering the morphological and molecular processes driving embryonic heart development (Rickert-Sperling et al, 2016). However, despite these efforts and the discovery of almost 100 genes associated with human CHD, routine whole genome sequencing of large numbers of CHD patients and their families has only provided genetic explanations for ~30% of CHD cases (Szot et al. 2018). Thus, scientific interest has returned to non-genetic cases and uncovering the environmental

causes of CHD. In this chapter we will discuss the best known environmental risk factors for CHD, and what is known about how they perturb embryonic heart development. We have divided these into two broad categories: *Extrinsic* factors, such as teratogen exposure and nutrient deficiencies; and *Intrinsic* factors, including maternal disease and illness.

Types of congenital heart disease

Congenital heart disease (CHD) is a general term for a structural or functional defect of the heart that is present at birth. The heart is a complex organ formed from cells derived from at least four distinct progenitor cell types, termed the “first heart field”, “second heart field”, “cardiac neural crest” and the “proepicardial organ” (reviewed in Rickert-Sperling et al, 2016). Perturbation of any stage of this process can cause a variety of effects, depending on the timing and nature of the perturbing factor. As a result, there are more than 20 specific types of CHD. These range in severity from mild defects, which may have very little effect on the patient, through to critical CHD, which will require immediate surgery and carries a high rate of morbidity and mortality. Here we summarise the most important forms of CHD that may be induced by exposure to environmental factors. In general, CHD can be divided into several broad classes, based on their embryological origins.

Conotruncal defects result from an abnormal formation of the outflow tract of the heart. These include: (i) ventricular septal defects (VSD), where a hole is present between the ventricles (Fig 1B); (ii) double outlet right ventricle (DORV), which is a defective alignment of the aorta and pulmonary trunk, such that both vessels drain the right ventricle. This is also commonly associated with a VSD (Fig 1C); (iii) overriding aorta (OA), a defective alignment of the aorta directly over a VSD (Fig 1D); (iv) transposition of the great arteries (TGA), where the aorta connects to the right ventricle and the pulmonary trunk to the left ventricle (Fig 1E); and (v) persistent *truncus arteriosus* (PTA), also called common arterial trunk (CAT), where the septum dividing the aorta and pulmonary trunk fully or partially fails to form (Fig 1F). Also in this group, tetralogy of Fallot (TOF) is a commonly observed association of four heart defects: VSD, pulmonary artery stenosis, overriding aorta, and right ventricular hypertrophy (Fig 1G). **Venous Pole defects** occur where the inflow tract fails to form correctly. These include: (i) atrial septal defect (ASD), where a hole is present between the atria; (ii) atrioventricular septal defect (AVSD), complete failure of the atrioventricular septum, resulting in a hole that allows communication between all four cardiac chambers. **Left ventricular obstructive lesions** occur when the aorta fails to form correctly. These include: (i) hypoplastic left heart syndrome (HLHS), where there is no outlet to the left ventricle (Fig 1H); (ii) aortic coarctation and/or stenosis, where the lumen of the aorta is abnormally thin (Fig 1I); and (iii) bicuspid aortic valve (BAV), where the aortic valve fails to form the normal three leaflets. Another important **valve defect** is Ebstein’s anomaly, a malformation of the tricuspid valve between the right atrium and right ventricle. Finally, CHD can present with abnormalities of the smaller vessels leaving the heart, in particular: (i) aortic arch anomalies (AAA) or interrupted aortic arch (IAA) are where there is a discontinuity between the ascending and descending aortae. There are three types of IAA: type A occurs distal to the left subclavian artery; type B between the left carotid artery and the left subclavian artery; and type C between the right and left carotid artery; (ii) persistent *ductus arteriosus* (PDA), a connection between the aorta and pulmonary artery that is open in the fetus, but normally closes a few days after birth; and (iii) branch pulmonary artery stenosis, where the branching arteries entering the lungs are narrow or blocked. For reference, the estimated birth prevalence of these forms of CHD (taken from Bruneau, 2008) are given in Table 1.

Extrinsic factors

Normal embryonic development is a carefully choreographed and robust process that is nonetheless vulnerable to perturbation by external factors. Such factors can be either an excess of a toxic substance, or the lack of an essential nutrient. In both cases, the factor can act directly on the embryo itself, or indirectly, for example by perturbing placental development and altering the nutrient supply to the embryo.

Teratogens

Thalidomide. The most notorious human teratogen is thalidomide. This was introduced in the 1950s to treat influenza, then used as a sedative, and finally as an anti-emetic for morning sickness in pregnancy. It was withdrawn in the early 1960s, following widespread reports of its association with severe limb defects (Lenz 1961). It is less well known that thalidomide also caused CHD in ~30% of cases (Ruffing 1977). In recent years, thalidomide usage in the clinic has resumed for treatment of Hansen's disease (leprosy) and relapsed-refractory multiple myeloma, albeit with tight contraceptive control in young women. The experience with thalidomide emphasises one potential problem with the use of animal models to test the teratogenicity of drugs. Orally-administered thalidomide is not teratogenic in rats, but it is highly teratogenic in both humans and rabbits (Schumacher et al. 1968). To further complicate matters, thalidomide forms two optical isomers, only one of which is teratogenic (Blaschke et al. 1979). The precise mechanism by which thalidomide causes birth defects has only been identified in recent years. Thalidomide binds to the normal cellular protein CRBN and potentiates its action (Ito et al. 2010). CRBN is a subunit of the CRL4^{CRBN} E3 ubiquitin ligase that targets a number of proteins for degradation by the ubiquitin pathway. Amongst its many protein targets is the transcription factor SALL4 (Donovan et al. 2018; Matyskiela et al. 2018). This protein is required in the embryo for both limb and heart development. In humans, deleterious mutants in this gene cause Duane Radial Ray and Holt-Oram syndromes that both phenocopy the limb and heart defects of thalidomide exposure (Kohlhase et al. 2003). Furthermore, mouse embryos heterozygous for null mutations in this gene have a high frequency of miscarriage and VSDs (Sakaki-Yumoto et al. 2006). Remarkably, this research has also uncovered a mechanism to explain the difference in teratogenicity between humans and rabbits, and rodents. Both rats and mice have amino acid changes in both CRBN and SALL4 that compromise the ability for thalidomide to activate SALL4 degradation (Donovan et al. 2018; Matyskiela et al. 2018).

Retinoic acid and vitamin A. Retinoic acid (RA) is an important signalling molecule synthesised from dietary vitamin A that has many roles in embryonic development (Niederreither and Dolle 2008). An excess of RA can have dramatic effects on human embryonic development. This can occur in either the offspring of women undergoing therapeutic treatment with the synthetic retinoid isotretinoin (13-*cis*-retinoic acid), or in the offspring of women with excess dietary vitamin A supplementation. Isotretinoin was introduced in 1982 as a clinical treatment for severe cystic acne (Peck et al. 1982). Because it was well known from animal experiments that both vitamin A and RA were highly teratogenic (Cohlan 1953; Shenefelt 1972), isotretinoin was labelled a category X medication (i.e. contraindicated for use during pregnancy). However, it was not long before clinicians were reporting cases of inadvertent use during pregnancy, leading to the frequent occurrence of a range of severe congenital defects, including conotruncal heart defects and aortic arch anomalies (Lammer et al. 1985). Even today, despite the introduction of a variety of risk reduction programmes around the world, there are still significant numbers of pregnancies affected by isotretinoin exposure (Khiali et al. 2018). In embryonic development RA acts as a morphogen, and it is crucial in such processes as heart development, anterior-posterior patterning, limb development, differentiation of

mesenchymal tissues, and fine-tuning the left-right signals required for asymmetric organ patterning. In the diet, vitamin A occurs as retinol and is metabolised into retinal and RA using alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH). Some studies suggest that taking vitamin A supplements during the first 12 weeks of gestation (Rothman et al. 1995) or even in the year prior to pregnancy (Botto et al. 1996), results in a more than 4-fold increased risk of conotruncal heart defects. However, these results have not been replicated by others (Mills et al. 1997). This may be due to differences in the source of vitamin A in supplements: isotretinoin was used historically, before being replaced with β -carotene (pro-vitamin A) more recently. As described above, isotretinoin is highly teratogenic, whereas β -carotene is not (Shaw et al. 1996; Mastroiacovo et al. 1999). The reason for this difference is unknown. The mechanism by which excess RA can cause CHD has been investigated using animal studies. These show that RA signalling has multiple roles throughout embryonic heart development (Nakajima 2019). These include: (i) forming the anterior/posterior boundaries of the first- and second heart field cardiogenic mesoderm (FHF and SHF) in the gastrulating embryo prior to heart tube formation; (ii) patterning and maintenance of heart progenitors in the anterior SHF, as well as for regulation of their migration and differentiation into the outflow tract and pharyngeal arches; (iii) induction of proliferation of ventricular cardiomyocytes via the epicardium; and (iv) formation and patterning of the coronary vasculature, also via the epicardium. Thus, exposure to excess RA can have a variety of effects on embryonic heart development, depending on the timing of the exposure.

Alcohol. It has been recognised since the 1970s that high dose alcohol consumption (i.e. ≥ 50 g in a single episode) during pregnancy can lead to foetal alcohol spectrum disorder (FASD, (Jones and Smith 1973). Heart defects are common in FASD, with up to 67% of cases reported with CHD, mostly VSD, ASD and conotruncal defects (Burd et al. 2007; Yang et al. 2015). Alcohol is commonly consumed by pregnant women, with up to 30% of pregnant women, and up to 66% of women who are planning a pregnancy, drinking alcohol (Ethen et al. 2009; Cameron et al. 2013; Green et al. 2016; Reynolds et al. 2019). As a consequence, FASD affects 0.8% of individuals worldwide (Lange et al. 2017) and up to 5% of children in the USA (May et al. 2014). However, in the absence of a FASD diagnosis, there may only be a slightly increased risk of CHD in offspring of mothers drinking peri-conceptionally (Zhu et al. 2015). Similar to excess RA exposure, animal experiments suggest that the timing and amount of alcohol dictate the nature and severity of the outcomes (Ungerer et al. 2013). The mechanism of alcohol-induced heart defects is not well understood. There are several hypotheses. Firstly, alcohol may act by competitive inhibition of RA synthesis from vitamin A. Alcohol metabolism and RA synthesis both require ADHs and ALDHs. These enzymes preferentially detoxify alcohol (Kot-Leibovich and Fainsod 2009), and thus in the presence of alcohol there will be less RA synthesised. In addition, the first breakdown product of alcohol metabolism, acetaldehyde, can inhibit ADH activity, which may further reduce RA synthesis (Shabtai et al. 2018). However, as discussed below, there is little clinical evidence that *reduced* RA levels affect human embryonic development. Secondly, ethanol exposure may have a directly deleterious effect on cardiac neural crest cells (Karunamuni et al. 2014). These are cells that arise in the neural tube, but during early development migrate into the cardiac outflow tract and pharyngeal arches, and contribute to the valves, septa and aortic arch arteries. Alcohol is proposed to either causing increased apoptosis and/or impeding migration. Lastly, it has been suggested that acute alcohol exposure may cause broad epigenetic changes to the embryo by directly affecting DNA methylation, histone modification and/or non-coding RNA regulation (Ungerer et al. 2013).

Hypoxia. In the early 1950s, the first reports of an increased rate of ASD and patent ductus arteriosus cases in infants born at high altitude (Alzamora et al. 1953; Chavez et al. 1953; Espino-Vela 1967). At the same time, the animal experiments in mouse reported adverse effects of hypoxia on embryonic heart development (Ingalls et al. 1952). Maternal exposure to a broad range of reduced oxygen levels for even a short period mid-gestation causes conotruncal heart defects (Shi et al. 2016; Kenchegowda et al. 2017; Yuan et al. 2017; Moumne et al. 2018). Here, hypoxia reduces the proliferation of second heart field cardiac progenitor cells, whilst first heart field differentiated cardiomyocytes are unaffected (Shi et al. 2016; Yuan et al. 2017). By E10.5, this causes a shorter distal outflow tract (OFT) and an altered angle between proximal and distal OFT. This leads to mal-alignment and mal-rotation of the OFT, and ultimately manifests as conotruncal CHD at E15.5-17.5. In other studies, mid-gestation exposure to 8-14% hypoxia reduces levels of the cardiac transcription factor *Nkx2-5*, resulting in heart defects (Moumne et al. 2018; Moreau et al. 2019). Exposure of *Nkx2-5* null heterozygous embryos to hypoxia *in utero* exacerbates this effect, resulting in an increased penetrance of heart defects (Moumne et al. 2018) or embryonic death (Moreau et al. 2019). This gene-environment interaction may explain the curious clinical observation that complex CHD rates are significantly reduced in high altitude populations than in those sea level (Zheng et al. 2017). This perhaps suggests that at altitude there is an increased mortality rate of fetuses with complex CHD. Another clinically relevant source of embryonic hypoxic exposure is maternal smoking. However, despite the above evidence, this appears to only carry a mildly increased risk of CHD (Correa et al. 2015; Sullivan et al. 2015). The reasons for this disparity are unclear.

Other therapeutic drugs

Many women have serious medical conditions that rely on continuous drug treatment. However, some such drugs including anti-convulsants, anti-arrhythmics and anti-depressants are also teratogenic. As a result, during pregnancy difficult decisions may have to be made to balance the risks of damage to the embryo and the risks to the mother. In many cases, understanding how therapeutic drugs cause CHD is complicated by the fact that many drugs have off-target effects. For example, phenytoin is primarily used in the clinic as an anti-convulsant, but it also has anti-arrhythmia properties and causes folate deficiency (Lewis et al. 1995). If such a drug is identified as a teratogen, it could cause birth defects via any of these biological activities.

Anti-convulsants. A potential link between maternal epilepsy and an increased risk of having offspring with birth defects was first proposed in the 1960s. Whether this was due to the disease itself, or to the medications taken to control seizures, was a topic of much debate (Janz and Fuchs 1964). However, by 1970 it was clear that many of the anti-convulsant drugs in use at the time, including phenytoin, carbamazepine and valproic acid, were highly teratogenic (Meadow 1970). These drugs result most commonly in VSDs, occurring at a rate three times higher than the general population (Meador et al. 2008). More recently, second- and third-generation anti-convulsants have been developed. Some of these, such as lamotrigine, appear to be less teratogenic (Pariente et al. 2017), others are no better than the older drugs (e.g. topiramate, (Vajda et al. 2019), and comprehensive clinical studies are yet to be completed for others (Singh and Verma 2019). The mechanism by which these drugs effect heart development is not well understood as few animal studies have been carried out. The main candidates are perturbation of folic acid metabolism, induction of embryonic hypoxia or creation of excess oxidative stress (Etemad et al. 2012).

Anti-arrhythmics. Some women have heart conditions causing abnormal heart rhythms that require treatment. In addition, during pregnancy, the mother is subject to characteristic

changes in cardiac physiology, including increased heart rate and cardiac output, and changes in hormonal balance and may develop cardiac arrhythmias requiring treatment. However, some anti-arrhythmic drugs have been shown in animal models to be highly teratogenic, especially potassium channel blockers (also known as IKr or hERG channel blockers, or class III anti-arrhythmics, Danielsson et al. 2001). In addition, a significant number of drugs and natural medicines used for treating other conditions have hERG channel blocking activity as an unwanted side-effect (Rampe and Brown 2013; Kratz et al. 2017). For this reason, compounds with hERG channel blocking activity are contra-indicated for use during pregnancy (Merino and Perez Silva 2011). It is not entirely clear how these drugs effect embryonic development. One attractive theory is that anti-arrhythmia drugs reduce embryonic cardiac output, resulting in embryonic hypoxia. In the case of class III anti-arrhythmia drugs, there is some evidence in favour of this hypothesis. HERG-channel inhibitors like dofetilide, when administered to pregnant rats have been shown to cause embryonic bradycardia and hypoxia (Danielsson et al. 2003; Ritchie et al. 2015).

Anti-depressants. Lithium treatment was first used as a treatment for bipolar disorder in the 1870s, but it took 100 years for such treatment to become mainstream. Today, it is still regarded as the best treatment for long-term relapse prevention (Geddes and Miklowitz 2013). However, it is well established that maternal lithium treatment increases offspring CHD risk (Nora et al. 1974). Modern estimates suggest the increased risk is up to 3-fold at the highest doses of lithium, and Ebstein's anomaly (tricuspid valve malformation) is particularly prevalent (Patorno et al. 2017). It is thought that lithium causes CHD via aberrant induction of Wnt signalling. Lithium is a well-described inducer of canonical Wnt signalling, via inhibition of the enzyme GSK3 β (Stambolic et al. 1996), and this signalling pathway is crucial for multiple stages of embryonic heart development (Ruiz-Villalba et al. 2016). In mouse, dosing with lithium at E8-9 induces neural tube defects, but not heart defects (Jurand 1988; Giles and Bannigan 1997). However, a single intraperitoneal injection of lithium treatment at gastrulation (E6.5) results in AV and semilunar valve defects by E18.5 (Chen et al. 2008).

Selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed second-generation anti-depressant drugs. These drugs have also been implicated in a small increased risk of offspring CHD (Berard et al. 2017; Gao et al. 2018). However, this is somewhat controversial, as this association may disappear when corrected for confounding factors (Huybrechts et al. 2014). In the brain, SSRIs increase extracellular serotonin levels, by blocking reuptake into neurons. However, serotonin is not just a neurotransmitter. Serotonin signalling is also required for several different aspects of embryonic heart development. These include neural crest cell migration, proliferation and survival, and endocardial cushion and trabeculae formation (Yavarone et al. 1993; Choi et al. 1997). Thus, perturbation of serotonin levels by SSRIs may have a direct effect on embryonic heart development. Alternatively, SSRIs might cause heart defects indirectly, as in cultured rat embryos these drugs cause heart block and/or bradycardia, leading to hypoxia (Ababneh et al. 2012).

Nutritional deficiencies

In addition to maternal and embryonic exposure to teratogens, there is also evidence that reduced maternal levels of essential nutrients can also increase the risk of CHD in her offspring. However, in many cases these observations have been made in animal models, and as yet there is little conclusive evidence that these factors are also relevant in humans.

Vitamin A. As described above, dietary vitamin A is metabolised by the embryo to form RA, a molecule that is intimately involved in many aspects of development. In rodent models, a low vitamin A diet during pregnancy causes highly penetrant heart defects and aortic arch anomalies (Wilson and Warkany 1950; Kalter and Warkany 1961). This has been confirmed more recently by genetic perturbation of RA synthesis or RA signal transduction in mice, which leads similar embryonic defects. For example, mouse embryos heterozygous for a null *Aldh1a2* (formerly *Raldh2*) allele die by E10.5 with an unlooped, dilated heart (Niederreither et al. 1999), while embryos homozygous for a hypomorphic *Aldh1a2* allele have persistent truncus arteriosus, VSDs and aortic arch abnormalities (Vermot et al. 2003). Likewise, mouse embryos lacking various combinations of the $RAR\alpha1$, $RAR\beta$, and $RXR\alpha$ RA receptors develop VSDs, conotruncal defects and aortic arch abnormalities (Lee et al. 1997). However, despite these results, there is no epidemiological evidence of a similar effect in human populations where vitamin A deficiency is prevalent (Azais-Braesco and Pascal 2000). Therefore, more research needs to be done to determine the clinical importance of Vitamin A deficiency and CHD risk.

Folic acid (Vitamin B9). Folic acid is a vitamin essential for purine and thymidine synthesis, and as a methyl donor for DNA methylation (Stover 2004). It has been known for over 25 years that low maternal folate levels are associated with a significantly increased risk of neural tube defects (NTD, MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992). This is the basis for mandatory folic acid fortification of foodstuffs in the USA and Canada, and public health advice in Europe to take folic acid supplements preconceptionally and throughout pregnancy. As a consequence, NTD rates worldwide have dropped by at least 20% (Honein et al. 2001). It has been suggested that low maternal folate levels may also result in CHD. This has been studied extensively using epidemiology in a variety of human populations. Overall, there is some evidence suggesting that maternal folic acid supplementation during pregnancy might result in lowering CHD risk by as much as 50% (Mao et al. 2017; Parnell and Correa 2017; Feng et al. 2018). However, this conclusion is still somewhat controversial, as other studies have not replicated these results (Jenkins et al. 2007; Oyen et al. 2019). In addition, some widely used therapeutic drugs, such as aminopterin and methotrexate, are dihydrofolate reductase inhibitors. These block the conversion of folate to its more active metabolites (Lambie and Johnson 1985). Epidemiological studies show that use of these drugs in the second and third months of pregnancy increases risk of offspring CHD by 3-fold (Hernandez-Diaz et al. 2000; Czeizel et al. 2001). The hypothesis that folate deficiency increases CHD risk has also been tested using human genetics and animal studies. In humans, epidemiological studies have examined whether deleterious sequence variants in genes encoding folate transporters and metabolic enzymes in either mother or offspring increase risk of CHD. Although some studies suggest a small increase in CHD risk, the largest meta-analyses suggest there is no increase (Mamasoula et al. 2013). Similarly, experiments in mice with reduced folate levels due to genetic and/or dietary deficiency have given inconclusive results (Li et al. 2005a; Deng et al. 2008). Here, low maternal and embryonic folate levels leads to higher resorption rates and smaller embryos and placentas. However, it is not clear whether the small increase in VSD rate at E14.5 is a genuine disruption of heart development, or merely the result of developmental delay. The potential role of folate deficiency in CHD is further complicated by its role in DNA methylation and epigenetics. For example, a recent mouse study showed that genetic folate deficiency in a maternal grandparent resulted in two separate effects. Firstly, this had adverse effects on their daughter's uterine environment, leading to growth defects in some grandprogeny; and secondly, transgenerational epigenetic effects that resulted in congenital malformations independent of maternal environment (Padmanabhan et al.

2013). Such epigenetic effects may well confound epidemiological studies. In summary, it is unclear whether or not folate deficiency is a clinically-important risk factor for offspring CHD; nor is it clear the mechanism by which this might occur.

Hyperhomocysteinemia. Homocysteine (Hcy) is a sulfur-containing amino acid formed during the metabolism of methionine to cysteine (Kumar et al. 2017). In humans, elevated maternal Hcy levels are associated with an approximately 3-fold increased risk of having a child with CHD (Kapusta et al. 1999; Verkleij-Hagoort et al. 2006). Hyperhomocysteinemia (HHcy) can be caused by dietary deficiencies in folic acid, vitamin B6, and/or vitamin B12; or by genetic disorders in methionine, homocysteine or transcobalamin metabolism (Kumar et al. 2017). Since HHcy can often be symptomatic of folate deficiency, it has been proposed that the mechanism by which heart defects are induced in HHcy are the same as for folate deficiency. However, animal studies in both chick and mouse embryos shows that ectopic administration of homocysteine by itself is sufficient to induce septal and valve defects (Rosenquist et al. 1996; Han et al. 2009). Therefore, more work needs to be done to understand the mechanism.

Nicotinamide adenine dinucleotide (NAD) and vitamin B3. In humans, mutations in either of two genes encoding enzymes in the kynurenine pathway result in a spectrum of congenital abnormalities, including CHD (Shi et al. 2017). This pathway is the *de novo* NAD synthesis pathway, generating NAD from dietary tryptophan. NAD is an essential co-factor for cell function, and thus it is hypothesised that these congenital abnormalities arise as a direct result of NAD deficiency. This has been successfully modelled in embryos homozygous for null mutations in kynurenine pathway genes, in combination with a low-niacin diet (Shi et al. 2017). Oxidised NAD (NAD⁺) acts as an electron carrier or acceptor for at least 500 different cellular reactions (Kirkland 2012). These include ATP production, biosynthetic pathways and cellular responses to stress. Because of this complexity, it is currently unclear how NAD deficiency causes birth defects. When NAD⁺ accepts electrons, it is reduced to NADH. There is only a small pool of NAD⁺, so it must be constantly regenerated by transferring the electrons from NADH to another substrate, such as oxygen or pyruvate. NAD is generated either from dietary tryptophan by the *de novo* pathway, or from dietary niacin or nicotinamide (two forms of vitamin B3) via the salvage pathway. Interestingly, a small epidemiological study showed an increased risk of TGA (but not TOF) in offspring of mothers with the lowest quartile of dietary vitamin B3 intake (Shaw et al. 2010). In embryos with kynurenine pathway mutations, adequate NAD levels during development can be restored with vitamin B3 supplementation, preventing embryonic defects. This suggests that such dietary supplementation with vitamin B3 may reduce the risk of congenital malformations in humans with NAD deficiency.

Vitamin D. Recently, a small epidemiological study suggested that even moderate maternal vitamin D deficiency significantly increased offspring CHD risk (Koster et al. 2018). However, this study was flawed as vitamin D levels were measured 15 months after birth. The idea that vitamin D signalling might be important for embryonic heart development is supported by a study in a zebrafish model, where knockdown of both vitamin D receptors simultaneously during embryonic development resulted in abnormal atrio-ventricular boundary specification and cardiac laterality defects (Kwon 2016). However, it is currently unclear whether vitamin D deficiency is a significant contributor to CHD worldwide.

Intrinsic factors

In addition to extrinsic factors, maternal diseases and infections that change the uterine environment can also impact embryonic development. These factors can result in the

accumulation of a teratogenic substance, such as glucose or phenylalanine. Alternatively, they can alter the uterine environment more directly, as in maternal hyperthermia.

Diabetes

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia as a result of poor insulin secretion, detection or action (National Diabetes Action Group 1979). It is estimated that 425 million people have diabetes, and it is predicted that this will rise to 629 million by 2045 (International Diabetes Federation 2017). There are three main types of diabetes. Type I typically arises via an autoimmune-mediated destruction of pancreatic β -cells, and culminates in a rapid decline in insulin production and persistent hyperglycaemia. Type II is usually associated with obesity and ageing, and results from a lack of insulin secretion and diminished insulin sensitivity due to obesity-related β -cell loss. Gestational diabetes is a transient disease similar to type II that develops during pregnancy, but resolves following parturition. Offspring of mothers with pre-existing diabetes (types I and II) have an approximately 3-fold increased risk of any type of CHD (Hoang et al. 2017), and gestational diabetes carries an approximately 1.5-fold increased CHD risk (Hoang et al. 2017). This is also the case when the data is adjusted for potential confounders such as maternal body mass index, alcohol use and age. In addition, these children have elevated risks for other developmental abnormalities such as spina bifida, anencephaly, craniofacial anomalies and macrosomia. When broken down into specific sub-types, it is clear that some of these carry a particularly increased risk, for example the risk of persistent truncus arteriosus is increased 14-fold (Hoang et al. 2017). The mechanism by which maternal diabetes increases CHD risk is far from clear (Basu and Garg 2018). This is because it is a complex metabolic disease. In addition to hyperglycaemia, patients may also present with hyperlipidaemia (Abbate and Brunzell 1990); protein misfolding and glycation (Scheuner and Kaufman 2008; Singh et al. 2014); and impaired glucose tolerance and impaired insulin sensitivity. However, prevalence of offspring CHD correlates with increasing or poorly-controlled maternal blood glucose levels (BGL, measured by glycated haemoglobin, HbA1c, (Priest et al. 2015), strongly suggesting that hyperglycaemia is the primary teratogen. Despite this assumption, there is no consensus on how hyperglycaemia causes CHD. Extensive investigation in a variety of animal models has led to many disparate hypotheses including: hypoxia and/or increased oxidative stress (Li et al. 2005b); activation of the polyol or hexosamine pathways (Sussman and Matschinsky 1988; Horal et al. 2004); increased apoptosis (Gareskog et al. 2007); or endoplasmic reticulum stress (Wang et al. 2013). However, because diabetes is such a multifaceted disease, it is also possible that a combination of these events results in CHD.

Obesity

Maternal obesity during pregnancy (antenatal body mass index >30) is associated with many pregnancy-related adverse outcomes including reduced fertility, miscarriage and stillbirth (Guelinckx et al. 2008), as well as an increased risk of CHD and NTDs (Stothard et al. 2009). This is a health concern of increasing importance, as currently $>15\%$ of women of child-bearing age are obese, a further 40% are overweight, and these numbers are rapidly growing (WHO 2016). Like *diabetes mellitus*, obesity is a complex metabolic disease. In particular, it is often accompanied by type 2 diabetes, or impaired glucose tolerance at the very least, making the two diseases difficult to separate. Therefore, the prevailing hypothesis is that obesity and diabetes-induced congenital malformations may share a common aetiology. Human and animal studies rarely account for impaired glucose tolerance during obese pregnancies and, therefore, fail to provide an unbiased account of the effect of obesity alone. One exception concluded that increased maternal weight was associated with increased risk for CHD after adjusting for glucose tolerance (Brite et al.

2014). However, exactly how increased maternal weight in isolation might affect embryonic heart development is as yet unknown.

Phenylketonuria

Phenylketonuria (PKU) is an inborn error of metabolism, where dietary phenylalanine (Phe) cannot be metabolised (Blau et al. 2010). Patients have very high blood Phe levels, which result in cognitive decline, growth failure, poor pigmentation, developmental delay and seizures. These phenotypes are due to an accumulation of toxic by-products of improper Phe metabolism. Clinically, PKU can be managed using a low Phe diet from birth. This keeps the majority of the complications under control. Originally, such treatment was only continued until puberty. However, due to a growing awareness of continued complications of PKU in adults, it is now recommended that such treatment is lifelong (National Institutes of Health Consensus Development, 2001). One major complication for women is a highly increased risk of having offspring with intrauterine growth restriction and/or birth defects including microcephaly and CHD. This is likely to be a direct consequence of increased Phe levels, as women with poor dietary control have significantly higher rates of offspring with defects (Platt et al. 2000). Overall, there is a >6-fold increased risk of CHD in offspring of mothers with PKU (Platt et al. 2000; Levy et al. 2001). This has been mimicked in studies in mouse and chick (Seagraves and McBride 2012; Watson and Seagraves 2019). Transcriptomic analysis of the chick model suggests that phenylalanine teratogenicity may act through dysregulation of RA signalling (Watson and Seagraves 2019). This is supported by the observation that the characteristic facial phenotype of PKU-affected patients is often very similar to those of FASD (Levy and Ghavami 1996), which has also been associated with perturbed RA signalling (see above). Exactly how elevated Phe levels might affect RA signalling or metabolism awaits further investigation.

Viral infection and hyperthermia

Maternal infection with Rubella during the first 10 weeks of pregnancy causes birth defects in up to 90% of cases, and may result in stillbirth or miscarriage (Gregg 1941). Cardiac defects are present in about half of these cases, with branch pulmonary artery stenosis, patent ductus arteriosus and VSD the most prevalent forms of CHD observed (Vince 1970; Oster et al. 2010). This risk factor has largely been controlled by mass vaccination programs, but in recent years falling vaccination rates have led to increased numbers of Rubella outbreaks worldwide. First trimester infection with other viruses, and fever in general, also carry an increased risk of offspring CHD (Dreier et al. 2014; Luteijn et al. 2014; Shi et al. 2014). Although exactly how maternal viral infection causes birth defects is not clear, there is some evidence that the increased body temperature (hyperthermia) is the teratogen, rather than the virus itself. This is supported by animal studies, the earliest of which was the serendipitous observation from a guinea pig colony housed in an uninsulated shed during a Sydney heatwave that resulted in spontaneous abortions and limb defects (Edwards 1967). Subsequent studies in many species have confirmed that maternal hyperthermia can cause a range of embryonic defects, including heart defects (Edwards 2006). Various mechanisms have been proposed, but there is little evidence for most of these. Two of these seem most likely. Firstly, hyperthermia results in activation of the heat shock response, which is a subset of the UPR (Barna et al. 2018). Therefore, it is possible that hyperthermia acts by the same mechanism as hypoxia (Shi et al. 2016), see above). Alternatively, a recent study very elegantly showed that two temperature-activated ion channels expressed in neural crest cells might be responsible (Hutson et al. 2017). They show that activation of these channels by temperature or chemical agonist treatment replicated hyperthermia-induced defects in chick embryos. Furthermore, they showed that treatment with antagonists of these ion channels protected against the effects of

hyperthermia. Hyperthermia can also potentially occur via physical activity or exposure to high environmental temperatures. Such non-viral hyperthermia may also result in CHD. For example, one epidemiological study found that maternal exposure to 3-11 cumulative days or more of extreme heat (≥ 95 percentile daily maximum temperature) between weeks 3-8 of pregnancy carries an increased CHD risk of ~50% (Lin et al. 2018).

Concluding remarks

A significant proportion of CHD cases are likely to be caused by environmental risk factors, however it has been relatively difficult to clearly identify specific factors or their mechanism of action. Although the embryonic consequences of maternal Rubella infection and thalidomide use were identified readily through observation and anecdotal report, large scale epidemiological studies have been required to identify other potential risk factors (Erickson 1991). Such studies are complicated by the fact that the period of human embryonic heart development that is vulnerable to teratogenic perturbation is gestational weeks 3-8. However, even with the advent of routine ultrasonography, detection of CHD may only occur perinatally, when accurate details of the mother's health status or teratogen exposure details 6 months previously may be difficult to ascertain and collect. In addition, differences in CHD recording and classification between different countries may further complicate analysis (EuroCAT). Animal studies have also been useful in identifying and confirming environmental risk factors. However, the experience with thalidomide, which does not cause birth defects in rodents (Schumacher et al. 1968), underlines the difficulties of relying on animal experimentation as a sole measure of cardiac teratogenicity. Therefore, there may be other significant environmental risk factors for CHD that are yet to be identified. These may include factors identified from demographic studies that suggest differing CHD rates in populations stratified by age, ethnicity, socio-economic grouping and geographical location (van der Linde et al. 2011).

Advances in understanding how embryonic heart development occurs now provides tools for understanding how extrinsic and intrinsic factors acting on the mother can perturb the formation of the heart. This could potentially make it possible for the first time to significantly reduce the prevalence of CHD worldwide. Studies identifying genetic causes of CHD have often resulted in useful diagnostic and predictive capacity in a case-by-case manner for genetic counselling in individual families. However, genetic studies cannot suggest strategies for reducing the general population-wide risks of CHD. By contrast, studies of the environmental causes of CHD potentially could be directly translated to provide clinical impact. Understanding the molecular mechanisms of induction of CHD by a range of environmental and genetic factors, and identifying the stages in heart formation that are most vulnerable to these factors will aid the design of epidemiological studies to assess the extent of these risks in human CHD. It will also guide the formulation of health policy recommendations to aid women planning pregnancy to minimise their exposure to such environmental risks.

Acknowledgments

DBS and JIK are supported by funding from the British Heart Foundation (FS/17/55/33100 and RE/13/1/30181). NV is supported by a Novo Nordisk postdoctoral fellowship run in partnership with the University of Oxford.

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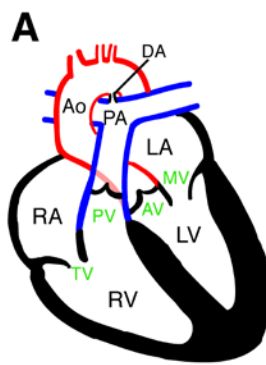
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Table 1

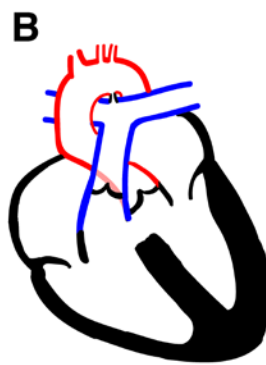
CHD type	Estimated prevalence (per 1000 births from Bruneau 2008)
Ventricular septal defect	4.0
Double outlet right ventricle	0.2
Overriding aorta	na
Transposition of the great arteries	0.2
Persistent <i>truncus arteriosus</i>	0.1
Tetralogy of Fallot	0.4
Atrial septal defect	1.0
Atrio-ventricular septal defect	0.3
Hypoplastic left heart syndrome	0.2
Aortic coarctation/stenosis	0.8
Bicuspid aortic valve	14.0
Persistent <i>ductus arteriosus</i>	0.8
Branch pulmonary artery stenosis	na

Figure 1 legend.

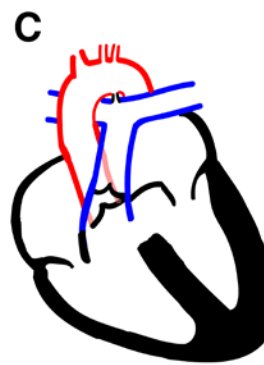
Schematic diagrams showing the structures of some of the most important forms of CHD induced by exposure to environmental factors.



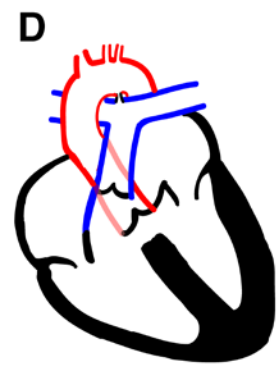
normal



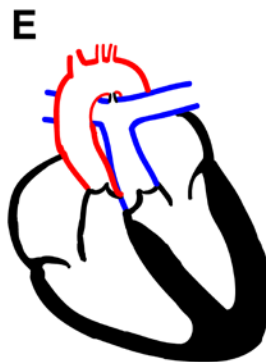
ventricular septal defect



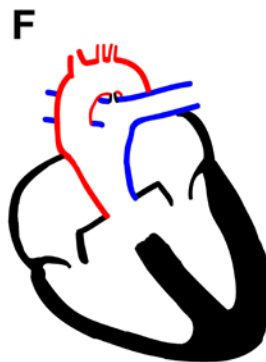
double outlet right ventricle



overriding aorta



transposition of the great arteries



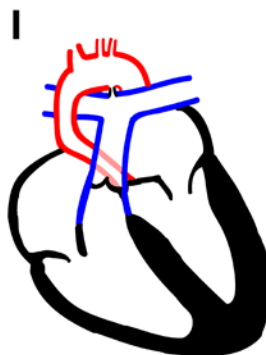
persistent truncus arteriosus



tetralogy of Fallot



hypoplastic left heart syndrome



aortic coarctation

RV: right ventricle
RA: right atrium
LA: left atrium
LV: right ventricle
TV: tricuspid valve
MV: mitral valve

PV: pulmonary valve
PT: pulmonary trunk
AV: aortic valve
Ao: aorta
DA: ductus arteriosus