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Environmental mechanisms of orofacial clefts

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Abstract

Orofacial clefts (OFCs) are among the most common birth defects and impart a significant burden on afflicted individuals and their families. It is increasingly understood that many non-syndromic OFCs are a consequence of extrinsic factors, genetic susceptibilities, and interactions of the two. Therefore, understanding environmental mechanisms of OFCs is important in the prevention of future cases. This review examines the molecular mechanisms associated with environmental factors that either protect against or increase the risk of OFCs. We focus on essential metabolic pathways, environmental signaling mechanisms, detoxification pathways, behavioral risk factors, and biological hazards that may disrupt orofacial development.

1. Introduction

Orofacial cleft (OFC) is among the most common birth defects affecting newborns and occurs at an average frequency of 1 out of 700 live births. Craniofacial structures are formed within the first ten weeks of human development, involving a tight coordination between molecular signaling pathways (Reynolds et al., 2020) (in this issue) and cellular processes such as proliferation, migration, differentiation, transition, and apoptosis (Ji et al., 2020) (in this issue). Perturbation of any of these processes could offset the delicate balance required for normal craniofacial morphogenesis and results in birth defects such as OFCs. There are significant personal, social, and financial burdens associated with OFCs among afflicted individuals and their families. Further, those suffering from OFCs may be at increased risk of associated morbidities (Silva et al., 2018). It is therefore a priority to understand all aspects of OFC etiology so that it may be prevented when possible. Syndromic OFCs are

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA SHARING AND DATA ACCESSIBILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

typically associated with genetic mutation or chromosomal abnormalities. Non-syndromic OFCs (to which "OFC" refers from here on) are believed to be multifactorial and frequently involve genetic susceptibility to environmental factors (gene-environment interactions, or GxE). Epigenetic processes such as DNA methylation or histone modification, as well as non-coding microRNAs that regulate gene expression, are also influenced by environmental factors and may account for OFCs (Garland et al., 2020) (in this issue).

Generally, OFCs are divided into two classes: cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). There is also evidence that cleft lip only (CLO) is a distinct class, although this can be difficult to discern in severe CLO cases since palatogenesis may depend on lip fusion (Harville, Wilcox, Lie, Vindenes, & Abyholm, 2005). Orofacial development involves several morphogenetic steps, molecular signaling pathways, and epithelial-mesenchymal interactions that can be affected by extrinsic factors (Ji et al., 2020; Reynolds et al., 2020) (in this issue). Lip development precedes palatogenesis, the latter of which can be generalized to involve palatal shelf growth, elevation, and fusion. During fusion, cells of the medial edge epithelium (MEE) from both shelves merge, becoming the medial epithelial seam (MES) and disappearing through apoptosis, migration, and epithelial-mesenchymal transition (EMT). These processes involve signaling through various pathways such as Wnt, fibroblast growth factor (Fgf), transforming growth factor beta (Tgf-β), epidermal growth factor (Egf), Notch, Hedgehog, and others. In some cases, these processes can be perturbed by environmental factors that alter gene expression and offset the delicate balance of developmental signaling (X. Z. Liu et al., 2020). In other cases, genetic susceptibilities can adversely affect orofacial development but only in the context of an environmental stressor. For example, a recent study found that deficiency of the transcription factor *Msx1* does not affect murine lip fusion unless the mother endures hypoxic stress (Nakatomi et al., 2020). Other stressors, like oxidative stress mediated by reactive oxygen species (ROS), can directly damage biomolecules and do not necessarily require genetic susceptibility to cause OFCs (Larouche & Hales, 2009).

Understanding the downstream molecular effects of environmental risk factors may be informative for health professionals that issue guidance for protecting human developmental health. In this review, we present a survey on recent studies examining environmental effects on the risk of oral clefts. We begin with metabolism of the essential nutrients, folates and retinoids, and their role in orofacial development. We then describe three major environmental signaling pathways and their potential interactions in mediating teratogenicity. Next, we describe how detoxification pathways and associated genetic variants could influence OFCs. Behavioral risk factors, such as smoking tobacco, drinking alcohol, or occupational exposures, and possible mechanisms are also reviewed. Finally, we consider evidence for the involvement of pathogenic factors in OFC etiology.

2. Metabolism of folates and retinoids in orofacial clefts

2.1. Folic acid metabolism

Folate (vitamin B9) is an essential hydrophilic nutrient required for 1-carbon reactions in various cellular processes. Animals are unable to produce folate *de novo* and must obtain it through the diet. Some folate-dependent bioactivities include the synthesis of nucleotides,

methionine, and polyamines, as well as the methylation of biomolecules including DNA, RNA, proteins, lipids, and other small molecules (Crider, Yang, Berry, & Bailey, 2012). Folate is therefore required for cell proliferation, differentiation, repair, and other processes necessary for development such as chromatin remodeling and genetic imprinting (Loenen, 2006). It is particularly critical for neural tube development, as folate deficiency is a well-described risk factor for neural tube defect (NTD) pathologies like spina bifida. There is increasing laboratory and epidemiological evidence that supplementation with folate, usually through the synthetic form of folic acid, is also protective against OFCs. For the average adult, the recommended daily intake of folate is 400 μg, while for pregnant women the recommended amount is > 450 μg per day based on meta-analysis (Marchetta et al., 2015).

Folate metabolism is a complex process with various regulatory mechanisms, feedback loops, and interacting pathways. While this is reviewed in greater detail elsewhere (Lucock, 2000), we provide a brief overview of the folate cycle and its relationship with the methionine cycle. Once obtained through the diet, folic acid is typically metabolized in the small intestine and liver (Crider et al., 2012). Metabolism starts with the sequential reduction of folic acid to dihydrofolate and tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR). THF is subsequently converted into 5,10-methylene-THF by serine hydroxymethyltransferase (SHMT), which uses pyridoxine (vitamin B_6) as a cofactor. Finally, methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylene-THF into 5-methyl-THF, the primary folate metabolite utilized by non-hepatic cells.

Once in circulation, 5-methyl-THF is brought to the membrane of target cells where it enters the cytoplasm through the activities of folate carrier and binding proteins (Lucock, 2000). The 1-carbon cycle begins with conversion of 5-methyl-THF to THF by methionine synthetase, which uses cobalamin (vitamin B_{12}) as a cofactor (Crider et al., 2012). This reaction creates methionine by transferring a methyl group from 5-methyl-THF to homocysteine. Methionine, in turn, can be converted to S-adenosylmethionine (SAM) by SAM synthetase (Loenen, 2006). Through the activities of methyltransferases, SAM is the methyl donor for most biomolecules and is required for epigenetic modifications such as DNA methylation and the post-translational methylation of histone residues. SAM becomes S-adenosylhomocysteine (SAH) after donating its methyl group, which is subsequently converted to homocysteine by S-adenosylhomocysteine hydrolase (SAHH). Homocysteine again becomes available as a substrate for methionine synthesis by using THF, thus completing the methionine cycle. Choline/betaine metabolism also interacts with the methionine cycle by providing trimethylglycine, which can donate methyl groups to homocysteine through the activity of betaine-homocysteine methyltransferase (BHMT) (Barak, Beckenhauer, Junnila, & Tuma, 1993).

In 1998, the United States Food and Drug Administration implemented mandatory fortification of grains with folic acid (Food & Administration, 1996). The goal was to protect the population against NTDs, but the decision may also have been beneficial for preventing OFCs. A cross-sectional study among the Californian population found a decreased prevalence of both CL/P (PR = 0.91, 95% CI: 0.82, 1.00) and CPO (PR = 0.81, 95% CI: 0.70, 0.93) following mandatory grain fortification with folic acid (Yang, Carmichael, & Shaw, 2016). However, a study on voluntary fortification in Australia

observed a decrease risk only for NTDs and not for other birth defects among the West Australian population (Bower, Miller, Payne, & Serna, 2006). Similarly, a Cochrane review found no evidence for a protective effect of folate supplementation against OFCs, although the result was considered low quality evidence (De-Regil, Pena-Rosas, Fernandez-Gaxiola, & Rayco-Solon, 2015). Despite disagreement in the literature, there is some additional evidence that folate is protective against OFCs. A recent systematic review concluded that folic acid could be protective in high dosages (Jayarajan, Natarajan, & Nagamuttu, 2019). Folate appears to be most protective when supplement starts early relative to conception. Recent meta-analysis found that folic acid supplementation was only protective when administered periconceptionally (OR = 0.64, 95% CI: 0.56, 0.74) versus during pregnancy (OR = 0.90, 95% CI: 0.71, 1.14) (Jahanbin, Shadkam, Miri, Shirazi, & Abtahi, 2018). Similarly, another study found that supplementation during the first trimester was protective (OR = 0.14, 95% CI: 0.05, 0.41), but not during the second trimester (OR = 9.79, 95% CI: 3.47, 27.50) (Figueiredo et al., 2015). Because the metabolism and utilization of folate requires co-factors, it is likely that folic acid supplementation is augmented by the intake of multivitamins. A study in the Netherlands found that thiamine and pyridoxine protected against orofacial clefts, but only in the context of folic acid supplementation (Krapels et al., 2004). This is consistent with the role of pyridoxine in THF metabolism (Crider et al., 2012), while thiamine may be related to folates by common transport proteins (Zhao & Goldman, 2013).

Dozens of studies on genetic variants related to the folate cycle and risk for OFCs have been conducted, as previously reviewed (Bhaskar, Murthy, & Babu, 2011). Although evidence is often equivocal, human polymorphisms in folate-associated genes may either increase or decrease the risk of OFCs. Genetic variation has been hypothesized to explain why folic acid fortification has been protective against OFCs is some regions but not others (Nazer & Cifuentes, 2014). Recent studies in the Chilean population serve as an example of how GxE interactions affect the relationship between OFCs and folate metabolism. Among CL/P cases, a polymorphism of the *SHTM1* gene was less frequent than in control cases (Salamanca et al., 2020b). The authors postulated that the mutation protected against CL/P by decreasing enzymatic activity, thereby increased cellular folate levels. Three intronic alleles of MTR, involved in SAM metabolism, may also be protective against CL/P (Salamanca et al., 2020a). In contrast, the most widely documented polymorphism in folate metabolism, MTHFR c.677C>T, decreases enzymatic activity and has been associated with increased risk for CL/P (Ramirez-Chau, Blanco, Colombo, Pardo, & Suazo, 2016). Interaction between maternal folic acid intake and this mutation has been previously described (I. A. van Rooij, Swinkels, Blom, Merkus, & Steegers-Theunissen, 2003). Meta-analysis of 15 studies indicated that maternal 677C>T mutation increased risk of CL/P, demonstrated an important role for maternal metabolism of folates in OFC pathology (Pan et al., 2015). Polymorphisms of other folate-related genes are associated with OFCs, as described in a companion review (Reynolds et al., 2020) (in this issue).

There are several possible mechanisms by which folates can be protective against OFCs. One is that the folate cycle supports nucleotide synthesis, allowing for the rapid genome replication and cell proliferation required in orofacial development. Because of this role, folate deficiency is also linked with aberrant DNA repair due to misincorporation of uracil,

leading to a catastrophic repair cycle and destabilization of the genome (Duthie, Narayanan, Brand, Pirie, & Grant, 2002). Additionally, many folate-dependent downstream processes are mediated by SAM. Some of these include the synthesis of polyamines, which are involved in cell proliferation, differentiation, and apoptosis (Bjelakovic et al., 2017). SAM is also a substrate for DNA methyltransferases involved in epigenetic modifications, specifically DNA methylation and post-translational methylation of histone residues (Garland et al., 2020) (in this issue). Folate deficiency could additionally result in the accumulation of homocysteine to toxic levels (hyperhomocysteinemia), which has been linked to OFCs (Lonn et al., 2006; Wong et al., 1999). Hyperhomocysteinemia can increase cellular asymmetric dimethylarginine which generates ROS and oxidative stress (Tyagi et al., 2005). Thus, the protective effects of folate against OFCs likely stems from its various roles associated with cell proliferation, differentiation, and physiological homeostasis.

2.2. Retinoic acid metabolism

Retinoic acid (RA), a metabolite derived from retinol (vitamin A), is a small lipophilic molecule with critical roles in tissue patterning and cell fate specification during embryogenesis (Rhinn & Dolle, 2012). In most animals, retinoids must be obtained environmentally through the diet as retinol, retinyl esters, or carotenoids (e.g., β-carotene) (Finnell et al., 2004). Retinol, the primary circulating retinoid, is obtained by embryos through transplacental circulation in placental mammals (Spiegler, Kim, Wassef, Shete, & Quadro, 2012). Retinoid metabolism involves several steps mediated by binding proteins, transporters, and metabolic enzymes, many of which are controlled through feedback mechanisms intrinsic to RA-mediated signaling (Okano, Udagawa, & Shiota, 2014; Rhinn & Dolle, 2012). Overabundance (Dekker et al., 1994) or knockout (Halilagic et al., 2007) of these proteins has perturbed orofacial development in laboratory studies.

Once in circulation following dietary or pharmacological exposure, retinol is bound by retinol binding protein 4 (RBP4) and transported to the cell membrane (Rhinn & Dolle, 2012). Because maternal RBP4 cannot cross the placenta, retinol must diffuse across the yolk sac and placenta to bind with embryonic RBP4, which is synthesized in the latter structure (S. J. Ward, Chambon, Ong, & Bavik, 1997). Embryonic RBP4 facilitates retinol circulation to a target cell, where the transmembrane protein signaling receptor and transporter of retinol (STRA6) transports retinol across the cell membrane and into the cytoplasm (Kawaguchi et al., 2007). At this stage, the pathway splits—retinol may be processed for storage, or it may continue through the RA metabolic pathway for bioactivation. For storage, retinol is processed into retinyl ester by lecithin retinol acyltransferase (LRAT) (Ruiz et al., 1999). In target cells, retinol is instead oxidized into retinaldehyde by enzymes belonging to either of two enzyme families: cytosolic alcohol dehydrogenases (ADHs) or microsomal retinol dehydrogenases (RDHs) (Pares, Farres, Kedishvili, & Duester, 2008). Retinaldehyde serves as a substrate for one of three tissue-specific isoforms of retinaldehyde dehydrogenases (RALDHs; RALDH1, RALDH2, and RALDH3) which oxidize it into bioactive RA (Niederreither & Dolle, 2008).

RA signaling includes feedback mechanisms that alter the abundances of proteins that control the cellular RA pool across tissues. An important negative feedback loop is the

increased expression of cytochrome P450 family member 26 enzymes (CYP26A1, CYP26B1, and CYP26C1), which oxidize RA into metabolites with increased polarity such as 4-hydroxy-retinoic acid and 4-oxo-retinoic acid (Chithalen, Luu, Petkovich, & Jones, 2002). While these are still bioactive metabolites, they are subject to conjugation reactions that facilitate their elimination (Rhinn & Dolle, 2012). In mice, double knockout of *Raldh2* and *Raldh3* resulted in severe craniofacial malformations including failure of frontonasal fusion (Halilagic et al., 2007), while knockout of *Cyp26b1* prevented tongue depression and horizontal elevation of the palatal shelves (Okano et al., 2014). Upstream of these enzymes, knockout of *Rdh10* demonstrated a role for RA synthesis in spontaneous neuromuscular movement of the tongue and mandible, which facilitates palatal shelf elevation (Friedl et al., 2019). Thus, the cellular pool of RA must be tightly regulated for appropriate orofacial morphogenesis.

In human populations, there is some evidence for polymorphisms of genes associated with RA metabolism and signaling that could increase the risk for OFCs. A study on Han and Uyghur populations in China found an association between offspring *RBP4* polymorphism and increased risk for CL/P using a machine learning model (S. J. Zhang et al., 2018). Two other studies in Chinese populations and a study in an Indian population found correlation between *RARA* polymorphism and increased risk for CL/P (Fan, Li, & Wu, 2007; Peanchitlertkajorn, Cooper, Liu, Field, & Marazita, 2003; Xavier et al., 2013).

Teratogenic levels of retinoids have been reached during pregnancy through pharmacological and dietary sources. Exposure to the retinoid-based acne medication isotretinoin, for example, was associated with birth defects including cleft palate (Lammer et al., 1985). Excess vitamin A (hypervitaminosis A) or deficiency (hypovitaminosis A) can both adversely affect orofacial development. Daily supplementation of vitamin A in excess of 10000 IU (approximately 3000 μg) has been associated with cleft lip in humans (Rothman et al., 1995). High levels of dietary vitamin A have also induced cleft palate in mice (Inomata et al., 2005), rats (Mineshima et al., 2012), cats (Freytag, Liu, Rogers, & Morris, 2003), and dogs (Wiersig & Swenson, 1967). Conversely, some recent studies have found that vitamin A consumption is protective against OFCs. A population study in Norway found a decreased risk of CPO among pregnant women with daily intake of vitamin A ranging between 1911.1 - 9640.0 μg (OR = 0.47, 95% CI: 0.24, 0.94) (Johansen, Lie, Wilcox, Andersen, & Drevon, 2008). Similar protective effects were observed for β-carotene intake ranging between 4,531 – 26,903 μg (OR = 0.50, 95% CI: 0.31, 0.80). Consumption of cod liver oil, which contains large quantities of retinol as well as antioxidants like vitamin E and omega-3 fatty acids, is reportedly recommended to pregnant women in Norway (Johansen et al., 2008). This guideline is supported by evidence from a study in China that found cod liver oil supplementation during the first trimester had protective effects against both CL/P (OR = 0.12, 95% CI: 0.03, 0.52) and CPO (OR = 0.16, 95% CI: 0.02, 1.21) (Hao et al., 2015). The effect was even stronger among women who took it before conception (CL/P: OR = 0.04, 95% CI: 0.01, 0.31; CPO: OR = 0.11, 95% CI: 0.01, 0.82). It is unknown how exactly cod liver oil may protect against OFCs, but it may involve a combination of support for RA signaling and antioxidant activity. A study in Denmark similarly observed a protective effect of vitamin A, either through multivitamins use or liver consumption (Mitchell, Murray, O'Brien, & Christensen, 2003). Approaches using proteomic and

metabolite analyses may better assess the relationship between vitamin A status and OFCs. One study found that serum levels of RBP4 were significantly lowered in CL/P cases versus controls (sample size n = 10-14) (J. Zhang et al., 2014). Metabolite analysis verified that vitamin A levels were correspondingly lower in the CL/P group.

Because hyper- and hypovitaminosis A are both associated with birth defects including OFCs, there is risk associated with the choice of supplementing during pregnancy. The World Health Organization (WHO) has stated that prenatal vitamin A supplementation is not recommended in populations where a well-balanced diet can be maintained during pregnancy (McGuire, 2012). However, in regions where hypovitaminosis A is a known public health issue, WHO advises that pregnant women take an oral liquid, oil-based supplement containing retinyl palmitate or retinyl acetate at either 10,000 IU daily or up to 25,000 IU weekly (McGuire, 2012).

3. Environmental signaling pathways in orofacial clefts

While environmental stressors like ROS can disrupt orofacial development by causing cellular damage (Larouche & Hales, 2009), some xenobiotics serve as ligands for receptors that modulate gene expression. Environmental signaling pathways can be inappropriately activated or blocked to disrupt tightly coordinated developmental processes. Among the best studied pathways interacting with orofacial development are those controlled by the retinoic acid receptor (RAR), aryl hydrocarbon receptor (AHR), and glucocorticoid receptor (GR). Extrinsic factors interacting with these pathways have helped to characterize potential environmental hazards that exist for OFCs. Additionally, they have been useful means of chemically inducing OFCs in the laboratory to better understand the molecular and cellular processes of orofacial development. In this section, we describe the signaling pathways mediated by RAR, AHR, and GR, as well as their downstream mechanisms that could cause OFCs. We also discuss how these signaling pathways can interact to increase teratogenicity.

3.1. Retinoic acid receptor signaling

Once retinol is metabolized into RA, cellular retinoic acid binding protein (CRABP) transports RA into the nucleus (Okano et al., 2014) (Figure 1). There it serves as a ligand for retinoic acid receptors (RARs), a group of transcription factors consisting of three isotypes: RARα, RARβ, and RARγ. The RARs function as heterodimers by partnering with the retinoid X receptors (RXRs), similarly consisting of three isotypes: RXRα, RXRβ, and RXRγ. The RAR-RXR heterodimers modulate the expression of specific genes through binding of retinoic acid response elements (RAREs) in gene promoter regions. In the RA-unbound state, the RAR-RXR heterodimers recruit co-repressor proteins and prevent transcription. Upon binding RA, they alter conformation and recruit co-activator proteins to act as transcription activators.

The importance of RA signaling in orofacial development is conserved in vertebrates, as demonstrated in studies using zebrafish (Laue, Janicke, Plaster, Sonntag, & Hammerschmidt, 2008), Xenopus (Kennedy & Dickinson, 2012), chicks (Shimomura et al., 2015), and mice (Rhinn, Schuhbaur, Niederreither, & Dolle, 2011). In rodents, studies on the role of RA signaling have utilized various approaches such as targeted knockout of RARs

and RXRs, knockout or inhibition of enzymes involved in RA metabolism, or by addition of exogenous RA (Rhinn & Dolle, 2012). Laboratory exposures have utilized excess all-trans retinoic acid (ATRA) in the mouse model to generate cleft palate phenotypes with high frequency. These exposures could perturb development by disrupting concentration-dependent changes in gene expression and activating RA signaling beyond a normal spatiotemporal context. RA signaling via ATRA exposure can be mutually antagonistic toward Tgf-β, Wnt, and Hedgehog signaling during palatogenesis. The specifics of these interactions are discussed in a companion review (Reynolds et al., 2020) (in this issue). Additionally, ATRA exposure can modulate Notch signaling in palatal epithelial and mesenchymal cells, possibly affecting p21 signaling in both tissues. One study showed that ATRA exposure at embryonic day (E) 12 increased the abundance of Notch1 in MEE cells at E15, which was hypothesized to inhibit normal apoptotic processes (Y. D. Zhang et al., 2016). Another study found that ATRA exposure at E10 increased abundance of Notch2 and decreased that of CyclinD1 in mesenchymal cells between E12.5-14.5, thereby inhibiting proliferation (Y. D. Zhang et al., 2017). Because RA signaling intersects with several pathways, the balance of the endogenous RA gradient must be carefully managed through feedback mechanisms.

RA signaling can modulate the expression of transcription factors to control developmental processes, in some cases through its interactions with other pathways. In the upper jaw of chick embryos, ATRA exposure decreased expression of *Lhx8*, *Msx1*, and *Msx2*, the former of which is controlled by Fgf signaling (Shimomura et al., 2015). These genes appear to be similarly controlled through endogenous RA signaling. Knockdown of *raldh2* and *rary* during orofacial development in *Xenopus* resulted in median facial cleft as well as decreased expression of *lhx8* and *msx2* (Kennedy & Dickinson, 2012). In mice, ATRA exposure may affect Lef1-mediated periderm trajectory toward EMT by decreasing expression of *Ambra1* in a lncRNA-transcription factor-target gene regulatory network (Shu, Dong, & Shu, 2019). In mice, Cyp26b1 is particularly important for palatogenesis and is required for horizontal elevation of the palatal shelves as well as tongue depression.

3.2. Aryl hydrocarbon receptor signaling

Activation of the aryl hydrocarbon receptor (AHR) during orofacial development is a reliable method for inducing cleft palate in the laboratory. Also known as the dioxin receptor, the AHR is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors (Nebert, 2017). It is a promiscuous receptor that binds several xenobiotic chemicals as well as some endogenous compounds (Figure 2), as reviewed in (Denison & Nagy, 2003). Major ligands include industrial products and byproducts such as dioxins and polychlorinated biphenyls (PCBs); combustion products such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzofurans (PCDFs); and phytonutrients such as flavonoids and carotenoids. The AHR also responds to endogenous ligands including tryptophan derivatives, tetrapyrroles, and arachidonic acid metabolites. The AHR exhibits a structure-activity relationship (SAR) with its ligands (P. Shankar et al., 2019). While dioxins and PCBs are known to induce OFCs (Courtney & Moore, 1971; Watanabe & Sugahara, 1981), the endogenous ligand 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) does not appear to affect orofacial development (Henry,

Bemis, Henry, Kende, & Gasiewicz, 2006). Additionally, some AHR ligands are non-activating and can prevent AHR signaling through competitive inhibition (Brenerova et al., 2016; Guyot, Chevallier, Barouki, & Coumoul, 2013). AHR ligands often occur in the environment as a mixture of activating and non-activating ligands, each competing for AHR with different binding affinities and SARs (Geier et al., 2018). It is therefore challenging to understand how relevant exposure scenarios will affect human development including how they may impact the risk for OFCs.

While it has endogenous roles, the AHR acts as an environmental sensor. Its transcriptional activities usually result in an adaptive response ultimately intended for detoxification and/or elimination of the ligand(s) (Denison & Nagy, 2003; Nebert, 2017). To this end, its activation as a transcription factor strongly induces expression of genes encoding phase I and II metabolic enzymes (described in Section 3). During inactivity, the AHR resides in the cytosol in association with a chaperone complex. Upon binding a ligand, it translocates to the nucleus where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AHR-ARNT heterodimer subsequently regulates gene expression through binding of dioxin response elements (DREs) within gene promoters, resulting in activation or repression of transcription. The phase I metabolic enzyme CYP1A1, along with a negative feedback regulator called the aryl hydrocarbon receptor repressor (AHRR), are well-described biomarkers for AHR activation. Polymorphisms in the *ARNT* (Kayano et al., 2004) and *AHRR* (Elizabeth J Leslie et al., 2017) (Linnenkamp, Raskin, Esposito, & Herai, 2020) genes have been associated with OFCs, indicating that allelic variation of genes in the AHR pathway may perturb orofacial development through GxE interactions (Kayano et al., 2004).

Much of what is known about the role of the AHR in OFCs is derived from dioxin exposure studies. The most potent is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is often regarded as the most toxic anthropogenic substance known (Gallo, Scheuplein, & Heijden, 1991; Kao, Chen, Liu, & Wu, 2001; McAdams & Aquino, 1994). TCDD has been experimentally demonstrated to induce cleft palate and hydronephrosis in both mice and rats at dose-dependent levels (Huuskonen, Unkila, Pohjanvirta, & Tuomisto, 1994; R. M. Pratt, Dencker, & Diewert, 1984), as well as in *ex vivo* human embryonic palate cultures (Abbott & Birnbaum, 1991; Abbott, Probst, Perdew, & Buckalew, 1998). TCDD exposures do not increase the incidence of cleft lip in mice, even in the A/J strain that is susceptible to spontaneous cleft lip during development (Yamada, Mishima, Fujiwara, Imura, & Sugahara, 2006). Mouse embryos that lack functional AHR do not develop cleft palate following TCDD exposure, demonstrating absolute dependency of TCDD-induced cleft palate on AHR-mediated effects (Mimura et al., 1997; Peters et al., 1999). In contrast, the developing pups of *Ahr*-null dams are up to five times more sensitive to TCDD exposure, likely as a consequence of increased uptake by the pups (Thomae, Glover, & Bradfield, 2004).

TCDD exposure can affect gene expression in both epithelial and mesenchymal tissues, disrupting several stages of palatogenesis including palatal growth and fusion. Histological examination of developing TCDD-exposed mouse palate found that the MEE of the separate palatal shelves make contact, but this is followed by a post-fusional split (Yamada et al., 2006). One explanation for this could be the disruption of epithelial cell development.

Rupture of the epithelial basement membrane has been observed in TCDD-exposed palates, suggesting that cell adhesion or integrity of the palatal shelves were compromised (Sakuma et al., 2018). AHR signaling also appears to disrupt growth factor signaling during palatogenesis. TCDD exposure reduces expression of transforming growth factor alpha (TGF-α), which normally is highly expressed in the epithelial cells of human palatal cultures (Citterio & Gaillard, 1994). This indicated that TCDD targets the Egf pathway, which was confirmed using knockout mice (Abbott et al., 2003), although the effect on Egf signaling appears to be counterintuitive to decreased ligand expression. In human fetal palatal epithelial cells (hFPECs), TCDD stimulated EGF receptor phosphorylation, resulting in the phosphorylation of ERK/p38 and subsequent cell proliferation (Z. Gao, Bu, Liu, et al., 2016). TCDD also promoted entry into the S and G2/M phases at least partly through the PI3K/AKT pathway (Z. Gao, Bu, Zhang, et al., 2016). While TCDD exposure decreased abundance of epithelial markers E-cadherin and keratin-14, it increased abundance of mesenchymal markers vimentin and fibronectin, signifying inappropriate progression through EMT. A likely mechanism was the increased expression of *Slug*, an inducer of EMT and repressor of E-cadherin, which has DREs in its promoter that could allow direct modulation by AHR. Co-immunoprecipitation demonstrated that, in addition to Slug induction, TCDD also promoted physical interaction between AHR and Slug. These studies provided strong evidence that a key mechanism of AHR-mediated cleft palate is inappropriate induction of EMT, with Slug as a likely intermediary. This is consistent with previous observations that exogenous Tgf-β3, a mediator of EMT during palatogenesis, protects against cleft palate following TCDD exposure (Thomae, Stevens, & Bradfield, 2005). A caveat is that Tgf-β signaling can repress expression of the AHR (Wolff et al., 2001), so the protective effect of Tgf-β3 may be independent of its effects on EMT. However, TCDD has been observed to decrease expression of Tgf-β3 in the MEE, concurrent with loss of filopodia and cell polarity that may interfere with apoptosis (Li, He, Meng, Lu, & Shi, 2014).

AHR can additionally disrupt mesenchymal development during palatogenesis. In mice, an immunohistological survey found that TCDD exposure prevented osteogenesis (decreased abundance of Runx2 and osteopontin) and myogenesis (decreased expression of MyoD and desmin) (Yamada et al., 2006). In human fetal palatal mesenchymal cells (hFPMCs), TCDD caused decreased proliferation, alkaline phosphatase activity, and deposition of calcium onto mineralizing tissue (X. Z. Liu et al., 2020). Further, TCDD exposure decreased TGF-β (SMAD2/3) and BMP (SMAD1/5/8) signaling, altering cellular function and fate. Co-treatment of hFPMCs with TGF-β1 and BMP2 restored appropriate SMAD signaling for both pathways and rescued the impaired osteogenesis.

Taken together, these studies demonstrated that AHR activation, as mediated by TCDD, disrupt both epithelial and mesenchymal development during palatogenesis. Specifically, AHR activation can increase proliferation and EMT of epithelial cells, while decreasing proliferation and preventing differentiation of mesenchymal cells. EMT and osteogenesis appear to be major processes affected by AHR activation. Additional studies have identified other potential mechanisms of AHR-mediated cleft palate, including altered expression of genes implicated in OFCs such as the lncRNA *H19* (L. Y. Gao et al., 2017) and gamma aminobutyric acid type A receptor beta-3 subunit (*GABRB3*) (Lei et al., 2019). AHR

activation can also alter the epigenetic state of palatal cells, inducing promoter hypermethylation of stemness genes like *Oct4* (Tao et al., 2020; Wang, Yuan, Fu, & Zhai, 2016; Wang et al., 2017). While much has been characterized about the effect of AHR signaling on OFCs, more remains to be discovered. As an example, little is known of the full range of possible AHR ligands that could contribute to OFCs, as well as their relative potencies for inducing cleft palate. For example, in toxicity assays using the embryonic zebrafish, the screening of over a hundred AHR ligands revealed varying teratological effects based on chemical structure, ranging from craniofacial malformations to appendage duplication (Reynolds et al., 2020; Prarthana Shankar, Dasgupta, Hahn, & Tanguay, 2020; P. Shankar et al., 2019). Characterizing these hazards will be important for evaluating environmental risks for craniofacial defects mediated by AHR signaling. Additionally, a better understanding of AHR signaling could lead to therapeutic approaches toward preventing OFCs in higher risk cases, such as those who may be genetically or occupationally predisposed to having offspring with an OFC.

3.3. Glucocorticoid receptor signaling

Glucocorticoids (GCs) are a class of corticosteroid hormones that include both endogenous and exogenous chemicals. Homeostatic levels of endogenous cortisol are required for appropriate craniofacial morphogenesis, but addition of exogenous GCs can cause cleft palate (Hu, Gao, Liao, Tang, & Lu, 2013; R. Pratt, 1980). Although excess of cortisol itself can cause cleft palate in mice (Jaskoll, Choy, Chen, & Melnick, 1996), some synthetic GCs may have a more potent effect of cleft palate induction. As an example, the synthetic GC dexamethasone is approximately 300 times more potent than cortisol for inducing cleft palate (Pinsky & Digeorge, 1965). The transcriptional response to GCs is mediated by the glucocorticoid receptor (GR), a member of the nuclear receptor family of transcription factors that is encoded by the gene *NR3C1* (Weikum, Knuesel, Ortlund, & Yamamoto, 2017). Monomeric GR resides in the cytosol with a chaperone complex and becomes activated as a transcription factor upon binding a ligand (Vandevyver, Dejager, & Libert, 2012; Weikum et al., 2017) (Figure 3). After translocating to the nucleus, the GR homodimerizes and subsequently binds glucocorticoid response elements (GREs) within gene promoters to modulate gene expression. It can activate or repress the transcription of thousands of genes involved in immune response, inflammation, wound healing, energy metabolism, and others (Weikum et al., 2017).

An early observation in GR-mediated cleft palate was decreased proliferation of mesenchymal cells and secretion of the ECM (Diewert & Pratt, 1981). This hypoplasia prevented contact between the shelves and blocked palatal fusion. One hypothesis was that GCs induced cleft palate through the same mechanism by which the GR regulates the inflammatory response. Activation of the GR induces expression of macrocortin and other anti-phospholipases, thereby preventing the Phospholipase A2-mediated cleavage of arachidonic acid from phospholipids (Grove, Willis, & Pratt, 1985). This prevents downstream metabolism of arachidonic acid into physiologically active derivatives such as leukotrienes, thromboxanes, and prostaglandins, the latter of which are believed to regulate cyclic AMP levels in palatal cells (Greene & Garbarino, 1984). However, a study investigating this hypothesis in mice showed that addition of exogenous arachidonic acid did

not rescue pups from GC-mediated cleft palate (Grove et al., 1985). This demonstrated two possibilities: 1) inhibition of arachidonic acid does not affect palatogenesis; or 2) inhibited metabolism of arachidonic acid is still a valid mechanism, but additional mechanisms are involved that prevented rescue.

Most subsequent efforts have not considered the arachidonic acid hypothesis, instead focusing on GC-mediated changes to cellular properties or modulation of developmental signaling. Previous observations for decreased production of ECM proteins have been corroborated in mouse studies. Dexamethasone acetate was found to decrease the palatal area covered by glucosaminoglycan content (Fu, Li, & Huang, 1997). Both hyaluronate and collagen had decreased abundance following exposure to dexamethasone (Montenegro, Rojas, Dominguez, & Rosales, 1998). Each of these ECM component types is important for palatal shelf elevation and fusion (Ji et al., 2020) (in this issue). Additionally, dexamethasone increased the abundance of E-cadherin, which is normally suppressed during palatal fusion and EMT (Xiaoxiao, Li, Li, Qian, & Chenghao, 2015). Transmission electron microscopy of mouse palates exposed to dexamethasone revealed cellular swelling and loose cell connection, along with decreased abundance of mitochondria and extracellular matrix (Lan et al., 2016).

Mesenchymal proliferation may be inhibited by the GR through modulation of Tgf-β2, which inhibits cell proliferation by targeting G_1 phase cyclins and cyclin-dependent kinases (Derynck, 1994; Jaskoll et al., 1996). During palatogenesis, exposure to exogenous cortisol maintained elevated levels of Tgf-β2 during a stage where it normally decreases at E14 (Jaskoll et al., 1996). The authors postulated that this sustained expression of Tgf-β2 occurred during a sensitive time for proliferating, perturbing cell cycle progression through the above mechanism. Another possible mechanism of reduced proliferation is through modulation of β-catenin, which was inappropriately upregulated in palatal tissues of mice exposed to dexamethasone (Xiaoxiao et al., 2015). In addition to exhibiting decreased proliferation, cells in the palatal shelves also undergo apoptosis following dexamethasone exposure with increased abundance of Bax and caspase-3 (Lan et al., 2016). Bmp2, which suppresses apoptosis, also had decreased abundance. This may have been due to decreased levels of Gata-6, which regulates *Bmp2* expression.

Activation of the GR can have specific effects on the epithelium that contribute to the inability of palates to fuse. An *in vitro* study on cultured murine palatal cells found that dexamethasone induced the differentiation of MEE cells into squamous epithelial cells, perturbing development of the palate epithelium (Huang, Shi, Sun, & Wang, 2005). An *in vivo* study found that dexamethasone exposure decreased expression of *Fgf10* in the mesenchyme as well as *Fgfr2b* and *sonic hedgehog* (*Shh*) expression in the epithelium, thereby perturbing Fgf-Hedgehog signaling axis (He et al., 2010). Interestingly, this study found that co-exposure with vitamin B_{12} restored gene expression and mesenchymal proliferation. More recently, a study found that dexamethasone exposure disrupted the PAR complex by decreasing transcription and translation of PAR3 and PAR6 in the epithelium, and aPKC in both epithelial and mesenchymal cells (Ma, Shi, & Zheng, 2018). This adversely affected epithelial cell polarity and contributed to the inability of palatal shelves to fuse.

These studies demonstrated that GR signaling can modulate several aspects of palatogenesis, particularly mesenchymal proliferation and epithelial differentiation. In general, GR signaling prevents palatal shelves from ever making contact, but fusion would likely be inhibited by GC exposure due to the altered developmental state of epithelial cells. The GR appears to modulate several developmental pathways important for palatogenesis including Wnt, Fgf, Tgf-β, and Bmp. The exact mechanisms behind this are not well understood, but at least in the case of Bmp signaling, this is a consequence of altered *Gata6* expression. A better understanding of the mechanisms by which GR induces cleft palate would provide additional methods of protecting against OFCs. There is evidence from mouse studies that vitamins B_6 and B_{12} could protect factor against OFCs caused by GCs, and it would be advantageous to know whether these or other compounds would be protective in a clinical setting (Lu et al., 2008; Yoneda & Pratt, 1982). There is also evidence for vertebrate-conserved SAR in GR signaling, which guides the development of novel GCs with the goal of preventing unwanted side effects (I. H. Song, Gold, Straub, Burmester, & Buttgereit, 2005). For example, some GCs like beclomethasone dipropionate increase expression of the feto-oncogene *Cripto-1/Tdgf1* in embryonic mouse cells, which could perturb several developmental pathways relevant to orofacial development (Garland et al., 2019). The development of GCs with lower risk for teratogenicity would be beneficial for pregnant women, especially for those who may unexpectedly require immunosuppression in emergency situations.

3.4. Signaling interactions in orofacial development

There is evidence for substantial crosstalk between environmental signaling pathways and orofacial development, particularly in association with AHR signaling. For example, a mouse study found that the expression of *Ahr* the nasal mesenchyme depends on the expression of *Raldh3* in the nasal epithelium (Jacobs et al., 2011b). The resulting model is that epithelium-derived RA transduced mesenchymal expression of *Ahr* through activation by RAR-γ. Knockout studies demonstrated that this regulatory network was required for TCDD-induced cleft palate. AHR signaling appears to interact with multiple hormone signaling pathways as well. TCDD exposure increased abundance of the GR in both epithelial and mesenchymal tissue in the palate (Abbott, Perdew, Buckalew, & Birnbaum, 1994). Co-exposure of TCDD and hydrocortisone during palatogenesis induced cleft palate in mice at 100% incidence using concentrations that could not induce cleft palate following exposure to either chemical alone (L. S. Birnbaum, Harris, Miller, Pratt, & Lamb, 1986). The AHR may also interact with thyroid signaling. Compared to TCDD exposure alone (approximately 8% incidence), co-exposure of TCDD and either of the thyroid hormones triiodothyronine (T_3) or thyroxine (T_4) increased incidence of cleft palate in mice to 31% and 27% at the maximum tested dosages, respectively (Lamb, Harris, McKinney, & Birnbaum, 1986). T_3 or T_4 alone induced cleft palate, but only at a dose-independent rate of 1%, indicating a synergy between TCDD exposure and thyroid hormone signaling. Additionally, during murine palatogenesis, TCDD exposure reduced abundance of estrogen receptor alpha (ER-α) (Yamada et al., 2014). Given that AHR has previously been associated with ER-α and osteoblast differentiation (Ge, Cui, Cheng, & Han, 2018), TCDD exposure may further promote cleft palate by antagonizing estrogen signaling and downstream palatal bone formation.

The GR, in addition to its interactions with AHR signaling, may also influence RA signaling. Polymorphisms associated with glucocorticoid-induced cleft palate appear to influence the role of vitamin A in exacerbating the risk (Tyan & Tyan, 1993). This indicates that sensitivity to excess retinoids could be linked to genetic variants affecting pathways other than retinoid metabolism.

4. Biotransformation and transport in OFCs

Developmental processes rely on the biotransforming activities of metabolic enzymes for synthesis, modification, and degradation of endogenous compounds. Similar activities are critical for detoxification and subsequent elimination of exogenous chemicals that may be teratogenic. Exposure to chemicals incidentally (e.g. solvents and biocides) or behaviorally (e.g. smoking, drinking, and diet) can elicit and/or interfere with chemical metabolism, as described below. Dysfunction and misregulation of biotransformation enzymes and transporters are associated with OFCs in the laboratory and in human populations (Beaty et al., 2010; Shi et al., 2007). Metabolic activity may inadvertently result in the generation of toxic intermediates that cause oxidative stress or other harmful effects (Zangar, Davydov, & Verma, 2004). Here we describe what is currently known about the involvement of biotransformation enzymes in orofacial development with a focus on their roles in detoxification.

Exposure to xenobiotics can trigger increased expression of metabolic and transport enzymes. Enzymatic activities can result in structural modifications that facilitate detoxification and subsequent renal or biliary excretion, usually through the addition of polar groups that also increase hydrophilicity (Gonzalez, 2005). This process is often divided into three parts: phase I (oxidation), phase II (conjugation), and phase III (transport). A review by Xu et al. (2005) provides an overview of these detoxification phases and mechanisms of their induction (C. J. Xu, Li, & Kong, 2005). Metabolic enzymes outside of this paradigm, such as aldehyde dehydrogenases, are also involved in the detoxification of some xenobiotics. Dysfunction of any of these processes have been linked with OFCs.

4.1. Phase I: Oxidation

Phase I metabolic enzymes include cytochrome P450s (CYPs), epoxide hydrolases (EHs), and flavin-containing monooxygenases (FMOs). The CYP and EH classes have been associated with OFC pathology through human population or knockout/knock-in studies. Within the human genome, there are 57 genes encoding putatively functional CYP enzymes that are categorized into subfamilies based on sequence similarity (Fujikura, Ingelman-Sundberg, & Lauschke, 2015). CYPs are primarily expressed in hepatic tissue but are also found in the intestine, kidney, lung, and hematopoietic tissues (Gonzalez, 2005). Some can be also induced in orofacial tissue, such as *Cyp1a1* in mice (Jacobs et al., 2011a). While some CYPs can promiscuously metabolize thousands of endogenous or exogenous substrates, others are more specialized and direct their activities toward few chemicals. In general, CYPs oxidize carbon and nitrogen groups resulting in the formation of alcohol groups (Gonzalez, 2005). CYP-mediated oxidation of aromatic substrates can yield an epoxide, although these groups spontaneously hydrolyze into diols or are catalytically

hydrolyzed into trans-dihydrodiols by EHs. In contrast, FMOs are not yet directly implicated in OFCs. Because they are not induced by xenobiotic exposure and must compete for substrates with CYPs (Krueger & Williams, 2005), it is possible that FMOs may have less importance for OFC pathology. However, because FMOs can yield metabolites distinct from CYP products, they warrant consideration in future studies.

Although CYPs generate electrophilic metabolites that may be more toxic than the original substrate, evidence suggests that CYPs ultimately play a protective role in OFC pathology. This could be through the detoxification pathway or by altering toxicokinetics via sequestration. Polymorphisms in both fetal and maternal CYP genes have been associated with OFCs, often in the context of smoking or drinking. CYP1A1 is considered one of the most important enzymes for metabolizing several combustion products in smoke, particularly polycyclic aromatic hydrocarbons (PAHs). In the context of maternal smoking, fetal polymorphisms in *CYP1A1* had protective associations with CL/P in French (Chevrier et al., 2008), Danish, and Iowan (Shi et al., 2007) populations, at least one of which is thought to increase enzymatic function by stabilizing mRNA. Maternal polymorphism was also associated with protective effects in the French population (Chevrier et al., 2008). A knockout study demonstrated that altered expression of *Cyp1a1* or *Cyp1b1* does not significantly alter dioxin-induced OFCs during murine development (Dragin, Dalton, Miller, Shertzer, & Nebert, 2006). The same study showed that *Cyp1a2* (or *CYP1A2* in a humanized model) in the maternal liver had a protective effect, likely due to altered toxicokinetics. Thus, while there is evidence that CYP enzymes may be protective, the functional relevance for *CYP1A1* or *CYP1B1* in OFCs remains to be better characterized.

Fetal polymorphism of another CYP gene, *CYP2E1*, may also have a protective effect against OFCs as observed in a French population (Chevrier et al., 2008; Chevrier et al., 2007). CYP2E1 metabolizes organic solvents such as ethanol and benzene (Ingelman-Sundberg, 2004), as well as other toxicants including nitrosamines and tannins (Lieber, 1997). It may also convert retinoic acid into a less active metabolite (C. Liu, Russell, Seitz, & Wang, 2001; Stoilov, Jansson, Sarfarazi, & Schenkman, 2001), potentially offsetting the delicate balance required for appropriate retinoid signaling (Okano et al., 2014). This suggests that smoking or drinking could indirectly affect development by stimulating endogenous metabolism via phase I induction, although it is unclear how exactly the polymorphism affects this process (Chevrier et al., 2008; Shi, Wehby, & Murray, 2008).

CYP-metabolized PAHs may have reactive epoxide functionalities that are subsequently hydrolyzed by the microsomal EH enzyme, *EPHX1*. Data from an Iowan population suggested a protective interaction between maternal smoking and a fetal *EPHX1* polymorphism that increased enzymatic activity (Shi et al., 2007). Since this implied that *EPHX1* activity protects against OFCs, it is tempting to speculate that epoxidized chemicals derived from cigarette smoke are teratogenic. However, two other studies observed no interaction between smoking and *EPHX1* polymorphisms in OFCs (Hartsfield et al., 2001; Ramirez et al., 2007). Despite mixed conclusions, a demonstrated role for *EPHX1* in OFC pathology could improve our understanding of smoke-induced OFCs and deserves further investigation.

4.2. Phase II: Conjugation

Phase II enzymes covalently add small organic donor molecules to xenobiotics and intermediate metabolites. These conjugations are usually detoxifying mechanisms that may also facilitate excretion by polarizing the xenobiotic and increasing hydrophilicity. While several types of donor groups are used, some major modifications include arylamine N-acetylation, glutathionylation, UDP-glucuronidation, and sulfonation. Respectively, these are catalyzed by arylamine N-acetylases (NATs), glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and sulfotransferases (SULTs), with the former two families having implications in human OFCs.

Cigarette smoke is known to contain toxicants such as arylhydroxylamines (Lammer, Shaw, Iovannisci, Van Waes, & Finnell, 2004), and conjugating enzymes such as NATs provide an important mechanism for detoxifying these compounds. Humans possess two NAT genes, *NAT1* and *NAT2*, that each have over two dozen known polymorphisms that may affect arylamine detoxification and OFCs (Shi et al., 2008). A study on a California population linked two fetal polymorphisms of *NAT1* that increased the risk of CL/P by 4-fold when mothers smoked during early pregnancy (compared to reference genotype infants with non-smoking mothers) (Lammer et al., 2004). A study on a Danish population identified a protective effect of a maternal *NAT2* allele (Shi et al., 2007), while maternal polymorphism imparting a slow acetylator phenotype increased risk of CL/P in an Argentine population (Santos, Ramallo, Muzzio, Camelo, & Bailliet, 2015). In a Chinese population, polymorphisms of both NAT1 and NAT2 were found to increase the risk of CL/P, with increased risk among those having polymorphisms in both isozymes (T. Song et al., 2013). These studies suggest that detoxification mediated by NATs may be an important protective factor in OFCs. Outside of its role in detoxification, *NAT1* (and its murine ortholog, *NAT2*) may be involved in folate metabolism (R. P. Erickson, 2010; A. Ward, Summers, & Sim, 1995), implicating a possible endogenous function during orofacial development that could be altered by environmental factors.

GSTs, another class of conjugating enzymes that are associated with OFCs, detoxify and confer water solubility to compounds by covalently adding reduced glutathione. Human cytosolic GSTs are categorized into eight classes: alpha, kappa, mu, omega, pi, sigma, theta, and zeta (Strange, Spiteri, Ramachandran, & Fryer, 2001). In human populations, GST genes are highly polymorphic for alleles that can adversely affect enzyme function. Null mutations in two genes, *GSTM1* and *GSTT1*, are rather frequent and can vary based on population ethnicity. One study, using an international database of Caucasians (n = 12,525), Asians (n = 2,136), and Africans and African-Americans (n = 996), found that the sex-averaged frequencies *GSTM1* homozygous null genotypes were approximately 53% in both Caucasians and Asians, and 28% of Africans/African-Americans (Garte et al., 2001). *GSTT1* is similar, having null alleles at sex-averaged frequencies of 20% in Caucasians and 52% in Asians (Malaysian population).

GSTM1 and GSTT1 are capable of conjugating reduced glutathione to epoxidized PAHs (Alexandrie et al., 2000; Guengerich, 1993) and are therefore important for detoxification of CYP-metabolized smoke products (Shi et al., 2008). A relationship between maternal smoking (at least 20 cigarettes per day) and fetal null *GSTM1* genotype was identified in a

California population (Lammer, Shaw, Iovannisci, & Finnell, 2005). On the other hand, maternal null *GSTM1* genotype increased the risk of CL/P in a Polish population independent of maternal smoking (Hozyasz, Mostowska, Surowiec, & Jagodziński, 2005). This may signify a more ubiquitous role in detoxifying unspecified compounds, or perhaps an endogenous housekeeping role, for GSTM1 in the prevention of OFCs. Maternal and fetal null alleles for *GSTT1* are also linked with OFCs. Dutch children whose mothers carried the *GSTT1*-null allele and smoked during pregnancy were at 3-fold increased risk for CL/P compared to smoking mothers with the reference genotype (I. A. L. M. van Rooij et al., 2001). When both fetal and maternal *GSTT1* genotypes were null, there was a 5-fold increased risk of CL/P when compared to the reference group. Maternal *GSTT1*-null genotype with smoking during pregnancy were also linked with CL/P in a California population (Lammer et al., 2005), while fetal *GSTT1*-null genotype with maternal smoking was linked to CL/P in Iowan and Danish populations with skewed risk toward CPO (Shi et al., 2007). Shi et al. (2008) note that fetal *GSTT1*-null genotype also increased risk of CPO in mothers occupationally exposed to chemicals such as dyes, propellants, and insecticides (G. M. Shaw, Nelson, Iovannisci, Finnell, & Lammer, 2003; Shi et al., 2008). Fetal polymorphisms in other GST genes, such as *GSTA4* and *GSTP1*, have also been linked with increased OFC risk in the context of maternal smoking (Krapels et al., 2008; Shi et al., 2007) with increased chance of CPO associated with the latter gene in a Danish population.

4.3. Phase III: Transport

Different families of efflux transporter proteins may be induced as part of a response to environmental exposure or may be constitutively expressed. Transporters are expressed in the placenta and have an important role in protecting the fetus from toxicants during development. Members of the ATP-binding cassette (ABC) family of proteins are involved in transporting chemicals across extra- and intracellular membranes. So far, two ABC members have been implicated in OFC pathology: *ABCA4*, involved in retinoid transport; and *ABCB1*, involved in folic acid and drug transport. ABCA4 is a flippase transporter of retinoids including retinaldehyde and N-retinyl-phosphatidylethanolamine (Beharry, Zhong, & Molday, 2004). Polymorphism mapping to the *ABCA4* region was associated with CL/P in a GWAS study using case-parent trio data from individuals in the United States, Europe, China, Taiwan, Singapore, Korea, and the Philippines (Beaty et al., 2010). Association between *ABCA4* polymorphism and OFCs has been corroborated in several studies using data from various populations (Beaty et al., 2013; Fontoura, Silva, Granjeiro, & Letra, 2012; Lennon et al., 2012; Mi et al., 2015; Wu et al., 2018; Wu-Chou et al., 2020; Yuan, Blanton, & Hecht, 2011; Z.-w. Zhou et al., 2013). Using in situ hybridization, the authors of the initial study noted that *Abca4* is not expressed in the developing murine palate at E13.5-14.5 (Beaty et al., 2010).

Evidence to support involvement of ABCA4 in orofacial development is lacking, as it is a photoreceptor-specific transporter (Beharry et al., 2004; Lennon et al., 2012). A mouse study demonstrated that dysfunction of an adjacent gene, *Arhgap29*, causes CL/P likely through interacting with an Irf6 regulatory network (E. J. Leslie et al., 2012). The authors of that study proposed *ARHGAP29* as the true etiological gene in *ABCA4*-associated

polymorphisms that increase CL/P risk. Given these circumstances, further work is needed to determine whether *ABCA4* truly plays a role in OFCs.

ABCB1, encoding the P-glycoprotein efflux pump, is expressed in the placenta and can regulate the movement of several drugs (Hitzl et al., 2004; von Richter et al., 2009), making it an important transporter for protecting embryo development. In a case-control study of a Dutch population, children whose mothers had the 3435TT genotype and took any medication during pregnancy had an over 6-fold risk of CL/P compared to children whose mothers had the 3435CC and did not take medication. (Bliek et al., 2009). Notably, folic acid supplementation around the time of conception ameliorated these effects by 30%. Previous work has established that P-glycoprotein can mediate the export of folates, and folate-rich conditions can increase the capacity of P-glycoprotein to transport substrate (Hooijberg et al., 2004). Consistent with a role for ABCB1 in orofacial development, mouse pups with an *Abcb1*-null genotype are susceptible to cleft palates following maternal exposure to the macrocyclic lactone, avermectin (Lankas, Wise, Cartwright, Pippert, & Umbenhauer, 1998; Macdonald & Gledhill, 2007).

5. Behavioral risk factors for OFCs

Behavioral risk factors are among the most modifiable, making them a useful target in the prevention of OFCs. Known behavioral risk factors include smoking, drinking alcohol, and consumption of pharmacological agents. Here we describe studies examining the correlation between these factors and OFC with a focus on tobacco smoke and alcohol consumption.

5.1. Tobacco smoke

Historically, the consensus regarding an association between maternal exposure to tobacco smoking during pregnancy and OFC was divergent. In 2004, meta-analysis of 24 case-control and cohort studies published in the World Health Organization Bulletin identified associations between maternal smoking and either CL/P [RR = 1.34; 95% CI: 1.25, 1.44] or CPO (RR = 1.22; 95% CI: 1.10, 1.35) (Little, Cardy, & Munger, 2004). Ten years later, the 50th Anniversary United States Surgeon General's report declared sufficient evidence for a causal link between maternal active smoking and OFC ("The health consequences of smoking—50 years of progress: a report of the Surgeon General," 2014; Honein, Devine, Grosse, & Reefhuis, 2014). Since then, several studies in various regions of the world have implicated maternal active smoking in OFC pathology (Table 1). Even though the magnitude of the association was not large in many studies, increased statistical power through meta-analyses provides further validation of the risks associated with smoking. Including 29 case-control studies, meta-analysis by Xuan et al. (2016) identified increased risk for CL/P (OR = 1.37, 95% CI: 1.26, 1.49) and CPO (OR = 1.24, 95% CI: 1.12, 1.38) as a consequence of maternal active smoking (Xuan et al., 2016). There is also substantial evidence that environmental tobacco smoke (ETS, also called second-hand smoke or maternal passive smoking) poses an OFC risk which in some cases was found to exceed that of active smoking. Two meta-analyses identified increased risk for OFC due to maternal passive smoking (OR = 1.87, 95% CI: 1.47, 2.39 (Zheng, Xie, Yang, & Qin, 2019); OR = 1.51, 95% CI: 1.16–1.97 (Oldereid et al., 2018)). A case-control study on Norwegian and American

populations found that combined maternal active and passive smoking elevated the risk of either CL/P, CPO, or CLO relative to active or passive smoking alone (Kummet et al., 2016), indicating an additive effect of tobacco smoke toward OFC risk. Consistently, the number of cigarettes consumed appears to be an additional factor and indicates a dose-response relationship between smoking and OFCs. In an American population, Chung et al. identified elevated risk of OFC when pregnant women smoked 21 or more cigarettes per day (OR = 1.78, 95% CI: 1.22, 2.59) relative to 1-10 per day (OR = 1.50, 95% CI: 1.28, 1.76) (Chung, Kowalski, Kim, & Buchman, 2000). A similar effect was observed in a Chinese study on passive smoking, where developing offspring of mothers exposed to ETS > 6 times/week (OR = 2.6, 95% CI: 1.8, 3.8) were at greater risk for OFC compared those whose mothers were exposed 1-6 times/week (OR: 1.2, 95% CI: 0.9, 1.7) (Pi et al., 2018). Biomarkers, such as the nicotine metabolite cotinine, may help elucidate the dose-relationship between tobacco smoke and OFCs. A retrospective case-control study on the cotinine levels of pregnant women found no dose-response relationship between cotinine and OFC risk (G. M. Shaw et al., 2009). The authors noted that a prospective study, with a larger sample size and consideration for the relatively fast rate of cotinine metabolism, would provide a better measure of the dose-response relationship.

Because smoking is an established risk factor for OFCs, efforts must be made on individual and societal levels to prevent future incidence of tobacco smoke related OFCs. Health care providers and policymakers can advocate more effectively by knowing how many incidences of OFC can be prevented through modified behavior. Population attributable fraction (PAF) is a measure of the proportion of incidents that can be attributed to a given risk factor. Honein et al. found that that up to 6.1% of OFC cases in the United States could be prevented by ceasing maternal active smoking in the periconceptual period of pregnancy (Honein et al., 2014). A recent prospective case-control study on a Japanese population indicated that the PAF of CL/P due to maternal active smoking is 9.9%, and 10.8% for passive smoking (Sato et al., 2020).

There are thousands of compounds present in tobacco smoke (Rodgman & Perfetti, 2016). Correspondingly, there are several hypothesized mechanisms for how tobacco smoke increases the risk of OFCs. The expression of genes involved in cell cycle regulation, DNA repair, and oxidative stress response has been affected by tobacco smoke in fetal mouse tissue (Izzotti et al., 2003). Additionally, tobacco smoke can induce proteasome-mediated degradation of proteins that mediate DNA methylation in 1st branchial arch (BA1) cells (Mukhopadhyay, Greene, & Pisano, 2015). The smoke is known to contain endogenous tobacco plant compounds, such as nicotine, as well as pyrolysis products and added chemicals (Rodgman & Perfetti, 2016). Several teratogens such as polycyclic aromatic hydrocarbons (PAHs), dioxins, carbon monoxide, pesticides, and heavy metals such as cadmium may be present in tobacco smoke. One possible mechanism for smoke-induced OFC is AHR activation. Exposure to heavy metals can cause OFCs in rodent models and are believed to act teratogenically through induction of oxidative stress and perturbation of redox-sensitive signaling pathways (W. L. Ni et al., 2018). Both nicotine and carbon monoxide can cause fetal hypoxia, a condition known to induce CL/P in mouse embryos (Bailey, Johnston, & Billet, 1995). Nicotine is a vasoconstrictor that can impair uterine vascular function and affect blood flow and oxygen delivery to the fetus (Xiao, Huang,

Yang, & Zhang, 2007), while carbon monoxide can prevent oxygen transport by forming carboxyhemoglobin in red blood cells. Fetal hemoglobin has greater affinity for carbon monoxide compared to adult hemoglobin, forming 10-15% more carboxyhemoglobin than the mother (Farrow, Davis, Roy, Mccloud, & Nichols, 1990; Omaye, 2002). Pesticide exposure has been associated with OFCs (Yang et al., 2014), and some biocides can cause OFCs through inhibiting steroid anabolism (Giavini & Menegola, 2010).

Genetic susceptibility plays a major role in the risk of tobacco smoke-induced OFCs. Polymorphism of genes encoding developmental signaling ligands, such as transforming growth factor alpha (*TGFA*), transforming growth factor beta 3 (*TGFB3*), and bone morphogenetic protein 4 (*BMP4*), have been associated with OFCs in the context of maternal smoke exposure. TGFA, or transforming growth factor alpha, is a ligand for epidermal growth factor receptors. It is expressed in the MEE cells during palatal fusion (Ebadifar, Hamedi, KhorramKhorshid, Kamali, & Moghadam, 2016; Mitchell, 1997; Vieira & Orioli, 2001), and is known to regulate extracellular matrix synthesis and mesenchymal migration in palatal cultures (Ebadifar et al., 2016; Holder, Vintiner, Farren, Malcolm, & Winter, 1992; Machida et al., 1999; Qian, Feingold, Stoll, & May, 1993; Shiang et al., 1993). Variants effecting *TGFA*-smoking GxE have been observed in several populations (Ebadifar et al., 2016; Hwang et al., 1995; Junaid, Narayanan, Jayanthi, Kumar, & Selvamary, 2018; Maestri et al., 1997; G. M. Shaw et al., 1996; Sull et al., 2009). TGFB3 encodes a ligand that regulates TGF-β signaling which is critical for lip and palatal development (Reynolds et al., 2020) (in this issue). Expressed in the epithelium, *TGFB3* regulates fusion at the MES and cell proliferation. Variants in *TGFB3*, in the context of smoking, have been associated with CL/P (Guo, Huang, Ding, Lin, & Gong, 2010; Lin et al., 2010; Romitti et al., 1999), CPO (Romitti et al., 1999), and submucous cleft palate (SMCP) (Reiter et al., 2012). *BMP4*, a ligand for BMP signaling, has polymorphism associated with CL/P and is involved in fusion of the medial and lateral nasal processes (Reynolds et al., 2020) (in this issue). Polymorphism of *BMP4*, in conjunction with smoking, has been associated with CL/P (Lin et al., 2010).

In addition to growth factors, polymorphisms in other OFC-related genes have been implicated in smoking and orofacial clefts. Fetal homozygous mutation (allele 4) in *MSX1*, a transcription factor important for neural crest specification, was found to impart the greatest susceptibility for OFC when mothers were exposed to smoke during pregnancy in one study (van den Boogaard et al., 2008). The authors speculated a relationship with genes involved in cell cycle regulation. This is consistent with a hypoxic mechanism, as *Msx1* deficiency in mice can induce cleft lip coinciding with maternal hypoxia (Nakatomi et al., 2020). Another possible hypoxic mechanism is through the depriving of oxygen from certain proteins with 20G oxygenase domains that have been associated with OFCs, such as the lysine demethylase *PHF8* (Loenarz et al., 2010). Polymorphism in another transcription factor involved in regulating TGF-β during palatogenesis (Bjork, Turbe-Doan, Prysak, Herron, & Beier, 2010), *PRDM16*, was associated with OFCs and either active or passive smoking (Yin, Li, Li, & Zou, 2019). Variants of genes involved in detoxification have also been associated with tobacco smoke exposure and OFCs as previously discussed.

5.2. Electronic nicotine delivery systems

Health concerns have emerged over the recent surge in electronic nicotine delivery system (ENDS) usage such as e-cigarettes and nicotine vaporizing (vaping) devices, with little known about how exposure during the periconceptional period could impact human development. The use of ENDS as a perceived safer alternative to (or a method to quit) cigarette smoking among girls and women of reproductive age is of particular concern (Mark, Farquhar, Chisolm, Coleman-Cowger, & Terplan, 2015; Oncken et al., 2017; Wagner, Camerota, & Propper, 2017). Vapor is derived from an "e-liquid" that is usually heated by a metal filament, and chemical composition of the e-liquid depends on flavorings and other constituents. Impurities may be generated by vaporizing the e-liquid, or they may already be present in liquid form as contaminants (Hahn et al., 2014). Typical components of e-cigarette vapor, some of which pose a suspected OFC hazard, include nicotine, nitrosamines, aldehydes, carbon monoxide, heavy metals, and free radicals (Hahn et al., 2014; Rowell & Tarran, 2015). Although little is known about the developmental toxicity associated with e-cigarettes, some laboratory evidence suggests that vaping aerosols are toxic to craniofacial development. One study using *Xenopus* identified an oral cleft hazard in e-cigarette aerosols (Kennedy, Kandalam, Olivares-Navarrete, & Dickinson, 2017). The authors found that aerosol from a minimal e-liquid containing only propylene glycol, vegetable glycerin, and nicotine caused microstomia in larvae, while aerosol from e-liquids containing certain flavorings produced medial oral cleft. Exposure of murine O9–1 neural crest cells to cleft-inducing aerosols decreased expression of vascular and chondral markers, indicating a potential hazard for mammalian craniofacial development. The risk for human OFC due to vape exposure remains to be determined.

5.3. Alcohol consumption

Unlike tobacco smoking, there is less substantial evidence for a role of maternal alcohol consumption in OFC etiology (Table 2). A recent study in Brazil found elevated but non-significant risk associated with alcohol consumption at the highest frequency measured (OR = 1.55, 95% CI: 0.56, 4.26). Another recent study in Japan also observed no relationship between maternal drinking and OFC by two metrics: the mother quit drinking after pregnancy (OR = 0.87, 95% CI 0.63, 1.22) versus current drinker during pregnancy (OR = 0.71, 95% CI: 0.22, 2.27) (Sato et al., 2020). Meta-analysis by Yin et al. similarly found no relationship between any drinking and CL/P (OR = 1.00, 95% CI: 0.87, 1.15) or CPO (OR = 1.02, 95% CI: 0.92, 1.14) (Yin et al., 2019). However, some studies have identified an OFC risk related to alcohol consumption in China (OR = 1.79, 95% CI: 1.03, 3.12) (D. P. Xu et al., 2018), Democratic Republic of the Congo (OR = 19.30, 95% CI: 1.89, 197.10) (Mbuyi-Musanzayi et al., 2018), and Mexico (OR = 1.90, 95% CI: 1.17, 3.08) (Angulo-Castro et al., 2017). A pooled study by Deroo et al. (2016) observed an increased risk for CLO (OR = 1.95, 95% CI: 1.23, 3.11), but only for instances where the mother binged (5+ drinks) in at least three different sittings during pregnancy (Deroo et al., 2016). Another study in Brazil found an increased risk of CL/P due to drinking relative to CPO (RR: 1.54, 95% CI: 1.07, 2.38) (Maranhao et al., 2020).

The exact mechanisms by which consuming alcoholic beverages increases the risk of OFC are not understood, but it has been intensely studied in the context of other developmental

pathologies. Several hypotheses exist for teratological mechanisms of ethanol (EtOH) that could plausibly account for OFCs, such as antagonism of retinoic acid (RA) signaling (Pullarkat, 1991), altered epigenetics (F. C. Zhou et al., 2011), and oxidative stress (Zima et al., 2001). These mechanisms involve the oxidation of EtOH into a toxic intermediate, acetaldehyde (AcAL), before ultimately being metabolized into acetyl-CoA by aldehyde dehydrogenase (ALDH) (Deitrich, Zimatkin, & Pronko, 2006). Enzymes that form AcAL from EtOH include catalase, ADH, and the CYP enzyme CYP2E1. In the RA-EtOH competition model, AcAL competes with retinaldehyde for enzymes that biosynthesize RA. A recent study using *Xenopus* found that embryonic exposure to EtOH prevented RA synthesis due to the competitive binding of AcAL with Aldh1a2 (retinaldehyde dehydrogenase 2), which synthesizes RA from retinaldehyde (Shabtai, Bendelac, Jubran, Hirschberg, & Fainsod, 2018). Chemical inhibition of ADH prevented formation of AcAL, permitting normal RA synthesis by Aldh1a2. Kinetic analysis of human ALDH1A2 identified a preference for AcAL as a substrate over retinaldehyde, providing a biochemical basis for the RA-EtOH competition model. Protective *CYP2E1* gene variants associated with decreased risk of OFC are consistent with differential ability to metabolize EtOH as well as RA (Chevrier et al., 2007).

While the RA-EtOH competition model seems like a straightforward mechanism, the effect of EtOH on orofacial development appears to be more complex. In zebrafish larvae, EtOH exposure disrupted hair cell development in the lateral line but these effects were partially rescued by co-exposure with RA or folic acid (Muralidharan, Sarmah, & Marrs, 2015). The rescue effects of folic acid could be manifold due to its supportive effects on cell proliferation, epigenetic changes, and oxidative stress. Indeed, EtOH exposure can alter the epigenetic state of neural stem cells by altering the DNA methylation of cell cycle genes (Hicks, Middleton, & Miller, 2010). Another zebrafish study found that craniofacial malformations in *pdgfra* mutants were exacerbated by EtOH, and *pdgfra* protected against EtOH through the PI3K-mTOR pathway (McCarthy et al., 2013). Additionally, *in vitro* studies found that metabolism of EtOH to AcAL by CYP2E1 can lead to formation of ROS to induce oxidative stress-mediated activation of JNK signaling, and apoptosis (Jin, Ande, Kumar, & Kumar, 2013). Vitamins C and E, like through antioxidant activity, could rescue these affects. In murine embryonic stem cells, EtOH and AcAL promoted differentiation through transcriptional activities of the RA receptor, RAR-γ, causing induction of target genes such as *Hoxa1* and *Cyp26a1* (Serio, Laursen, Urvalek, Gross, & Gudas, 2019). Thus, EtOH exposure may alter the stemness and/or fate trajectory of cells during embryogenesis (Serio & Gudas, 2020).

While these effects are seemingly consistent with disruption of orofacial development, there is little experimental evidence linking them together. Much work remains to be done regarding EtOH exposure and mechanisms of OFC, but there is enough knowledge to begin well-planned investigations.

5.4. Diet and pharmacological exposures

The relationship between maternal diet and risk for OFC has been studied intensively. Higher quality diet, from the standpoint of the Mediterranean diet and USDA Food Guide

Pyramid, is associated with better outcomes for orofacial development (Carmichael et al., 2012). Some research indicates that vegetarian diets increase the risk of OFCs (Neogi et al., 2017), possibly because of a lack of vitamins (specifically, cobalamin), minerals, and fatty acids that are more prevalent in animal products (Pistollato et al., 2015). In a Chinese study, maternal consumption of tea and pickled food was associated with higher risk of OFCs, whereas soy consumption was protective (Lili et al., 2017). More studies will be necessary to determine the safety of select food items in the context of orofacial development. In these studies, food contamination should be a major consideration. For example, a study in the Democratic Republic of the Congo found a substantially increased risk for OFC in mothers that consumed Kapolowe fish, which was substantially contaminated with heavy metal (OR: 29.47, 95% CI: 7.44, 116.72) (Mbuyi-Musanzayi et al., 2018).

There is also evidence that supplementation with vitamins and minerals can protect against OFCs, but not all studies agree. A study in China found that multivitamin use decreased risk of both CL/P (OR = 0.04, 95% CI: 0.01, 0.11) and CPO (OR = 0.08, 95% CI: 0.02, 0.25), but only when taken preconceptionally (Hao et al., 2015). A recent study in Brazil, however, found no significant decreased risk of OFC among those that took a multivitamin (Regina Altoe et al., 2020). In some cases, use of pharmacological agents during pregnancy could increase risk of OFC. One of the best studied is phenytoin, an anti-convulsant that was previously believed to induce cleft palate through its interaction with the GR (Inayoshi, Ohsaki, Nagata, & Kurisu, 1989). It is now understood that phenytoin teratogenicity is more likely mediated through its inhibition of delayed rectifier K^+ channels, causing cleft palate by altering heart pumping and inducing hypoxia (Azarbayjani & Danielsson, 2001).

5.5. Occupational and domestic exposures

Occupational exposure to solvents may be a risk factor for OFCs. An example is methanol, poisonous alcohol with the capacity for inducing cleft palate in mice (Siu, Shapiro, Wiley, & Wells, 2013). A recent meta-analysis found that glycol ethers specifically increased the risk for CL/P (OR = 1.95, 95% CI: 1.38, 2.75) (Spinder et al., 2019). Another study on a Chinese population associated unspecified solvent exposure to a large increase in risk for CL/P (OR = 6.07, 95% CI: 1.49, 24.76) (Hao et al., 2015). The evidence for an association is not always consistent, however, as multiple studies have failed to associated OFC risk with solvent exposure. In Texas, exposure to relevant environmental levels to any of the BTEX compounds (benzene, toluene, ethyl benzene, or xylene) was not associated with CL/P (Ramakrishnan et al., 2013). Maternal occupational exposure to chlorinated solvents was also not associated with OFCs based on data from the National Birth Defects Prevention Study (Desrosiers et al., 2012). If solvents are a risk factor for OFCs, it likely depends on the chemical class and whether the mother or fetus are genetically susceptible due to polymorphisms of detoxification genes (Chevrier et al., 2007).

Biocides, including agricultural pesticides, are potential OFC risk factors. One meta-analysis based on 19 studies found a slight increased risk associated with pesticide exposure (OR = 1.37; 95% CI: 1.04 to 1.81) (Romitti, Herring, Dennis, & Wong-Gibbons, 2007). A study in the Democratic Republic of the Congo identified a significantly increased CL/P risk associated with pesticides (OR = 130.306, 95% CI: 13.19, 1286.95) (Mbuyi-Musanzayi et

al., 2018). In China, a case-control study identified an increased risk for both CL/P (OR = 23.99, 95% CI: 9.58, 60.06) and CPO (OR = 17.20, 95% CI: 6.35, 46.60) among mothers exposed to pesticides (Hao et al., 2015). Little is known about how specific pesticides could influence orofacial development. One class of biocides, azole fungicides, can induce cleft palate through their inhibitory effect on CYP51 (Giavini & Menegola, 2010). CYP51, or sterol 14α-demethylase, is a cholesterogenic enzyme that catalyzes an initial step required for steroid hormone synthesis. Homozygous Cyp51 knockout mouse embryos, while suffering lethality at E15, present with phenotypes correlating with Antley-Bixler syndrome that includes cleft palate (Keber et al., 2011).

Residential proximity to chemical emission may contribute to OFC incidence. In Texas, mothers over 35 were at increased risk to bear offspring with CL/P if they lived near sources of ethyl chloride (OR = 1.81, 95% CI: 1.06, 3.07), or CPO if they lived near sources of 1,2-dichloroethane (OR = 1.93, 95% CI: 1.05, 3.54) (Brender, Shinde, Zhan, Gong, & Langlois, 2014). A study in China found that residence near any factory also increased the risk for CL/P (OR = 1.90, 95% CI: 1.60, 2.25) (Lili et al., 2017). A possible contributing factor to the risk of OFCs for those who live near factories, or for those who work industrial professions, is the presence of AHR ligands in industrial or combustion products such as PAHs, polychlorinated biphenyls (PCBs), and polychlorinated dibenzofurans (PCDFs). Benzo[a]pyrene (BaP) is an example of a highly teratogenic and carcinogenic PAH that can induce cleft palate in mice (Shum, Jensen, & Nebert, 1979). A case control study using data from National Birth Defects Prevention Study identified a significant association between cleft lip with or without cleft palate and maternal occupational PAH exposure (Langlois et al., 2013). Another study in China measured PAH levels in umbilical cord samples from children with and without OFC as a surrogate for *in utero* exposure (W. Ni et al., 2019). In this analysis, the authors found no significant association between *in utero* PAH exposure and OFC. Few studies have examined the effects of PAH exposure on human orofacial development, and more are needed to understand if such an association exists.

Other persistent organic pollutants like PCBs and PCDFs interact with the AHR and are associated with cleft palate (Denison & Nagy, 2003). PCBs may have dioxin-like or non-dioxin-like structure (Van den Berg et al., 2006). They have been used primarily as electrical insulating, hydraulic, heat transfer, and lubricating fluids, with additionally utility as plasticizers, fire retardant mixtures, and carbonless copy paper (M. D. Erickson & Kaley, 2011). Although U.S. manufacturing of PCBs ended in 1977 due to concerns over their adverse health effects, they continue to be used and their environmental persistence is still a concern. In mice, subcutaneous injection of Kanechlor-500 (KC-500), a mixture of PCBs of which the main constituent was pentachlorobiphenyl, daily during E6-15 increased cleft palate incidence at doses of 10 – 50 mg total (not normalized to maternal body weight) (Watanabe & Sugahara, 1981). In another study, 2,2’, 4,4’, 5,5’-hexachlorobiphenyl (HCB) was found to cause hydronephrosis, but not cleft palate, in mouse embryos. Co-exposure of HCB and TCDD reduced the incidence of cleft palate compared to TCDD exposure alone, suggesting that HCB competes with TCDD for binding AHR (Morrissey, Harris, Diliberto, & Birnbaum, 1992). Non-dioxin-like PCBs have been shown to suppress the response to TCDD or PCB-126 (a dioxin-like PCB) exposure in rat liver cells in vitro (Brenerova et al.,

2016). PCB exposures are therefore a possible risk for OFCs, but this depends on exposure context including presence or absence of other AHR ligands.

PCDFs, which are structurally similar to dioxins, are often found as combustion products of organic materials that contain chlorine such as PCB-containing substances. They are not intentionally produced for industrial purposes and have no known industrial or commercial utility (Fiedler, 1996). Among those that induce cleft palate in mice include 2,3,7,8-tetrachlorodibenzofuran (TCDF), 1,2,3,7,8-pentachlorodibenzofuran (1-PeCDF), 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF), and 1,2,3,4,7,8-hexachlorodibenzofuran (HCDF) (L. Birnbaum, Harris, Barnhart, & Morrissey, 1987; L. S. Birnbaum, Harris, Crawford, & Morrissey, 1987; Hassoun, Dargy, Dencker, Lundin, & Borwell, 1984; Weber, Lamb, Harris, & Moore, 1984). In Japan during 1968, rice oil was contaminated with PCBs and associated degradation products (i.e., PCDFs) resulting in a mass poisoning. Congenital effects of *in utero* exposure to contaminated rice oil included altered pigmentation and was known as fetal PCB syndrome (Mitoma et al., 2015). While no cases of OFC have been reported in association with the Yusho incident, experimental evidence in mouse has indicated a cleft palate hazard for PCBs and PCDFs.

6. Pathogens and orofacial clefts

While chemical exposures and genetic predisposition can influence the risk of OFCs, there is evidence that biotic factors such as pathogens may also be involved. In some cases, such as with cytomegalovirus, specific molecular pathways likely contribute to OFC pathology. For others, however, it is unclear whether the pathogen acts through a specific mechanism or if the maternal or fetal immune response causes a teratogen effect. Here we discuss pathogenic associations with OFCs and, where possible, describe specific mechanisms affecting orofacial development.

6.1. Viruses

Viral infection during the first trimester is a suspected cause of OFC. While some viruses are known teratogens, the evidence for an association with OFC is divergent. Among the viruses considered potential risk factors for OFC are varicella, rubella, rubeola, Epstein-Barr virus, herpesvirus, cytomegalovirus, common cold, and influenza (Acs, Banhidy, Puho, & Czeizel, 2005; Banhidy, Puho, Acs, & Czeizel, 2007b; Cohen, 2000; A. E. Czeizel, Puho, Acs, & Banhidy, 2008; Luteijn, Brown, & Dolk, 2014; Metneki, Puho, & Czeizel, 2005; Molnarova et al., 2018; W. Shaw, 2004; J. Zhang & Cai, 1993). In 1963, Leck detected an elevated incidence of OFC in relation to influenza infection (Leck, 1963). Based on observations, he speculated that if an influenza-induced syndrome of birth defects existed, cleft lip would be among the four major congenital abnormalities most likely observed. However, several studies have disagreed that influenza infection is teratogenic (A. Czeizel, Tusnady, Domany, & Borsy, 1967; Doll, Hill, & Sakula, 1960; Walker & Mc, 1959; Widelock, Csizmas, & Klein, 1963; Wilson & Stein, 1969). Notably, different groups have reached divergent conclusions from the 1957 Asian influenza pandemic. While Coffey and Jessop (1959) identified an elevated incidence of congenital abnormalities in Dublin, Doll and Hill (1960) found no such correlation in London (Coffey & Jessop, 1959; Doll et al., 1960). The latter

group stated that if craniofacial malformations are a hazard of influenza infection during the first trimester, then the risk is so low that it is difficult to measure. One possible confounder of these types of studies is the consumption of drugs for symptom relief during acute illness (Saxén, 1975).

In spite of the divergent conclusions of the literature, more recent studies have identified a correlation between influenza and orofacial cleft (Acs et al., 2005; Metneki et al., 2005). The mechanisms involved are not fully understood, but research suggests that hyperthermia from fever induction may be an indirect driver of teratogenesis. A Hungarian case-control study by Acs et al. (2005) identified a significant correlation between influenza and cleft lip/palate among mothers that were diagnosed with influenza in the second and/or third month of the first trimester (Acs et al., 2005). Notably, this correlation disappeared among women that used antipyretics. Another study in Hungary found a correlation between OFC and other febrile infections that may be viral or bacterial, such as gastroenteritis, sinusitis, and bronchitis (Metneki et al., 2005). Herpesvirus infection during the second and/or third month of the first trimester has also been associated with congenital abnormalities including OFC, and antipyretic use in this context has been correlated with decreased OFC incidence (Banhidy, Puho, Acs, & Czeizel, 2007a). A separate study purposed for identifying non-pathogenic etiologies found a slight elevation of risk for OFC among mothers that used an electric blanket or heated waterbed in the periconceptional period (G. M. Shaw, Nelson, Todoroff, Wasserman, & Neutra, 1999). Further work must be undertaken to verify a hyperthermal etiology for OFC and pathogenic teratogenesis.

Cytomegalovirus (CMV), a herpes-related virus, is a suspected etiological agent of OFC and its mechanism of action appears to be independent of hyperthermia. CMV infection in pregnant women is a known teratogenic hazard. Weichert et al. (2010) reported a single case of cleft lip and micrognathia in a fetus whose mother was infected with CMV (Weichert, Vogt, Dudenhausen, & Kalache, 2010). In 2017, Divya et al. reported that CMV may be a potent inducer of cleft lip/palate, mental disability, and deafness (Divya et al., 2017). Animal models have shown that CMV impairs mesenchymal development, making orofacial development particularly vulnerable to CMV infection due to its effects on epithelial-mesenchymal interactions (Tsutsui, 1995; Weichert et al., 2010). Infection of murine BA1 culture demonstrated a tropism of CMV toward neural crest mesenchyme and disruption of the extracellular matrix (Melnick, Mocarski, Abichaker, Huang, & Jaskoll, 2006). The authors also suspected an EMT mechanism in CMV-exposed submandibular salivary glands, although this was difficult to ascertain in the *ex vivo* context. A follow-up study demonstrated that CMV infection of murine mandibular process culture from E11 resulted in mandibular hypoplasia stemming from decreased osteogenesis, as well as decreased chondrogenesis of Meckel's cartilage (Jaskoll, Abichaker, Sedghizadeh, Bringas, & Melnick, 2008; Melnick et al., 2006). Both studies described a disruption of nuclear factor kappa B (NFκB) signaling. The latter paper also demonstrated increased abundance of the Tgf-β signaling inhibitor Smad7 in infected osteoblasts and periosteal cells, as well as an absence of *Shh* expression in Meckel's cartilage. Given that both Smad7 and Shh are critical for normal orofacial development and are responsive to NFκB, the authors postulated that CMV may induce craniofacial malformations through an NFκB-dependent mechanism.

6.2. Bacteria

Like viral infections, there is some divergence as to whether bacterial infection can cause OFC. Some bacteria suspected of an association with OFC include mycoplasma (*Mycoplasma pneumoniae*), chlamydia (*Chlamydia trachomatis*), and syphilis (*Treponema pallidum pallidum*) (Gupta, Chotaliya, & Marfatia, 2012; LopezCamelo & Orioli, 1996; Molnarova et al., 2006). Based on clinical data from Campeche, Mexico, authors of a case-control study identified a possible association between bacterial infection during pregnancy and OFC, although information on specific pathogens was unavailable (Acuna-Gonzalez et al., 2011). A Slovak study published in 2006 compared the presence of antibodies to mycoplasma and chlamydia infection in newborns with OFC incidence (Molnarova et al., 2006). This study identified a high prevalence of mycoplasma antibodies in newborns with OFC and their mothers, in addition to a slightly elevated prevalence of chlamydia antibodies. The authors concluded that mycoplasma infection was more frequent in newborns with OFC and their mothers, and that chlamydia presented a comparatively lower and perhaps insignificant risk for OFC. They nonetheless state that this study could not identify a definitive association between infection with pathogen of OFC. Overall, the relationship between OFC and bacterial infection remains to be elucidated.

6.3. Protozoa

The only known protozoan associated with OFC is *Toxoplasma gondii*, the pathogen responsible for the disease toxoplasmosis. While usually asymptomatic, transmission of *T. gondii* from mother to fetus can result in serious birth defects, including craniofacial malformations such as microcephaly and hydrocephalus (J. Jones, Lopez, & Wilson, 2003). A survey published in 2001 indicated that 15% of women at childbearing age in the United States showed evidence of *T. gondii* infection (J. L. Jones et al., 2001). Latent maternal infection poses a low risk to the fetus since the parasites exist within tissue cysts as nonmigratory bradyzoites. However, ingestion of sporulated *T. gondii* oocytes from the environment is followed by transformation into the migratory tachyzoite stage. Only in this form can *T. gondii* cross the placental barrier to infect the fetus; thus, the hazard for congenital toxoplasmosis exists predominantly for women that are pregnant at the time of infection. Cats are the definitive host for the *T. gondii* reproductive stage, and congenital toxoplasmosis is one of the best-described hazards for maternal handling of used cat litter during pregnancy. Another entryway for *T. gondii* infection is through consuming contaminated food or water (Dubey, 2009).

Toxoplasmosis has been a suspected etiological agent of OFC since at least the 1950s (Gabka, 1953). In 1992, a hospital in Kunshan, China identified ten children with OFC that had *T. gondii* tachyzoites and bradyzoites within the cleft tissue (Gu, Lu, & Ma, 1992). A 1994 Slovak study found a correlation with serum antibodies for *T. gondii* in mothers and their children with OFC (Holková et al., 1994). In 2017, a hospital in Mexico City reported a single case of congenital toxoplasmosis where the infant presented with oblique oro-orbital cleft and associated anophthalmia (Arce-Estrada et al., 2017). While congenital rubella infection was also implicated, the defects were attributed to congenital toxoplasmosis. Despite this evidence, not all studies have identified an association between OFC and

congenital toxoplasmosis (Gylling, 1967). Further study is required to understand the full teratogenic potential of *T. gondii*.

7. Conclusions

Orofacial development is an intricate biological process involving rapid growth, differentiation, tissue fusion, transformation, and many other processes (Ji et al., 2020) (in this issue). The possible environmental influences on these activities are correspondingly complex and can interact with various aspects of development. This is further complicated by genetic variation that could perturb (or enhance) both endogenous processes and cellular capacity for dealing with environmental insults (Shi et al., 2007). Understanding the mechanisms by which environmental factors impact orofacial development could help in the prevention of future OFC cases.

Among the most important metabolic processes in orofacial development are folate and retinoid metabolism. Folates promote cellular proliferation, differentiation, epigenetics, and can prevent oxidative stress by preventing hyperhomocysteinemia (Wahl et al., 2015; Wong et al., 1999). There is increasing evidence that folic acid supplementation protects against OFCs, but its effectiveness may vary on a population or individual basis. A major influence on this outcome may be allelic variation in genes related to folate metabolism (Ramirez-Chau et al., 2016). Studies that integrate, genetic, dietary, and metabolic data would help clarify whether folates are truly protective against OFCs. Given the importance of maternal folate metabolism in OFCs, personalized guidance based on genotype may help guide expecting parents in managing their dietary needs. A similar approach may also clarify whether supplementing with vitamin A (i.e., consuming cod liver oil) is protective or an unnecessary risk.

To address the environmental mechanisms of OFCs, there are several challenges that must be met. These including the identification of hazards, genetic susceptibilities, and preventive measures. While many environmental factors are already known to increase the risk OFCs, there are likely many others for which the hazard has yet to be identified. Robust chemical screens using *in vivo* (S. Liu, Narumi, Ikeda, Morita, & Tasaki, 2020), *in vitro* (Belair et al., 2018), and *in silico* (Baker et al., 2020) approaches will be needed to identify OFC-inducing teratogens. Such approaches may include novel chemical screens or utilize *a priori* knowledge of bioinformatic relationships. These screens will also facilitate the identification of therapeutic supplements that protect against environmentally-induced OFCs (Muralidharan et al., 2015). Incorporating genetic models will add a layer of complexity to these approaches. Genome-wide surveys for gene-environment interactions can assist in the developmental of such models (O. A. Haaland et al., 2018; Ø. A. Haaland et al., 2019). Taking full advantage of these approach will help identify hazards, mechanisms, and therapeutic approaches in the effort to prevent OFCs.

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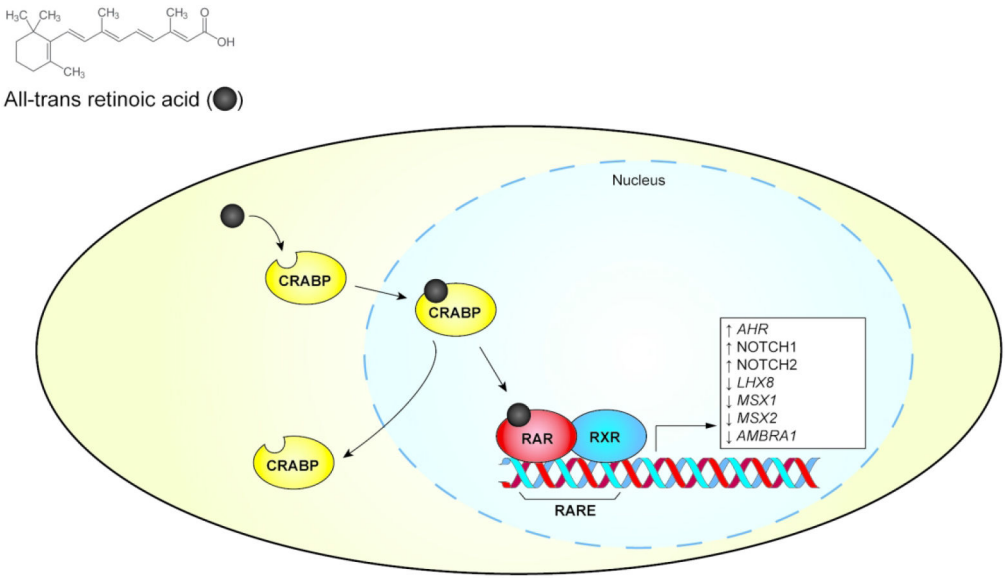

Figure 1.

Simplified retinoic acid receptor (RAR) signaling pathway. The pathway's modulation of gene expression and protein abundance associated with orofacial clefts is shown in the white box. CRABP, cellular retinoic acid binding protein. RARE, retinoic acid receptor response element. RXR, retinoid X receptor.

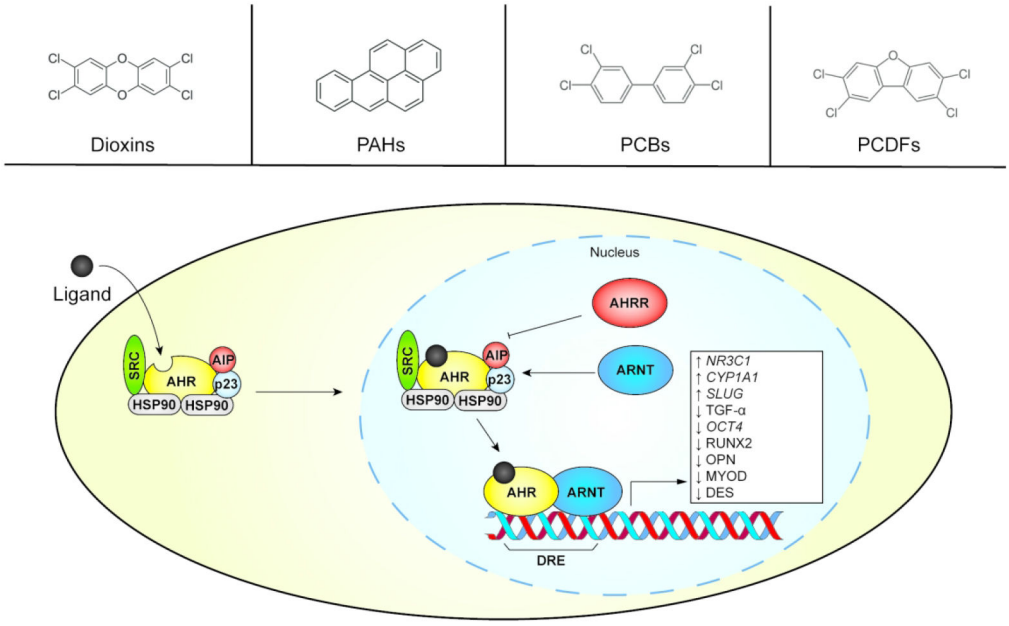

Figure 2.

Simplified aryl hydrocarbon receptor (AHR) signaling pathway. Representative members of ligand classes are presented in the top panels, while the AHR transcriptional activation pathway is illustrated below. The pathway's modulation of gene expression and protein abundance associated with orofacial clefts is shown in the white box. ARNT, aryl hydrocarbon receptor nuclear translocator. AHRR, aryl hydrocarbon receptor repressor. AIP, aryl hydrocarbon receptor interacting protein. DRE, dioxin response element. PAH, polycyclic aromatic hydrocarbon. PCB, polychlorinated biphenyl. PCDF, polychlorinated dibenzofuran.

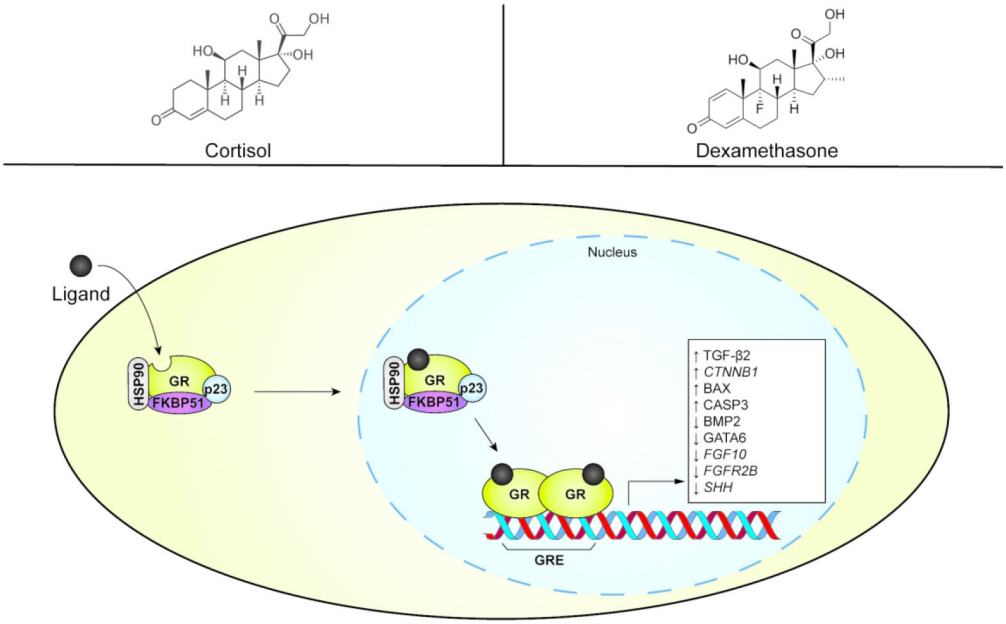

Figure 3.

Simplified glucocorticoid receptor (GR) signaling pathway. Examples of the endogenous ligand (cortisol) and a synthetic ligand (dexamethasone) are presented in the top panels. The pathway's direct or indirect modulation of gene expression and protein abundance associated with orofacial clefts is shown in the white box. FKBP51, FK506 binding protein 51 (representing immunophilins). GRE, glucocorticoid response element. HSP90, heat shock protein 90.

Table 1.

Recent population studies on the effects of maternal active and passive smoking on the risk for OFCs. Final adjusted regression model statistics used where available. Sample sizes reflect totals for overall study. CI, confidence interval. CLO, cleft lip only. CL/P, cleft lip with or without cleft palate. CPO, cleft palate only. OFC, any orofacial cleft. OR, odds ratio. PR, prevalence ratio. RR, relative risk.

Study	Design	Population	Sample	Exposure type	Statistic (95% CI)
(Regina Altoe et al., 2020)	Case-control (retrospective)	Brazil	OFC: n = 150 Control: n = 300	Maternal active smoking	OR: 1.53 (0.61, 3.87)
				Maternal passive smoking	OR: 2.18 (1.21, 3.93)
(Maranhao et al., 2020)	Relative risk	Brazil	CL/P n = 345 CPO: n = 148 CLO n = 153	Maternal active smoking	CL/P vs CPO RR: 0.94 (0.52, 1.71)
					CLO vs CPO RR: 0.61 (0.17, 2.12)
(L. Acs et al., 2020)	Case-control (retrospective; matched and population)	Hungary	CPO: n = 751 Matched control: n = 1196 Population control: n = 57231	Maternal active smoking	Matched control OR: 2.5 (1.9, 3.3)
					Population control OR: 2.3 (1.9, 2.8)
(Sato et al., 2020)	Case-control (prospective)	Japan	CL/P: n = 146 Control: n = 93987	Maternal active smoking	Smoked during pregnancy OR: 1.28 (0.77, 2.12)
				Maternal passive smoking	Every day OR: 1.49 (0.93, 2.39)
(Zheng et al., 2019)	Meta-analysis			Maternal passive smoking	OR: 1.87 (1.47, 2.39)
(Oldereid et al., 2018)	Meta-analysis			Maternal passive smoking	OR: 1.51 (1.16–1.97)
(Bui, Ayub, Ahmed, Taioli, & Taub, 2018)	Case-control (Retrospective)	Pakistan	CL/P: n = 225 CPO: n = 72 Control: n = 131	Maternal, paternal, or both parents smoking	OFC OR: 1.89 (1.10, 3.26)
					CL/P OR: 2.00 (1.13, 3.53)
					CPO OR: 1.51 (0.71, 3.19)
(Pi et al., 2018)	Case-control (retrospective)	China	OFC: n = 240 Control: n = 1420	Maternal passive smoking	1-6 times/week OR: 1.2 (0.9, 1.7)
					>6 times/week OR: 2.6 (1.8, 3.8)
(Mbuyi-Musanzayi et al., 2018)	Case-control (retrospective)	DR Congo	CL/P: n = 172 Control: n = 162	Maternal passive smoking	OR: 1.46 (0.38, 5.57)
(D. P. Xu et al., 2018)	Case-control (retrospective)	China	CL/P: n = 239 Control: n = 209	Maternal active smoking	Without drinking OR: 1.67 (1.01, 2.75)
					With drinking OR: 2.21 (1.09, 4.47)
(Angulo-Castro et al., 2017)	Case-control (retrospective)	Mexico	CL/P n = 24 Control: n = 24	Maternal active smoking	OR: 8.1 (1.6-39.3)
(Ly et al., 2017)	Case-control (retrospective)	Vietnam, Philippines, Honduras, Morocco	OFC: n = 392 Control: n = 234	Maternal passive smoking	Frequency 1-3 OR: 0.40 (0.11–1.42)
					Frequency 6-14 OR: 0.41 (0.11–1.49)
					Frequency 15-20 OR: 0.68 (0.19–2.52)

Study	Design	Population	Sample	Exposure type	Statistic (95% CI)
					Frequency 20+ OR: 0.33 (0.10–1.11)
(Lili et al., 2017)	Case-control (retrospective)	China	CL/P: n = 453 Control: n = 452	Maternal passive smoking	OR: 413.94 (24.54, 984.00)
(Xuan et al., 2016)	Meta-analysis			Maternal active smoking	CL/P OR: 1.37 (1.26, 1.49)
					CPO OR: 1.24 (1.12, 1.38)
(Campos Neves, Volpato, Espinosa, Aranha, & Borges, 2016)	Cross-sectional	Brazil	CL/P: n = 64 CPO: n = 22 CLO: n = 30	Maternal active smoking	PR: 1.33 (1.16, 1.52)
				Maternal passive smoking	PR: 1.21 (1.01, 1.45)
(McKinney et al., 2016)	Case-control (retrospective)	Thailand	CL/P: n = 95 Control: n = 95	Maternal passive smoking	OR: 6.52 (1.98, 21.44)
(Kummet et al., 2016)	Case-control (retrospective)	Norway, United States	OFC: n = 4508 Control n = 9626	Maternal active smoking	CL/P OR: 1.28 (1.09, 1.51)
					CPO OR: 1.25 (1.01, 1.55
					CLO OR: 1.52 (1.19, 1.94)
				Maternal passive smoking	CL/P OR: 1.11 (0.98, 1.27)
					CPO OR: 1.18 (1.00, 1.39)
					CLO OR: 1.14 (0.93, 1.39)
				Maternal active and passive smoking	CL/P OR: 1.55 (1.36, 1.77)
					CPO OR: 1.43 (1.20, 1.70)
					CLO OR: 1.63 (1.33, 2.00)
(Hoyt et al., 2016)	Case-control (retrospective)	United States	CL/P: n = 245 CPO: n = 125 CLO: n = 94 Control: n = 369	Maternal passive smoking	CL/P OR: 1.24 (1.05, 1.46)
					CPO OR: 1.31 (1.06-1.63)
					CLO OR: 1.41 (1.10, 1.81)
(Ebadifar et al., 2016)	Case-control (retrospective)	Iran	CL/P: n = 76 CPO: n = 29 Control: n = 210	Maternal active smoking	CL/P OR: 19.2 (6.2, 59.5)
					CPO OR: 48.7 (8, 293)
				Maternal passive smoking	CL/P OR: 0.45 (0.2, 1.05)
					CPO OR: 11.2 (3.1, 40.3)
				Both parents smoking	CL/P OR: 6.0 (2.2, 16.6)
					CPO OR: 55.6 (12, 256)

Study	Design	Population	Sample	Exposure type	Statistic (95% CI)
(Sabbagh et al., 2016)	Case-control (retrospective)	Saudi Arabia	CL/P: n = 112 Control: n = 138	Maternal Jorak smoking	OR: 14.07 (1.55, 128.1)
				Maternal passive smoking	OR: 2.05 (1.05, 4.01)
(Sabbagh et al., 2015)	Meta-analysis			Maternal passive smoking	OFC OR: 2.11 (1.54, 2.89)
					CL/P OR: 2.05 (1.27, 3.30)
					CPO OR: 2.11 (1.23-3.62)
(Hao et al., 2015)	Case-control (retrospective)	China	CL/P n = 362 CPO n = 137	Maternal active smoking	CL/P OR: 1.25 (0.72, 2.17)
					CPO OR: 1.14 (0.52, 2.47)
				Maternal passive smoking	CL/P OR: 2.52 (1.90, 3.33)
					CPO OR: 2.14 (1.45, 3.15)
				Maternal passive smoking (paternal active)	CL/P OR: 2.17 (1.65, 2.87)
					CPO OR: 1.87 (1.28, 2.75)
(Chung, Kowalski, Kim, & Buchman, 2000)	Case-control (retrospective)	United States	CL/P: 2207 Control: 4414	Maternal active smoking	1-10/day OR: 1.50 (1.28, 1.76)
					11-20/day OR: 1.55 (1.23, 1.95)
					21+/day OR: 1.78 (1.22, 2.59

Table 2.

Recent population studies on the effects of maternal drinking on the risk for OFCs. Final adjusted regression model statistics used where available. Sample sizes reflect totals for overall study. CI, confidence interval. CLO, cleft lip only. CL/P, cleft lip with or without cleft palate. CPO, cleft palate only. OFC, any orofacial cleft. OR, odds ratio. RR, relative risk.

Study	Design	Population	Sample	Exposure type	Statistic
(Regina Altoe et al., 2020)	Case-control (retrospective)	Brazil	OFC: n = 150 Control: n = 300	1-7 times/week	OR: 1.18 (0.67, 2.05)
				1-2 times/month	OR: 0.20 (0.03, 1.57)
				5+ drinks	OR: 1.55 (0.56, 4.26)
				1-4 drinks	OR: 0.85 (0.46, 1.55)
(Maranhao et al., 2020)	Relative risk	Brazil	CL/P n = 346 CPO: n = 237 CLO n = 151	Unspecified frequency	CL/P vs CPO RR: 1.54 (1.07, 2.38)
					CLO vs CPO RR: 1.25 (0.51, 3.07
(Sato et al., 2020)	Case-control (prospective)	Japan	CL/P: n = 146 Control: n = 93987	Quit after pregnancy	OR: 0.87 (0.63, 1.22)
				Current drinker	OR: 0.71 (0.22, 2.27)
(Yin et al., 2019)	Meta-analysis			Any drinking	CL/P OR: 1.00 (0.87, 1.15)
					CPO OR: 1.02 (0.92, 1.14)
(D. P. Xu et al., 2018)	Case-control (retrospective)	China	CL/P: n = 239 Control: n = 209	Unspecified frequency	OR: 1.79 (1.03, 3.12)
(Mbuyi-Musanzayi et al., 2018)	Case-control (retrospective)	DR Congo	CL/P: n = 172 Control: n = 162	Unspecified frequency	OR: 19.30 (1.89, 197.10)
(Angulo-Castro et al., 2017)	Case-control (retrospective)	Mexico	CL/P n = 24 Control: n = 24	Unspecified frequency	OR: 1.9 (1.17-3.08)
(Deroo et al., 2016)	Pooled study			Non-binge (<5 drinks), 1-2 times	OFC OR: 1.07 (0.96, 1.20)
					CL/P OR: 1.09 (0.93, 1.27)
					CPO OR: 0.97 (0.82, 1.15)
					CLO OR: 1.19 (0.98, 1.45)
				Non-binge (<5 drinks), 3+ times	OFC OR: 0.89 (0.79, 1.01)
					CL/P OR: 0.86 (0.72, 1.02)
					CPO OR: 0.88 (0.74, 1.06)
					CLO OR: 0.98 (0.79, 1.21)
				Binge (5+ drinks), 1-2 times	OFC OR: 1.00 (0.70, 1.45)
					CL/P OR: 0.91 (0.55, 1.51)
					CPO OR: 1.22 (0.73, 2.04)

Study	Design	Population	Sample	Exposure type	Statistic
					CLO OR: 0.94 (0.49, 1.85)
				Binge (5+ drinks), 3+ times	OFC OR: 1.23 (0.89, 1.69)
					CL/P OR: 1.02 (0.65, 1.60)
					CPO OR: 1.04 (0.63, 1.73)
					CLO OR: 1.95 (1.23, 3.11)
(Hao et al., 2015)	Case-control (retrospective)	China	CL/P n = 362 CPO n = 137	Unspecified frequency	CL/P OR: 0.75 (0.50, 1.12)
					CPO OR: 1.28 (0.77, 2.11)
(Bell et al., 2014)	Meta-analysis			Any drinking	CL/P OR: 1.00 (0.86, 1.16)
					CPO OR: 1.05 (0.92, 1.21)
				Binge (5+ drinks at any one time)	CL/P OR: 1.04 (0.87, 1.24)
					CPO OR: 0.94 (0.74, 1.21)