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Concomitant genetic defects potentiate the adverse impact of prenatal alcohol exposure on cardiac outflow tract maturation

Drayton C. Harvey[1], **Prashan De Zoysa**[1], **Omar Toubat**[1], **Jongkyu Choi**[2], **Jahnavi Kishore**[1], **Hidekazu Tsukamoto**[3,4,5], **S. Ram Kumar**[1,6]

[1]Department of Surgery, Keck School of Medicine of University of Southern California, Los Angeles, California, USA

[2]Department of Medicine, Keck School of Medicine of University of Southern California, Los Angeles, California, USA

[3]Department of Pathology, Keck School of Medicine of University of Southern California, Los Angeles, California, USA

[4]Southern California Research Center for ALPD and Cirrhosis, Los Angeles, California, USA

[5]Greater Los Angeles VA Healthcare System, Los Angeles, California, USA

[6]Department of Pediatrics, Keck School of Medicine of University of Southern California, Los Angeles, California, USA

Abstract

Background: Prenatal alcohol exposure (PAE) is associated with an increased incidence of congenital heart defects (CHD), in particular outflow tract (OFT) defects. However, the variability in the incidence of CHD following PAE has not been fully explored. We hypothesize that a concomitant, relevant genetic defect would potentiate the adverse effect of PAE and partially explain the variability of PAE-induced CHD incidence.

Methods: The OFT is formed by the second heart field (SHF). Our PAE model consisted of two intraperitoneal injections (3 g/kg, separated by 6 hr) of 30% ethanol on E6.5 during SHF specification. The impact of genetic defects was studied by SHF-specific loss of Delta-like ligand 4 (*Dll4*), fibroblast growth factor 8 (*Fgf8*) and *Islet1*.

Results: Acute PAE alone significantly increased CHD incidence (4% vs. 26%, $p = .015$) with a particular increase in OFT alignment defects, viz., double outlet right ventricle (0 vs. 9%, $p = .02$).

Correspondence: S. Ram Kumar, University of Southern California, 1441 Eastlake Avenue, NOR 5322, Los Angeles, California 90033., rsubrama@usc.edu.

AUTHOR CONTRIBUTIONS

D.C.H., H.T., and S.R.K. were responsible for the experimental design of this study. D.C.H. performed all ethanol and PBS injections, embryo dissections, and genotyping. Blood alcohol content experiments were performed by D.C.H., J.C., and J.K. Paraffin sectioning and hematoxylin and eosin staining were performed by D.C.H., P.D., O.T., and J.K. Diagnosing cardiac phenotypes for all samples was conducted by D.C.H. and S.R.K. Statistical analyses were conducted by D.C.H. Writing and editing of this manuscript was done by D.C.H., H.T., and S.R.K.

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

In embryos with a SHF genetic defect, acute PAE significantly increased CHD incidence (14 vs. 63%, $p < .001$), including double outlet right ventricle (6 vs. 50%, $p < .001$) compared to controls. PAE ($p = .01$) and heterozygous loss of *Dll4* ($p = .04$) were found to independently contribute to CHD incidence, while neither *Islet1* nor *Fgf8* defects were found to be significant.

Conclusions: Our model recapitulates the increased incidence of OFT alignment defects seen in the clinic due to PAE. The presence of a concomitant SHF genetic mutation increases the incidence of PAE-related OFT defects. An apparent synergistic interaction between PAE and the loss of DLL4-mediated Notch signaling in OFT alignment requires further analysis.

Keywords

cardiac outflow tract; congenital heart defect; Notch; prenatal alcohol

1 | INTRODUCTION

Congenital heart defects (CHD) are the most common birth defects in the world, with an incidence of approximately 1.8 cases per 100 live births (Zimmerman et al., 2020). Extensive efforts have focused on investigating the etiology of CHD. Genetic defects have been widely evaluated, yet only 10–15% of CHD can be explained by an entirely genetic etiology (Ferencz, Boughman, Neill, Brenner, & Perry, 1989; Richards et al., 2008). It has been suggested that environmental teratogens, including recreational and prescription drug use, as well as environmental toxins may play a role in the etiology of CHD (Wilson, Loffredo, Correa-Villasefior, & Ferencz, 1998). Prenatal alcohol exposure (PAE) is one such environmental teratogen of relevance in CHD. Approximately 29% of children diagnosed with fetal alcohol spectrum disorder have CHD and PAE is estimated to result in over 10,000 excess cases of CHD each year in the United States (Burd et al., 2007). The majority of PAE research in relation to CHD has focused on chronic exposure in the context of fetal alcohol spectrum disorder (Denny, Coles, & Blitz, 2017; Kalisch-Smith, Ved, & Sparrow, 2020; O'Leary et al., 2010; Yang et al., 2015; Zhang et al., 2020) despite the fact that the majority of pregnant individuals do not engage in repetitive consumption of high doses of alcohol such as what is observed in alcohol use disorder (Denny, Acero, Naimi, & Kim, 2019; Ethen et al., 2009). It is more common for individuals to consume alcohol in the periconceptual period, especially prior to their awareness of being pregnant (Branum & Ahrens, 2017; Ethen et al., 2009; Zhu et al., 2015). The impact of such acute or short-term exposure has not been fully evaluated.

Cardiogenesis commences during week 3 of human development well before many women are aware of their pregnancy (Branum & Ahrens, 2017; Gittenberger-De Groot, Bartelings, Deruiter, & Poelmann, 2005; MacGrogan, Münch, & de la Pompa, 2018). Compounded by the fact that nearly half of the pregnancies are unplanned (Finer & Zolna, 2016), unintentional acute exposure to significant amounts of alcohol early in pregnancy could be a common occurrence amongst pregnant individuals, and have important implications in early heart development. Yet, this model has not been widely studied. Whereas, it has been shown that PAE is associated with clinically relevant CHD, the incidence and severity of defects in children with PAE varies widely between studies (Yang et al., 2015; Zhang et al., 2020). The exact mechanisms underlying this phenotypic variability is as yet unknown,

although timing of exposure and amount of alcohol consumption are thought to play a role (Petrelli, Weinberg, & Hicks, 2018). In children with PAE, after atrial and ventricular septal defects (Burd et al., 2007; O'Leary et al., 2010), which represent the most common forms of CHD overall, cardiac outflow tract (OFT) defects are the next most represented CHD (Daft, Johnston, & Sulik, 1986; Yang et al., 2015; Zhang et al., 2020; Zhu et al., 2015). In a California study, it was found that 49% of women who gave birth to a child with an OFT defect consumed alcohol during pregnancy (Carmichael, Shaw, Yang, & Lammer, 2003).

During development, the cardiac OFT develops primarily from progenitor cells originating in the so-called second heart field (SHF) (Cai et al., 2003; Kelly, Buckingham, & Moorman, 2014). Initially, the OFT is connected exclusively to the right ventricle. Following cardiac assembly, through a complex, temporally and spatially regulated developmental process, this OFT is aligned over the two ventricles, septated and connected to the corresponding pulmonary and systemic circulations. This intricate mechanism is governed by a number of molecules with critical roles in SHF biology (Black, 2007; Park et al., 2006). Partial defects in these molecules may themselves not be sufficient to result in clinically significant CHD, but may serve to establish a genetically permissive environment in which an additional teratogen, such as PAE, may induce a more penetrant or severe phenotypic defect. Such a cooperation between genetic mutations and PAE has been well documented in fetal alcohol spectrum disorder research, centering on a variety of genes involved in alcohol metabolism, cholesterol synthesis, one-carbon metabolism, and more (Eberhart & Parnell, 2016; Ehrhart et al., 2019; Fernandes, Buckley, & Eberhart, 2018; Kaminen-Ahola, 2020; Liyanage, Curtis, Zachariah, Chudley, & Rastegar, 2017; Petrelli et al., 2018). PAE's interaction with risk alleles in platelet-derived growth factor genes have been studied in relation to obstructive heart defects, though not found to be significant (Tang et al., 2018). A significant relationship between acute, binge PAE, the presence of genetic mutations (*Sonic Hedgehog* or *Gli2*) and phenotype severity has been demonstrated in forelimb defects (Fish, Murdaugh, Sulik, Williams, & Parnell, 2017). Such an interaction, however, has not been studied in the context of OFT development. We hypothesized that a concomitant genetic defect of relevance in SHF biology, which may not be deleterious by itself, would potentiate the adverse effect of PAE and, in part, explain the variable impact of PAE on OFT maturation.

2 | MATERIALS AND METHODS

2.1 | Mice

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Southern California, ensuring protection of the animals used in this work and that this work met the ethical approval standards of the committee. *Islet1-cre* (Cai et al., 2003) and *Mef2c-AHF-cre* (Verzi, McCulley, de Val, Dodou, & Black, 2005) mice have been previously described, wherein expression of the *cre* gene is dependent on expression of *Islet1* and *Mef2c*, respectively. Expression of the *cre* gene thus begins at E7 in the *Islet1-cre* mice and E7.25 in the *Mef2c-AHF-cre* mice. The *cre* gene was maintained on the paternal side, eliminating risk of germline transmission. $Dll4^{F/F}$ mice were obtained from the Duarte lab (Benedito & Duarte, 2005; Duarte et al., 2004) and $Fgf8^{F/F}$ mice from the Moon lab (Park et al., 2006), both previously reported. CD1 dams (CD1, The Jackson Laboratory, Bar

Harbor, ME) were used to generate litters of *Islet1-cre* and *cre*-negative embryos to analyze the impact of heterozygous loss of *Islet1* alone. E0.5 was determined by the presence of a vaginal plug.

2.2 | PAE model

Our primary focus was to study the impact of PAE in the form of acute moderate alcohol exposure on OFT maturation. To that end, we elected to modify the existing model of acute PAE (Fish et al., 2017; Serrano, Han, Brinez, & Linask, 2010) in alcohol-naïve mice. In our model, ethanol (30% v/v ethanol in phosphate-buffered saline [PBS]) was injected intraperitoneally twice 6 hr apart at a 3 g/kg/dose. We chose to inject pregnant dams at E6.5, an important time-point for SHF progenitor specification and the first appearance of cardiac progenitor cells (Kokkinopoulos et al., 2015; Saba et al., 2019; Figure 1). Control mice received the equivalent volume of PBS. Blood alcohol content (BAC) was measured by analysis of serum obtained via intracardiac puncture, using colorimetric analysis tied to the oxidation of NAD+ to NADH by alcohol dehydrogenase (EnzyChrom™ Ethanol Assay Kit, BioAssay Systems). Blood was obtained at 3 hr before the first alcohol injection (n = 4), 30 min after the first injection (n = 4), 30 min before the second alcohol injection (n = 4), 30 min after the second injection (n = 4), and 3 hr after the second injection (n = 3). An additional cohort of pregnant mice (n = 4) was sampled for comparison at 30 min after the second injection.

2.3 | Experimental procedure

Embryos were dissected at E14.5 when development of the cardiac OFT was complete and genotyped by polymerase chain reaction using specific primers. Embryos were embedded in paraffin and serial transverse sections analyzed for defects by standard Hematoxylin–Eosin staining. *Cre*-negative and PBS-exposed embryos served as appropriate controls with multiple litters studied in each exposure group. No litters were excluded from analysis and no dead embryos or reabsorbed implantations sites were observed.

2.4 | Statistics

All statistical analysis was conducted in R. An independent student t test was conducted to compare the mean BAC of nonpregnant and pregnant mice 30 min after the second alcohol injection. Given that more than 20% of categories analyzed had a frequency less than 5, Fisher's exact test analysis was performed to calculate the odds ratio and identify significant associations between the incidence of CHD and both a subject's genetic background and exposure to alcohol. Logistical regression was then applied to identify whether PAE and/or genotype were associated with CHD.

3 | RESULTS

Following each alcohol injection, BAC spiked at 30 min (Figure 1b) to levels consistent with what would be observed following an acute, binge alcohol exposure in humans (0.43 ± 0.04 g/dL after first and 0.39 ± 0.04 g/dL after second), as defined by the National Institute of Alcohol Abuse and Alcoholism (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 2004). Baseline levels were observed 30 min before and 3 hr after the second

injection. There was no significant difference in the peak BAC 30 min after alcohol injection between nonpregnant and pregnant mice (Figure 1c).

We first began by analyzing the impact of PAE on OFT development in our model in wild-type mice (Figure 2a). Of the 54 wild-type embryos with acute PAE, 14 (26%) demonstrated CHD compared to one out of the 28 controls (4%, $p = .015$, odds ratio [OR] 9.27, 95% confidence interval [CI] 1.27, 413.29). The PBS exposed control embryo had an isolated, small, shallow ventricular septal defect. In embryos with PAE, isolated ventricular septal defects were observed in 5 (9%) and double outlet right ventricle in 9 (17%). The defects observed in our PAE model therefore reflect CHD encountered in clinical practice in children with PAE.

We then analyzed the combined impact of embryos carrying genetic mutations with relevance to SHF biology (hereafter referred as embryos carrying a SHF-mutation) and PAE. To this end, we used *Islet1-cre* mice, a *cre* recombinase knock-in mouse, thereby heterozygous (*Islet1* +/−) for the crucial SHF transcription factor, *Islet-1* (Cai et al., 2003; Srinivas et al., 2001). We also studied *Mef2c*-driven SHF-specific conditional heterozygous loss of *Fgf8* and *Dll4*, both proteins known to play an important role in SHF proliferation and differentiation (Chapman et al., 2020; De Zoysa et al., 2020; High et al., 2009; Kopan & Ilagan, 2009). Seven of 48 (14%) embryos carrying any SHF mutation without PAE had a heart defect—4 (8%) isolated ventricular septal defect and 3 (6%) double outlet right ventricle—with 1 of the isolated ventricular septal defects being an *Mef2c-cre*, $Dll4^{F/WT}$ embryo and the other 6 defects observed in *Islet1-cre*, $Dll4^{F/WT}$ embryos. Heterozygous mutations of *Islet-1*, *Fgf8*, and *Dll4* by themselves are thus not deleterious enough on their own to result in significant CHD. In contrast, when looking at all SHF-mutant embryos exposed to PAE, there is a significantly higher incidence of CHD (34/54, 63%) compared to wild-type/PBS (OR 43.96, 95% CI 6.29, 1912.28, $p < .001$), wild-type/PAE (OR 4.78, 95% CI 1.99, 12.05, $p < .001$), and SHF-mutant/PBS (OR 9.70, 95% CI 3.48, 30.69, $p < .001$) embryos. Six (11%) SHF-mutant/PAE embryos demonstrated an isolated ventricular septal defect, 27 (50%) double outlet right ventricle, and 1 (2%) embryo had a common arterial trunk that arose from the right ventricle.

We wanted to evaluate if any of the evaluated SHF mutations had greater relevance in PAE than the others (Figure 2). A total of 25 *Islet1-cre* (*Islet1* +/−) embryos (11 PBS, 14 PAE), 19 *Mef2c-cre*, $Fgf8^{F/WT}$ embryos (10 PBS, 9 PAE), and 31 *Mef2c-cre*, $Dll4^{F/WT}$ embryos (13 PBS, 18 PAE), were examined. Compared to wild-type/PAE embryos, *Mef2c-cre*, $Dll4^{F/WT}$/PAE embryos had a significantly higher incidence of CHD (OR 7.19, 95% CI 1.97, 30.71, $p < .001$), with a fourfold higher incidence of double outlet right ventricle. *Mef2c-cre*, $Fgf8^{F/WT}$, PAE did not demonstrate statistically significantly higher frequencies of CHD than wild-type/PAE embryos. While *Islet1* +/−/PAE embryos exhibited a CHD frequency nearly twofold greater than wild-type/PAE embryos (50 vs. 26%), the increase was not statistically significant ($p = .108$), potentially due to small sample size. The frequency of CHD in *Mef2c-cre*, $Dll4^{F/WT}$/PAE embryos (but not other SHF-mutations) appeared greater than the simple summation of the frequency of CHD in wild-type/PAE embryos and *Mef2c-cre*, $Dll4^{F/WT}$/PBS embryos. To formally test this, we undertook independent regression analysis of PAE and the individual SHF-mutations

or various combinations of two of the three mutations studied. PAE was found to be independently associated with CHD ($p = .01$). Heterozygous loss of *Dll4*, but not *Islet1* or *Fgf8*, resulted in a significant association with incidence of CHD (OR 3.07, 95% CI 1.23, 13.57, $p = .04$) after accounting for PAE. Together, these results demonstrate that heterozygous loss of *Dll4*, which by itself does not significantly increase the incidence of CHD, establishes a "permissive" genetic environment. The addition of PAE results in a significant increase in CHD incidence, more than what is observed with genetic defects or similar dose of PAE alone. Heterozygous loss of DLL4-mediated Notch signaling appears to be independently associated with CHD after accounting for PAE, indicating a potentially synergistic interaction between these two deleterious events.

Furthermore, we observed a gradation in the severity of CHD observed in the different subgroups. A normal heart at E14.5 displays fully septated ventricles (Figure 3a) and two separate OFTs—the aorta which arises from the left ventricle (Figure 3a′) and the pulmonary artery from the right ventricle (Figure 3a″). In embryos with an isolated ventricular septal defect, there was variability in the size of the defect, the smallest one observed in the wild-type/PBS embryo (data not shown). Furthermore, double outlet right ventricle was the most dominant phenotype in embryos with PAE and carrying an SHF mutation, *Mef2c-cre*, $Dll4^{F/WT}$/PAE embryos in particular, with very few instances of isolated ventricular septal defects. The degree of malalignment of the aorta was also found to vary. In milder form, the aortic valve straddled the ventricular septal defect (over-riding aorta), observed most frequently in the wild-type and *Mef2c-cre*, $Fgf8^{F/WT}$ PAE embryos (Supplemental Figure S1a,a′). Malalignment was greater in the more obvious double outlet right ventricle phenotype, where the aorta arose entirely from the right ventricle (Supplemental Figure S1b′) with lack of fibrous continuity between the aortic and mitral valves (Supplemental Figure S1b). This classic double outlet right ventricle phenotype was more dominant in *Mef2c-cre*, $Dll4^{F/WT}$/PAE embryos. To ensure that the observed phenotypes were not a result of PAE causing delayed embryonic development, we examined other organs in our embryos at E14.5, which were found to be developmentally appropriate (Supplemental Figure S2) (Catón & Tucker, 2009; Chen et al., 2017; Krishnan et al., 2014; Martin, 1990; Schittny, 2017; Wilding Crawford, Foley, & Elmore, 2010). To ensure that the observed cardiac phenotype was not a result of delayed closure of the ventricular septum, we evaluated *Mef2c-cre*, $Dll4^{F/WT}$ embryos with PAE at E15.5 and E16.5. The increased prevalence of CHD in alcohol-exposed embryos persisted in both E15.5 and E16.5 embryos. Compared to no CHD in wild-type/PBS embryos, 25% of wild-type/PAE embryos at E15.5 and 29% at E16.5 had a CHD. In contrast, 60% of *Mef2c-cre*, $Dll4^{F/WT}$/*PAE* embryos at E15.5 and 67% at E16.5 demonstrated CHD (Supplemental Figure S3). The defects observed at E15.5 and E16.5 were thus consistent with the defects observed at E14.5. These results indicate that the cardiac defects observed at E14.5 represent true CHD rather than delayed cardiac development.

We then evaluated if the extent of genetic burden played a role in CHD resulting from PAE. To mimic an oligogenic inheritance pattern, we generated heterozygous loss of *Dll4* in SHF induced by the more globally expressed *Islet1-cre*. Because this *cre* is a knock-in (unlike the transgenic *Mef2c-cre*), these mice also have heterozygous loss of *Islet-1* (+/−). In addition to being an oligogenic mutation, given the more global SHF expression of *Islet1*, the extent

of the genetic insult is greater in this model compared to the *Mef2c-cre* that is restricted to the anterior SHF (Black, 2007; Cai et al., 2003; Srivastava & Olson, 2000). We studied 27 *Islet1-cre*, $Dll4^{F/WT}$ embryos (Figure 2; 14 PBS, 13 PAE). Heterozygous loss of both *Islet1* and *Dll4* without PAE (*Islet1-cre*, $Dll4^{F/WT}$ /PBS) led to a statistically significant increase in CHD incidence (OR 18.54, 95% CI 1.86, 958.51, $p = .003$) compared to wild-type/PBS embryos. *Islet1-cre*, $Dll4^{F/WT}$/PAE embryos demonstrated significantly higher incidence of CHD compared to wild-type/PAE, *Islet1* +/−/PAE and *Mef2c-cre*, $Fgf8^{F/WT}$/PAE embryos. While the incidence of CHD was slightly greater than that of *Mef2c-cre*, $Dll4^{F/WT}$/PAE embryos, there was no statistically significant difference between the two. This may be a result of small sample size or alternatively indicate *Dll4* heterozygosity is the primary genetic factor that interacts with PAE during heart development. Over half the embryos (7/13) had double outlet right ventricle. One embryo demonstrated a large ventricular septal defect (Figure 3d; Supplemental Figure S1c) and a single common arterial trunk exiting the right ventricle (Figure 3d,d″). This vessel arose exclusively from the right ventricle with the ventricular septal defect being the only outlet for blood from the left ventricle (Supplemental Figure S1c). We interpret this to be a mal-aligned OFT that in addition did not undergo septation (Ma et al., 2016).

4 | DISCUSSION

Fetal alcohol spectrum disorder is a disease spectrum encountered in children chronically exposed to significant quantities of alcohol in utero (Denny et al., 2017; Wozniak, Riley, & Charness, 2019). Although neurologic and craniofacial abnormalities predominate in these children (Calhoun & Warren, 2007), 28.6% of them also harbor a CHD (Burd et al., 2007). The timing of cardiac specification and development early in the first trimester presents a context in which acute, yet significant, exposure to alcohol within this critical window of time may result in CHD, even in the absence of other features related to chronic, sustained heavy alcohol exposure. In order to model the OFT defects observed in children with PAE in the clinic, we set up an acute, binge PAE mouse model. Our model reflects alcohol exposure akin to that of a fetus of an individual who may drink a significant amount of alcohol in an acute setting, enough to clear the 0.08 g/dL benchmark set by the NIAAA for binge drinking, rather than behavior consistent with prolonged consumption of alcohol seen with alcohol use disorder (Grant et al., 2017; NIAAA, 2004). A very similar model has been previously studied in the context of limb development (Fish et al., 2017). The timing of alcohol exposure in our model was chosen to coincide with the specification of SHF cells that eventually contribute to the majority of cardiac OFT (Kokkinopoulos et al., 2015; Saba et al., 2019). Embryos with PAE in our model demonstrate cardiac defects akin to those observed in the clinical setting following periconceptual drinking (Zhu et al., 2015). This would provide credence to the fact that timing of PAE plays an important role in determining the nature of the developmental defect that ensues.

We hypothesized that the variability in the phenotypes of PAE-related CHD could be further explained by gene–environment interactions as has been shown in the case of limb defects and studied in obstructive heart defects, which ultimately was not found to be significant (Fish et al., 2017; Tang et al., 2018). There is growing evidence to suggest that multiple teratogenic insults, in particular genetic and environmental factors, act in concert to cause

CHD (Chapman et al., 2020; Wilson et al., 1998). We studied three different heterozygous mutations in molecules of relevance to SHF biology and show that PAE-induced CHD is increased in embryos with genetic mutations. These mutations by themselves do not result in significant CHD. As only heterozygous loss of *Dll4* was independently found to be associated with CHD frequency, we submit that its role is primarily to establish a genetically susceptible environment. In the clinical setting, these mutations may be inherited from a parent who is a genotypic carrier without overt phenotypic manifestations. Alternatively, they could be accrued as *de novo* somatic mutations during embryogenesis. Regardless, when such genetically vulnerable embryos are exposed to an additional genetic mutation (i.e., heterozygous loss of *Islet1)* or teratogenic event, PAE in our case, a higher incidence of more severe CHD ensues. The complexity of PAE-induced CHD is further enhanced in the setting of oligogenic mutations, wherein the genetic burden is increased, another well-described clinical entity in the etiology of CHD (Akhirome, Walton, Nogee, & Jay, 2017). It would be interesting to understand the mechanistic basis by which PAE impacts SHF biology and OFT maturation. Prior reports in zebrafish have suggested that PAE reduces the number of *Islet1*-positive SHF progenitors and results in disorganization of highly active Notch signaling cells in the endocardium (Sarmah & Marrs, 2017; Sarmah, Muralidharan, & Marrs, 2016). We have previously demonstrated that depletion of the SHF progenitor pool leads to inadequate cellular incorporation into the developing OFT. As such, the foreshortened OFT is incapable of appropriate alignment across the developing ventricles, resulting in double outlet right ventricle (Cai et al., 2003; De Zoysa et al., 2020; High et al., 2009; Park et al., 2006). Formal studies are required to confirm that this mechanism is relevant in PAE-induced CHD in mammals. If found to be concordant with the findings on SHF progenitors and Notch signaling in zebrafish models, it may suggest the mechanism by which PAE interacts with cardiac development is evolutionarily conserved. Alternatively, PAE has been well documented to adversely impact the neural crest population broadly (Rovasio, 1995; Smith, Garic, Flentke, & Berres, 2014; Wang & Bieberich, 2010). Signaling from cardiac neural crest cells to SHF progenitors, which exhibit incredible physical proximity during early cardiogenesis, also plays an important role in the elongation of the OFT (Kelly & Buckingham, 2002; Rovasio, 1995; Smith et al., 2014; Wang & Bieberich, 2010). It is therefore also possible that the observed effects of PAE in our model are a direct result of alcohol impacting cardiac neural crest cells, while the genetic injury is restricted to the SHF. We are currently pursuing mechanistic studies to address these questions.

Although both Islet-1 and FGF8 have crucial roles in OFT maturation (Park et al., 2006; Sarmah & Marrs, 2017), our data only indicate a potential synergistic relationship between PAE and DLL4 expression. PAE has previously been described to disrupt Notch signaling in the developing central nervous system and in the atrio-ventricular canal of the heart (MacGrogan et al., 2018; Sarmah et al., 2016; Tong et al., 2013). DLL4 was originally described as an arterial-specific Notch ligand (Duarte et al., 2004; Shutter et al., 2000). Haploinsufficiency of DLL4 results in embryonic lethality secondary to vascular maturation arrest (Duarte et al., 2004). DLL4-mediated Notch signaling has since been established to provide pro-proliferative cues essential to lymphocytic and retinal progenitor cells (Luo et al., 2012; Rocha, Lopes, Gossler, & Henrique, 2009; Yu et al., 2015). We are among the

first to show that DLL4 plays a similar role in the SHF (De Zoysa et al., 2020). Loss of DLL4 expression leads to loss of SHF proliferation and apoptosis, leading to reduction in SHF progenitor pool available for incorporation into the OFT. The resulting CHD is uniformly double outlet right ventricle (De Zoysa et al., 2020). Thus, the observed synergy between PAE and *Dll4* mutation could be a result of overlapping downstream functional effects. However, given that FGF8 also supports SHF proliferation, a molecular synergy between the Notch pathway and PAE cannot be ruled out. In such a case, mutations in Notch signaling could establish a genetic background that is uniquely susceptible to the teratogenic effects of acute PAE. Future studies to specifically address these hypotheses have significant clinical relevance. Notch mutations have been identified as pathogenic in human syndromes that include OFT defects, such as Adams–Oliver syndrome (exhibiting double outlet right ventricle, resulting from heterozygous loss of *Dll4*) and Alagille syndrome (exhibiting another alignment defect, tetralogy of Fallot, resulting from inactivating mutations in *Notch2* or *Jag1*), as well as in nonsyndromic cases of tetralogy of Fallot (De Zoysa, Toubat, Harvey, Choi, & Kumar, 2021; MacGrogan et al., 2018).

Environmental factors are modifiable teratogens, unlike genetic mutations. Awareness and education can modify behaviors and lead to avoidance of maternal alcohol consumption. The potential impact of dietary or nutritional modifications, such as folate ingestion, to counteract the effect of alcohol have been studied in the context of neurologic development (Young, Giesbrecht, Eskin, Aliani, & Suh, 2014; Zhang et al., 2008) and may have relevance in CHD as well (Liu et al., 2016; Serrano et al., 2010). If particular molecular pathway defects are identified as being at higher risk for PAE-induced CHD, similar to DLL4 in our current model, this may represent another opportunity for targeted intervention in families that may harbor silent mutations. In this regard, population studies identifying defects in specific genetic pathways in children with PAE-related CHD may be of particular relevance.

In summary, our animal model of acute, binge PAE recapitulates the increased incidence of OFT alignment defects seen in the clinic due to PAE. We also show that the concomitant presence of heterozygous loss of *Dll4* in the SHF, which by itself may not be deleterious, establishes a permissive genetic environment. PAE, at the time of SHF specification, in the background of this genetic vulnerability leads to a higher incidence of OFT defects. The next steps would be to unravel the mechanisms underlying this CHD Risk Triad—(a) a teratogenic exposure, (b) during a critical developmental window, and (c) in the context of a genetically permissive background (Chapman et al., 2020; Wilson et al., 1998). There appears to be a potentially synergistic interaction between PAE and loss of DLL4-mediated Notch signaling in OFT alignment, which also requires further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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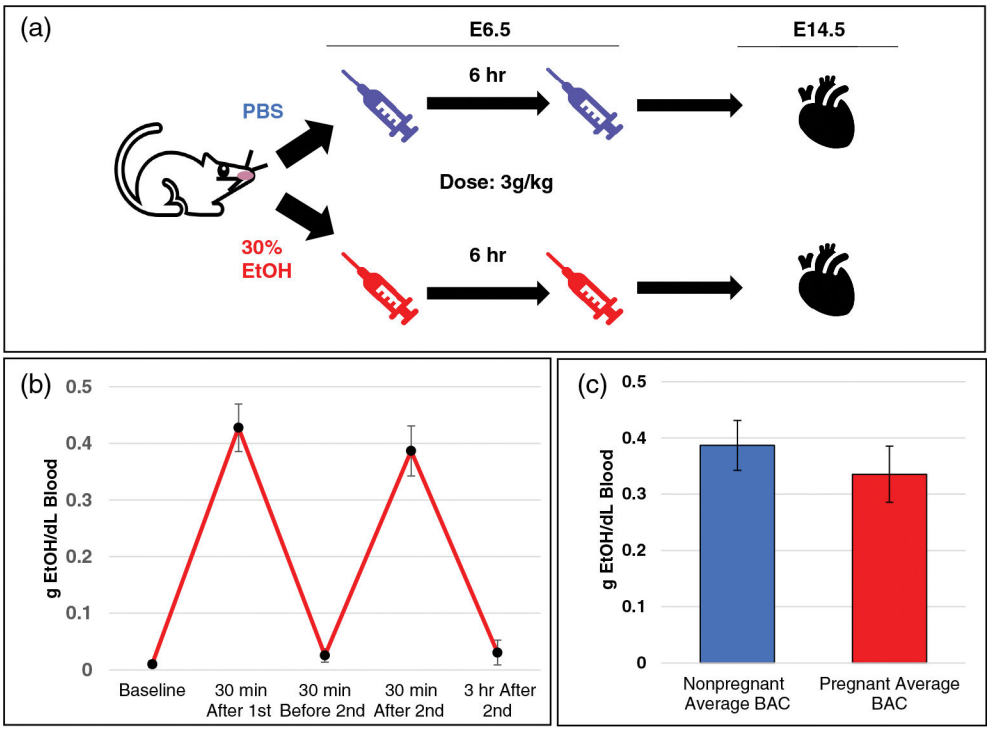

FIGURE 1.

Prenatal alcohol and phosphate-buffered saline (PBS) exposure protocol and blood alcohol content. Acute prenatal alcohol exposure protocol is depicted (a). At embryonic day 6.5 (E6.5) the pregnant dam was administered two intraperitoneal injections of either 3 g/kg PBS or 30% ethanol. The embryos were analyzed for cardiac phenotype on E14.5. Blood alcohol content was found to peak (b) 30 min after each injection. Baseline levels were achieved by 30 min prior to the second injection and 3 hr after the second injection. There was no significant difference in levels between nonpregnant (average peak 0.39 ± 0.04 g/dL after second injection) and pregnant mice (0.34 ± 0.05 g/dL after the second injection, c)

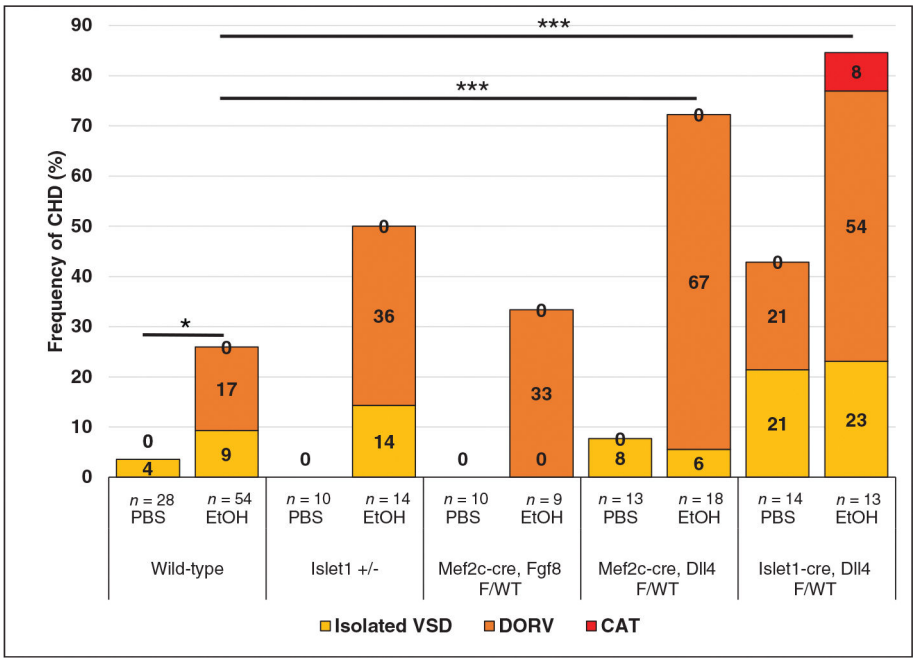

FIGURE 2.

The combined deleterious impact of prenatal alcohol exposure (PAE) teratogenicity and second heart field (SHF) genetic defects. Embryos with heterozygous loss of SHF genes were examined for the presence of congenital heart defects (CHD). Comparisons were made across genotypes and between those exposed to phosphate-buffered saline (PBS) or received acute PAE. Wild-type/PAE embryos had a significant ($p < .05$) increase in CHD frequency (26 vs. 4%). All embryos carrying a SHF mutation with PAE have a higher frequency of CHD than wild-type/PAE mice, but only *Dll4* mutations reached statistical significance. *Mef2c-cre*, $Dll4^{F/WT}$ and *Islet1-cre*, $Dll4^{F/WT}$/PAE mutants had the most significant increase in overall CHD ($p < .001$ and $p < .005$, respectively) and frequency of DORV (67 and 54 vs. 17%). CAT, common arterial trunk; DORV, double outlet right ventricle; Isolated VSD, isolated ventricular septum defect. *$p < .05$, ***$p < .005$

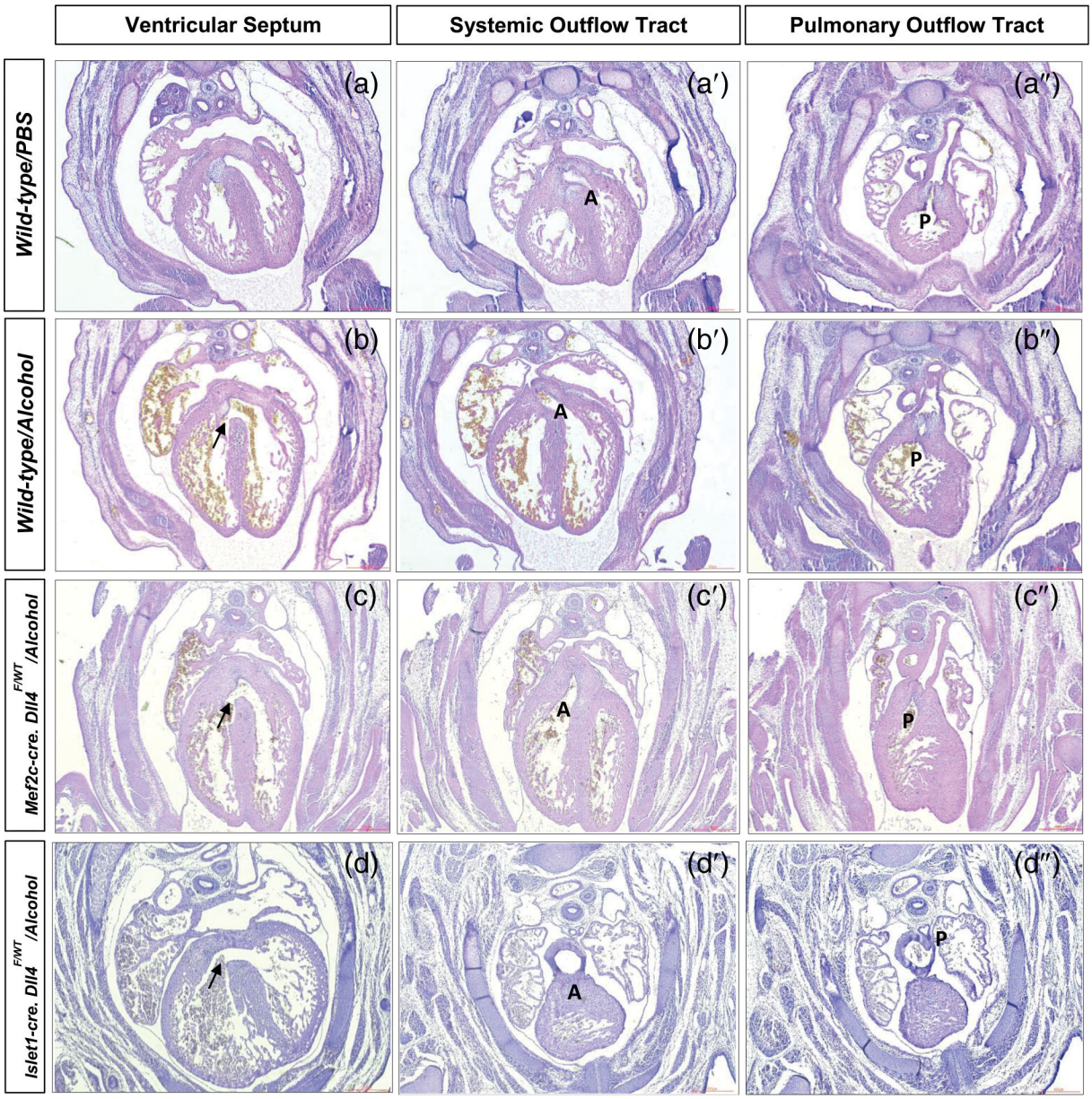

FIGURE 3.

Phenotypic spectrum of lesions observed with combined Notch mutation and prenatal alcohol exposure (PAE). Typical cardiac anatomy at embryonic day 14.5 (E14.5) is displayed in panels a-a″. The ventricular septum is fully intact (a), the aorta arises from the left ventricle (a′), and the pulmonary artery arises from the right ventricle (a″). PAE and mutations in the second heart field (SHF) result in a variety of cardiac phenotypes. The mildest lesion is an isolated ventricular septal defect (arrow in b) with normally aligned outflow tracts (b′ A indicates aortic valve, b″ P indicates pulmonary valve). Double outlet right ventricle consists of an obligate ventricular septal defect (VSD; arrow in a), observed to be larger than when the VSD was isolated, and malalignment of the outflow tract (OFT). The aorta is situated over the right ventricle (A in c′) as is the pulmonary valve (P in c″). The most severe phenotype observed was persistent truncus arteriosus in a PAE *Islet1-cre*,

Dll4$^{F/WT}$ embryo. This also consists of an obligate VSD (arrow in d) and is defined by the lack of septation of the OFT into two separate vessels, and instead continues as a single common arterial trunk (A in d′) from which the pulmonary artery arises (P in d″).Images were brightened in ImageJ, which was applied evenly to all parts of each image and equally to all subjects. Scale bar = 400 μm