Non-syndromic Cleft Palate: An Overview on Human Genetic and Environmental Risk Factors

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The epithelial and mesenchymal cells involved in early embryonic facial development are guided by complex regulatory mechanisms. Any factor perturbing the growth, approach and fusion of the frontonasal and maxillary processes could result in orofacial clefts that represent the most common craniofacial malformations in humans. The rarest and, probably for this reason, the least studied form of cleft involves only the secondary palate, which is posterior to the incisive foramen. The etiology of cleft palate only is multifactorial and involves both genetic and environmental risk factors. The intention of this review is to give the reader an overview of the efforts made by researchers to shed light on the underlying causes of this birth defect. Most of the scientific papers suggesting potential environmental and genetic causes of non-syndromic cleft palate are summarized in this review, including genome-wide association and gene–environment interaction studies.

Keywords: non-syndromic cleft palate, NSCPO, risk factors, etiology, GRHL3, FOXE1, PAX7

INTRODUCTION

Orofacial clefts are the most common orofacial malformations in humans and include cleft lip (CL), cleft lip with or without cleft palate (CL/P), and cleft palate only (CPO). CPO (MIM 119540) is a birth defect that occurs when only the secondary palate is involved and can affect the hard palate and/or the soft palate, sometimes limited to one cleft uvula. It represents one third of all oral clefts and affects about 1 to 25 per 10,000 newborns worldwide (Mossey et al., 2009). The incidence of CPO is highly influenced by ethnicity and race, with the highest rates observed in non-Hispanic Whites and the lowest in Africans (Mossey and Modell, 2012). Besides, females are more prone to the defect than males (1:1.075) (Mossey and Catilla, 2003).

Cleft palate only is a multifactorial disorder influenced by both genetic and environmental factors that act during palatogenesis (Meng et al., 2009; Dixon et al., 2011). Moreover, the local changes in growth factors, extracellular matrix (ECM), and cell adhesion molecules may also play a part in CPO onset. A variety of signaling pathways are involved in palate development and several mutations on developmental genes that have been found to contribute to CPO will be discussed.

Furthermore, the environmental contribution to CPO by tobacco, alcohol and multivitamin supplementation has been highlighted by epidemiological studies hereinafter detailed.

Classification

Cleft lip with or without cleft palate and CPO are considered as different congenital malformations, having distinct embryologic origins and recurrence risks. Hence, CPO refers to any cleft of the palate which is posterior to the palatine foramen, and which does not involve the alveolar process...
or lip. A number of different classifications have been proposed since Veau’s version in 1931 (Veau, 1931; Danescu et al., 2015). In Figure 1, a collection of different types of CPO, including the recently introduced submucous microform (Danescu et al., 2015).

Another kind of classification divides CPO into two major groups: (i) isolated CPO or non-syndromic CPO (NSCPO), when clefting is an isolated defect unassociated with any other additional anomalies, representing 54.8% of the total CPO cases; (ii) syndromic CPO, when clefting is associated with other anomalies in a recognizable (27.2%), or unrecognizable syndrome (18%) (Calzolari et al., 2004).

Although the presence of an evident pattern of anomalies makes the diagnosis easier, some features may not be fully expressed or could represent subclinical phenotypes more difficult to diagnose. Most frequently, the defects associated with CPO affect the heart (31.1%), the encephalon (hydrocephaly 11.2%), the urinary tract (9.7%), or digits (polydactyly 9.2%) (Mossey and Catilla, 2003). The number of syndromes involving cleft palate, among orofacial clefts, is subject to progressive implementation but already in 1978 it was quoted as 77 (Cohen, 1978).

The most common syndromes in which cleft palate deformity represents one of the features are: Apert Syndrome, Crouzon Syndrome, DiGeorge Syndrome, Loeys-Dietz Syndrome, Treacher Collins Syndrome, X-linked cleft palate Syndrome, Ankyloglossia, Bamforth-Lazarus Syndrome. An exhaustive overview of the syndromes that count cleft palate among their features, as well as the responsible genes, can be found in a paper by Burg et al. (2016).

**Palatogenesis**

The human palate separates the nasal and oral cavities and consists of a bony hard palate (the anterior two thirds) and a fibromuscular soft palate (the posterior one third). The incisive foramen divides the hard palate into primary and secondary portions. The secondary portion separates the nasal passage from the pharynx and is marked by a median palatine raphe. The soft palate, or velum, is a mobile muscular fold forming a curtain posterior to the hard palate, ending with a conical mass called the uvula. The soft palate separates the nasopharynx from the oropharynx.

Palatal development takes place between the fifth and twelfth embryonic weeks, with the most critical period occurring during the sixth to ninth weeks (Merritt, 2005), when the lateral palatine processes fuse, after the fusion of the secondary palate to the primary palate with a junction marked by the incisive foramen. The palate body is composed by mesenchymal cells, mainly derived from migrating neural crest cells, layered by epithelial cells that surround the mesenchyme (Ito et al., 2003). The correct growth, migration, transition, differentiation, and apoptosis of these cells form the basis of a regular development of the palate. Palatal shelves, initially grown in a vertical position, have to rotate from the sides to the top of the tongue in order to acquire a horizontal position. Then, shelves approach and fuse with each other in an antero-posterior direction in the transient midline epithelial seam (Ferguson, 1988; Meng et al., 2009).

Conceivably, there are several molecular, mechanical and morphological steps, including the key step of epithelial to mesenchymal transition, involved in the orchestra of palate development which could go wrong (Diewert and Lozanoff, 2002). Clefting of the secondary palate may then arise in any one of these critical steps, and may be caused by failure in growth, elevation, adhesion or fusion of the palatal shelves.

**Epidemiology**

Cleft palate affects newborns with a world prevalence of about 3.1/10,000, which significantly varies depending on the geographical location. According to the Global Burden of Disease (GBD) study, the highest rates of CPO are found in the Oceanic continent, and the lowest in Sub-Saharan Africa, with overall values ranging from 0.4 to 11.3 every 10,000 births (Mossey and Modell, 2012). Nevertheless, there are very few epidemiological studies about OFC in African populations and estimated values of CPO incidence may not be accurate. Moreover, even inside the same continent, CPO prevalence values vary among different geographical areas, as in Europe. In fact, according to the EUROCAT registries Finland and Malta show the highest rates while Portugal and Romania the lowest, with more than three-times difference in rates (EUROCAT).

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1. [https://www.ncbi.nlm.nih.gov/omim](https://www.ncbi.nlm.nih.gov/omim)
Besides geographical differences, there is also a disparity in the CPO incidence between genders. In fact, while for isolated CL/P cases the sex ratio was estimated at 1.81 (95% CI, 1.75–1.86), isolated CPO counts more females than males, with a sex ratio of 0.93 (95% CI, 0.89–0.96) in the world (Mossey and Catilla, 2003) and reported as 0.78 in Europe (Calzolari et al., 2004). This discrepancy is probably due to the delay of 1 week in the fusion of the palatal shelves in female embryos compared to males (Burd and Faist, 1967). Possibly, in this period of time the morphogenesis could be more influenced by teratogens, thus justifying the higher CPO rate in girls.

**ETIOLOGY**

**Environmental Factors**

The complex etiology of NSCPO can be explained, beside genetic factors, by the intervention of unmodifiable (e.g., race/ethnicity, sex, family history of clefts) and modifiable factors, acting in the period from 1 month before through 2 months after conception. Fundamentally, maternal factors such as health/disease status, lifestyle, medication, exposure to environmental teratogens can affect/influence the intrauterine environment, more heavily during embryo development, and have been the topic of a number of studies looking at their association with NSCPO risk.

Surprisingly, it is the older age of the father that has been reported to increase the risk of NSCPO in the offspring, rather than that of the mother (Savitz et al., 1991; Bille et al., 2005) as confirmed by a meta-analysis (Herkrath et al., 2012).

Among the maternal behavioral factors, alcohol consumption during the first trimester of pregnancy has been suggested to have a correlation with oral cleft onset (Romitti et al., 2007; DeRoo et al., 2008). However, there are few evidences supporting a role in the CP etiology (DeRoo et al., 2008; Sabbagh et al., 2015). A recent meta-analysis (Yin et al., 2019) concluded that there is no evidence of a correlation between moderate periconceptional alcohol consumption and the risk of both non-syndromic CL/P and CP in infants. However, authors suggest that the potential risk of binge-drinking cannot be excluded, because of the heterogeneity of the threshold level for alcohol consumption in the different investigations and of the limited sample sizes of heavy drinker mother cohorts.

Also maternal tobacco smoking has been deeply investigated as an NSCPO risk factor. The studies were recently considered in two meta-analyses, which concluded by supporting a role for both maternal tobacco smoking (Little et al., 2004) and passive smoking (Sabbagh et al., 2015) in NSCPO etiology. Nevertheless, it should be taken into account that the results of meta-analyses could be biased because the sample size, ethnicity, and consumption levels vary widely from one study to another. Moreover, the alcohol and tobacco teratogenic dose-response effect is still a matter of debate.

A recent study examined the effect of maternal exposure to water disinfection by-products (DBP), a heterogeneous group of compounds that can originate from a combination of chemical disinfectants (e.g., chlorine, chloramine) and organic matter present in water (Kaufman et al., 2018). Studying the association between DBP and the risk of craniofacial malformation, the authors obtained evidence that some DBPs (specifically: DBP9, HAA5, trichloroacetic acid, and dichloroacetic acid) can increase the risk of NSCPO. Similarly, a previous study suggested an increased risk of NSCPO as a result of maternal exposure to total trihalomethanes, but at lower ranges than those of the above-mentioned work (Hwang et al., 2008). Moreover, nitrate intake from drinking water was seen to increase the risk of NSCPO in a large cohort study (OR, 1.9; 95% CI, 1.17–3.09) (Brender et al., 2013).

Since the nutritional status of the embryo is fully dependent on maternal food intake and metabolism, unbalanced maternal nutrition during the first trimester of pregnancy can lead to birth defects. Several essential micronutrients, substances that cannot be synthesized by our body in sufficient amount, are needed for health maintenance, pregnancy progression and normal embryonic development. In case of maternal nutrition deficiencies, supplementation during pregnancy is strongly advised. It is commonly accepted that folic acid-fortified multivitamin supplementation before conception and continuing through the first trimester reduces the overall occurrence of several congenital anomalies. However, only few systematic reviews or meta-analyses regarding orofacial clefts have been conducted and findings on cleft palate are inconclusive. A systematic review analyzed data from five clinical trials in order to evaluate if supplementation with folic acid, alone or in combination with vitamins and minerals, can prevent the occurrence of neural tube defects (NTD) and other birth defects. No evidence of any preventive or negative effects on cleft palate by periconceptional oral folate supplementation was detected (RR, 0.73; 95% CI, 0.05–10.89) (De-Regil et al., 2015). A reduced risk of NSCPO resulted in a meta-analysis of case-control studies for mothers who took multivitamin supplementation starting from before pregnancy (OR, 0.76; 95% CI, 0.62–0.93) (Ingrid Goh et al., 2006). An observational study reported that after food grain fortification with folic acid, the prevalence of NSCPO in United States had a significant 12% reduction (Canfield et al., 2005). On the other hand, Sutton et al. (2011) measured levels of red cell folate (RCF), vitamin B12 and homocysteine (tHcy) in the blood of women who were pregnant with a malformed baby at a time when multivitamin supplementation or food fortification was still rare. They found that the level of B12, which plays a critical role in folate metabolism, was surprisingly higher in mothers expecting a baby with cleft palate than in mothers of un malformed offspring, with no differences in levels of RCF and tHcy (Sutton et al., 2011).

Retinoic acid (RA), a derivate of Vitamin A, is an important regulator of processes that occur during embryogenesis, such as proliferation, differentiation, and apoptosis (Finnell et al., 2004). Only few epidemiological studies on human maternal intake of Vitamin A are available (Ackermans et al., 2011). Nevertheless, an excess of Vitamin A is considered teratogenic, causing congenital malformations, including cleft palate. On the other hand, Johansen et al. (2008) evidenced a substantial protective association between high (1.91–9.64 mg) maternal intake of Vitamin A and risk of NSCPO (adjusted OR [aOR], 0.47;
including the use of inhaled β₂-specturm of congenital anomalies considered, cleft palate showed with an increased risk of adverse maternal outcomes. On the period has long been generally ascertained to be correlated the Philippines. is not compromised to a certain severity as commonly seen in probably attributable to the fact that zinc status in US mothers association in a sample study from Utah (Munger et al., 2009), whereas the same authors did not confirm the association in gestational diabetes mellitus and the subsequent hyperglycemia as a real cause of the embryo malformation (Correa et al., 2008). With regard to pregestational diabetes, a very large population-based birth defect case-control study, carried out this year in the United States, evidenced an association with cleft palate alone (OR, 4.3; 95% CI, 2.9–6.5), while a smaller increased risk was observed in association with gestational diabetes (OR, 1.4; 95% CI, 1.1–1.8) (Tinker et al., 2020).

Genetic Factors
Increased recurrence risk in relatives indicates a high level of heritability for NSCPO (Grosen et al., 2010). Hence, the evidence of a genetic component in the etiology of this birth defect and the challenge taken up by researchers to obtain a better understanding of the NSCPO molecular bases.

During secondary palate formation, the palatal shelves grow, approach and fuse. The cells need to activate a series of biological mechanisms, including cell migration, epithelial–mesenchymal transition, and apoptosis, necessary to remove epithelial cells from the palatal epithelia medial edge, leading to the continuity of the mesenchyme and to palatal formation. For this reason, it has been hypothesized that many genes that code for proteins involved in the formation of cytoskeleton filaments, for cell adhesion molecules, or for ECM components, may contribute, if altered, to the clefted palate phenotype (Gibbins et al., 1999; Tudela et al., 2002). Here, we browse through the association studies on genes involved in mechanisms crucial for palatal development (Table 1).

Cell–Cell Adhesion
The epithelial adhesion molecule cadherin (CDH1) participates actively in the epithelial–mesenchymal transition, the developmental step when the epithelial cells at the palatal midline disappear, allowing mesenchymal continuity and palatal fusion. Alterations in the CDH1 gene contribute to the complex events that drive orofacial cleft, as demonstrated for the onset of non-syndromic cleft lip with or without cleft palate (NSCL/P) (Letra et al., 2009). Some studies have recently hypothesized that this gene may also represent a risk factor for NSCPO (Song and Zhang, 2011; Rafighdoost et al., 2013; Song et al., 2017).

Another gene involved in cell adhesion and motility that is crucial for craniofacial development is CRISPLD2. It contains an LCCl domain, common to other proteins involved in cellular migration (Chiquet et al., 2007). CRISPLD2 product has also been shown to be important for normal migration and differentiation of neural crest cells during early palate formation (Swindell et al., 2015). A significant association between allelic variants at rs1546124 and rs4783099 in the CRISPLD2 gene and the risk for
| Gene | Locus | SNP information | Country | Sample size | P-value\(^1\) | OR (95% CI) | References |
|------|-------|-----------------|---------|-------------|--------------|-------------|------------|
| CDH1 | 16q22.1 | rs16260 | China | 26 cases – 107 controls | 0.004 | 6.90 (1.47–32.40)\(^6\) | Song and Zhang (2011) |
|      |       | rs11642413 | Iran | 31 cases – 100 controls | 0.019 | 3.70 (1.26–10.87) | Rafighdoost et al. (2013) |
|      |       | rs16260 | Latvia | 31 cases – 100 controls | NS |  |  |
|      |       | rs9929218 | Africa | 10 cases – 190 controls | NS |  | Krasone et al. (2014) |
|      |       | rs16260; rs11642413 | China | 115 cases – 271 controls | 0.036 | 0.62 (0.40–0.97) | Song et al. (2017) |
|      |       | rs1801552 | Asia | NS |  | NS | Gowans et al. (2016) |
|      |       | rs16260; rs9929218 | NS |  |  |  |  |
| COL2A1 | 12q13.11 | rs1793949 | Baltic regions | 104 cases – 606 controls | 7.26 \(\times\) 10\(^{-4}\) | 1.659 (1.235–2.229) | Nikopensius et al. (2010) |
|      |       | rs8823 | Brazil | 107 triads | NS |  | Machado et al. (2016) |
|      |       | rs12228854 | Ireland | 293 cases – 902 controls | NS |  | Carter et al. (2010) |
|      |       | rs12368284 | China | 118 cases – 463 controls | 5.4 \(\times\) 10\(^{-4}\); 1.1 \(\times\) 10\(^{-3}\) | 2.93 (1.69–5.07)\(^6\); 0.48 (0.30–0.77) | Sheng et al. (2011) |
|      |       | rs4783099 | Nigeria | 163 cases – 1078 controls | 0.02 | 0.74 | Gowans et al. (2016) |
|      |       | rs1546124 | Brazil | 236 cases – 693 controls | 0.01 | 1.31 (1.05–1.62) | Messetti et al. (2017) |
| CRISPLD2 | 16q24.1 | rs12051468; rs8061351; rs721005; rs1546124; rs16974880; rs4783099 | Ireland | 293 cases – 902 controls | NS |  | Carter et al. (2010) |
|      |       | rs1546124 | China | 118 cases – 463 controls | 5.4 \(\times\) 10\(^{-4}\); 1.1 \(\times\) 10\(^{-3}\) | 2.93 (1.69–5.07)\(^6\); 0.48 (0.30–0.77) | Sheng et al. (2011) |
|      |       | rs4783099 | Nigeria | 163 cases – 1078 controls | 0.02 | 0.74 | Gowans et al. (2016) |
|      |       | rs1546124 | Brazil | 236 cases – 693 controls | 0.01 | 1.31 (1.05–1.62) | Messetti et al. (2017) |
| FOXE1 | 9q22.33 | rs1867278 | Denmark, Norway, United States, Philippines | 524 triads | 4.1 \(\times\) 10\(^{-4}\) |  | Moreno et al. (2009) |
|      |       | Other 14 SNPs genotyped | NS |  |  |  |  |
|      |       | rs111846096 | Thailand | 77 cases – 90 controls | NS |  | Srichomthong et al. (2013) |
|      |       | rs4460498 | Germany, The Netherlands, Maya | 165 cases – 1500 controls | 0.017 | 0.81 (0.56–1.17) | Ludwig et al. (2014) |
|      |       | rs4460498; rs3758249 | Europe | 156 triads | 0.043 | 0.913 (0.51–1.65) | Ludwig et al. (2014) |
|      |       | rs894673; rs3758249 | China | 51 triads | NS |  | Liu et al. (2015) |
|      |       | rs6586 | Africa | 163 cases – 1078 controls | NS |  | Gowans et al. (2016) |
|      |       | rs4618817 | California (Hispanic) | 66 cases – 476 controls | 0.34 (0.13–0.90)\(^6\) | 0.34 (0.15–0.80) | Lammer et al. (2016) |
|      |       | other 11 SNPs genotyped |  |  |  |  |  |
| GRHL3 | 1p36.11 | rs2486668; rs455809 | China | 297 cases – 377 controls | NS |  | He and Bian (2016) |
|      |       | rs41268753 | Norway, Denmark, United States | 246 cases – 1685 controls | 2.81 \(\times\) 10\(^{-4}\) | 2.16 (1.43–3.27) | Leslie et al. (2016) |
|      |       | rs113965554 | Europe, Yemen | 288 cases – 725 controls | 6.82 \(\times\) 10\(^{-4}\) | 1.97 (1.33–2.91) | Mangold et al. (2016) |
|      |       | rs41268753 | Germany | 116 cases\(^5\) – 267 controls | 2.63 \(\times\) 10\(^{-5}\) | 2.46 (1.62–3.74) | Mangold et al. (2016) |
|      |       | Other 11 SNPs genotyped | NS |  |  |  |  |

(Continued)
| Gene | Locus | SNP information | Country | Sample size | P-value | OR (95% CI) | References |
|------|-------|-----------------|---------|-------------|---------|-------------|------------|
| IRF6 | 1q32.2 | rs4844880; rs669694; rs2235371; rs2235375; rs2013162; rs126280 | Norway | 117 triads | NS | | |
| | | rs2235371 | | | | | |
| | | rs2235371; rs2013162; rs7552506; rs2235377 | | | | | |
| | | rs17389541 | Baltic regions | 104 cases – 606 controls | 5.45 × 10⁻⁴ | 1.726 (1.283–2.358) | Nikopensius et al. (2010) |
| | | rs9430018 | China | 25 cases – 96 controls | 0.25 (0.061–0.57) | Tang et al. (2009) |
| | | rs4844880; rs2235371; rs2013162; rs861019; rs2073487; rs658860 | Ireland | 293 cases – 902 controls | NS | | |
| | | rs47433335; rs642961 | Ireland | 53 cases – 285 controls | NS | | |
| | | rs642961; rs77542756; rs2235371 | Ireland | 163 cases – 1078 controls | NS | | |
| | | rs2235371 | Brazil | 38 cases – 182 controls | 0.004 | 3.01 (0.97–8.97) | Bezerra et al. (2019) |
| | | rs642961; rs2236907; rs861019; rs1044516 | Brazil | 53 cases – 205 controls | NS | | |
| | | rs1106514 | India | 53 cases – 205 controls | NS | | |
| JAG2 | 1q32.33 | rs11624283 | Estonia | 53 cases – 205 controls | 0.0016 | | |
| | | rs10134946 | Baltic regions | 104 cases – 606 controls | 0.0318 | 1.384 (1.028–1.864) | Nikopensius et al. (2010) |
| | | rs1057744 | Brazil | 81 cases – 413 controls | 0.0318 | 1.384 (1.028–1.864) | Nikopensius et al. (2010) |
| MSX1 | 4p16.2 | rs1106514 | Estonia | 53 cases – 205 controls | 0.0037 | | |
| | | rs3821949; rs12532; rs104893854 – P147Q | Baltic regions | 104 cases – 606 controls | 0.0095 | 1.482 (1.100–1.998) | Nikopensius et al. (2010) |
| | | rs62636562 | China | 42 triads | NS | | |
| | | rs12532 | Brazil | 81 cases – 413 controls | NS | | |
| | | rs3775261 | Iran | 31 cases – 100 controls | 0.008 | 10.83 (2.38–49.38) | Rafighdoost et al. (2013) |
| | | rs12532; rs3821949 | China | 56 cases – 605 controls | NS | | |
| | | rs2073242 | Brazil | 75 cases – 823 controls | NS | | |
| | | rs1106514 | Brazil | 107 triads | NS | | |
| | | rs115200552 | Africa | 163 cases – 1078 controls | 0.01 | 1.81 | Gowans et al. (2016) |
| | | rs12532 | | | | | |
| PAX7 | 1q36.13 | rs472071 | Europe | 180 triads | NS | | |
| | | rs4920520; rs766325 | Europe | 266 triads | NS | | |
| | | rs742071 | Iowa, Asia | 94 triads | NS | | |
| | | rs742071 | China | 56 cases – 605 controls | NS | | |
| | | rs766325; rs472071 | Africa | 163 cases – 1078 controls | NS | | |
| | | rs742071 | China | 144 triads | 0.025 | 3 (1.09–8.25) | Duan et al. (2017) |
| | | rs4920522; rs766325; rs6695765 | | | | | (Continued)
| Gene     | Locus       | SNP Information                                                                 | Country                                      | Sample size                  | P-value† | OR (95% CI)          | References                                    |
|----------|-------------|----------------------------------------------------------------------------------|----------------------------------------------|------------------------------|----------|----------------------|-----------------------------------------------|
| ROCK1    | 18q11.1     | rs359968865                                                                      | Italy, Iran                                  | 189 triads                   | 0.006    | 0.63 (0.45–0.88)     | Palmieri et al. (2020)                         |
| SUMO1    | 2q33.1      | rs3769817; rs12470401                                                           | Ireland                                      | 293 cases – 902 controls     | 0.038    | 1.45 (1.06–1.99)     | Carter et al. (2010)                          |
| TBX22    | Xq21.1      | rs6523677; rs6621541; rs7055763; rs8147590; rs6621542; rs41307258; rs6621543       | Brazil, Europe, North America                | 126 cases – 295 controls     | NS       | NS                   | Pauws et al. (2009)                           |
| TCOF1    | 5q32-q33.1  | rs2255796                                                                        | Taiwan, Singapore, United States             | 81 triads                    | 0.033    | 2.08                 | Sull et al. (2008)                            |
|          |             | rs2748222; rs1864957; rs15251; rs28372960; rs2569062                              | Brazil                                       | 107 triads                   | 0.096    | 1.77                 | Machado et al. (2016)                         |
| TGFA     | 2p13.3      | Tag I polymorphism*; GGA4D07; rs2166975                                        | Iowa                                         | 62 cases – 251 controls      | NS       | NS                   | Lidral et al. (1998)                          |
|          |             | rs2166975; rs6743202; rs1058213; rs2166975; rs930655; rs1523305; rs2902345; rs377122 | Lithuania                                    | 18 triads                    | 0.045    | 1.42 (1.05–1.42)     | Morkuniene et al. (2007)                      |
|          |             |                                                                                  | Ireland                                      | 293 cases – 902 controls     | 0.041    | 1.42 (1.05–1.42)     | Carter et al. (2010)                          |
|          |             |                                                                                  | Baltic regions                               | 104 cases – 606 controls     | 0.0467   | 1.356 (1.004–1.831)  | Nikopensius et al. (2010)                     |
|          |             |                                                                                  | Brazil                                       | 53 cases – 285 controls      | NS       | NS                   | Letra et al. (2012)                          |
|          |             |                                                                                  | Brazil                                       | 28 triads                    | NS       | NS                   | Souza et al. (2012)                          |
|          |             |                                                                                  | Ireland                                      | 296 triads – 62 dyads – 15 NSCPO | 0.047    | 1.42 (1.05–1.42)     | Fan et al. (2013)                             |
|          |             |                                                                                  | China                                        | 62 cases – 150 controls      | NS       | NS                   | Xu et al. (2014)                              |

**Further information about the molecular function, biological process and relative pathway is given in Supplementary Table S1.**

†NS, not significant.

‡OR for homozygous variant genotype.

§Submucous cleft palate only.

*Deletion of 4 bp in intron V, creating a Tag I site.
NSCPO, has been reported in case–control studies (Shen et al., 2011; Gowans et al., 2016; Messetti et al., 2017).

One of the genes that participate in the signaling mechanism required for normal palate development is JAG2, which encodes a ligand for Notch family transmembrane receptors. JAG2 and NOTCH1 are spatio-temporally activated in the oral epithelia during palate development for the correct cell adhesion of the elevated palatal shelves, preventing their premature adhesion to other oral tissues (Casey et al., 2006). Nevertheless, only few studies investigated a possible involvement of JAG2 in the onset of NSCPO, with controversial results. JAG2 collaborates with IRF6 on the same molecular pathway during oral epithelial differentiation. This coordination is essential for the control of palatal adhesion and fusion competence (Richardson et al., 2009). IRF6 plays an important role in the formation and maintenance of the oral periderm, the spatiotemporal regulation of which is essential to ensure appropriate palatal adhesion (Richardson et al., 2009). Mutations in IRF6 cause two common forms of syndromic cleft, known as Van der Woude Syndrome (VWS) and popliteal pterygium syndrome (Kondo et al., 2002). The crucial role of IRF6 in the genesis of NSCL/P has been widely demonstrated in several studies. Zuccherò et al. (2004), in a vast NSCL/P sample study from different countries, first demonstrated that genetic variants in IRF6, underlying VWS, might also be involved in the etiology of isolated clefts. However, although a strong association between genetic variants at IRF6 and NSCL/P risk was successively confirmed by independent studies (Blanton et al., 2005; Ghassibe et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005), the existing results regarding NSCPO are contradictory (Table 1). This could be due to the low power of the studies because of small sample sizes, but also to the widely demonstrated different etiology of NSCPO with respect to NSCL/P.

A number of scientific evidences suggest that FOXE1 is one of the most consistent genetic factors in the NSCPO etiology. Mutations of FOXE1 cause Bamforth–Lazarus, a recessive syndrome characterized by cleft palate and congenital hypothyroidism (Castanet et al., 2002). Mice lacking Foxe1 express a similar phenotype (De Felice et al., 1998). The FOXE1 is a transcription factor expressed in the shelf epithelium of the secondary palate during the fusion and regulates both MSX1 and TGFβ3, which are required for proper palate formation (Venza et al., 2011). Various studies reported a strong association between FOXE1 and both NSCL/P or NSCPO (Moreno et al., 2009; Nikopenius et al., 2011; Lammer et al., 2016). No common missense mutations in FOXE1 can explain the association signal evidenced by different authors, suggesting the hypothesis of a common variant in a regulatory region (Lidral et al., 2015). Sequence analysis identified a novel non-coding polymorphism c.-1204C>G in the promoter of FOXE1 in 11 Italian NSCPOs (Venza et al., 2009), while Srichomthong et al. (2013) identified five non-synonymous mutations, none of which was present in the control population.

In craniofacial development, TGFA is expressed during the fusion of the palatine shelves at the level of the medial edge epithelium. Evidence of the involvement of TGFA in NSCPO etiology was found by several authors investigating different populations, but not by others, as detailed in Table 1.

**Cell Proliferation**

TCOF1 actively participates in the formation of neural crests. Alterations of this gene cause development anomalies that lead to craniofacial malformations (Dixon et al., 2006). Indeed, TCOF1 is a causative gene for Treacher Collins syndrome, characterized by hypoplasia of the facial bones, cleft palate, and middle and external ear defects. Sull et al. (2008) identified the SNP rs15251 variant as a possible risk factor for NSCPO.

The homeobox gene MSX1 actively participates in cell proliferation, differentiation, and apoptosis, all crucial processes for normal embryonic development. This gene plays a critical role in the epithelial–mesenchymal interaction during the formation of craniofacial bones (Satokata and Maas, 1994). Many studies on animal models have amply demonstrated MSX1 involvement in palatal, facial and dental development (Satokata and Maas, 1994; Jumlongras et al., 2001). Genetic studies conducted on humans have ascertained a specific role for MSX1 in the orofacial cleft (Jezewski et al., 2003; Modesto et al., 2006; Tasanarong et al., 2019). However, the association studies specifically dedicated to NSCPO cohorts obtained conflicting evidence, possibly due to the small sample sizes and the different ethnic groups considered (Table 1). A recent meta-analysis performed by Gu et al. (2018) of investigations published till 2017, indicated the SNP rs12532, able to affect mRNA expression and stability of MSX1, as a possible risk factor for NSCPO but not for NSCL/P.

**TBX22** encodes a transcription factor important for the mesenchymal proliferation and elevation of palatal shelves before their fusion. Its normal expression is related to a correct development of the palate, while TBX22 mutations cause the hereditary X-linked disorder, cleft palate with ankyloglossia (CPX, OMIM 303400) (Marcano et al., 2004; Stanier and Moore, 2004). Marcano et al. (2004) screened for TBX22 mutations in 238 NSCPOs from the Philippines, North America, and Brazil, and found 15 different variants: five mutations affecting the coding region, as well as several putative splice site mutations. Suphapeetiporn et al. (2007) by sequencing the TBX22 gene in 53 NSCPO cases in the Thai population, found three missense mutations and a deletion in four affected individuals. Fu et al. (2015) reported a functional -73G>A mutation in the promoter of TBX22, in all five affected males of a six-generation Chinese family of NSCPO (Fu et al., 2015). Both TBX22 and MSX1 are post-translationally modified by SUMO1, a regulating factor that participates in the modulation of many other genes, with evidence of a role in human oral clefts (Dobreva et al., 2003; Ghioni et al., 2005; Lee et al., 2006; Andreou et al., 2007).

Shi et al. (2009) identified a de novo deletion on chromosome 2 in a Danish child affected by NSCPO. The estimated size of the deletion is 124.2 kb and contains SUMO1 (Shi et al., 2009). In a large Irish study population composed by 383 NSCPO patients, the SNP rs3769817 located in SUMO1 intron 2 was associated with an increased risk for cleft palate (Carter et al., 2010).

The PAX transcription factors are critical for neural crest induction (Monsoro-Burq, 2015). In particular, Pax7 regulates...
the cell cycle of embryonic stem cells and mouse embryonic fibroblasts (Czerwinska et al., 2016). The intronic rs742071 PAX7 polymorphism, previously proposed as a risk factor for NSCL/P (Ludwig et al., 2012), was also found significantly associated with NSCPO in a family-based association study carried out with 266 European triads (Bohmer et al., 2013). The association with NSCPO was then confirmed in the Chinese and sub-Saharan African populations (Duan et al., 2017; Butali et al., 2019).

Another transcriptional factor widely involved in the processes of closure of the neural tube and craniofacial development is encoded by GRHL3. In families that do not show mutations at the IRF6 locus, GRHL3 seems to be the second candidate gene in VWS (Peyrard-Janvid et al., 2014). Functional analyses demonstrate that GRHL3 is a target of IRF6 in the processes of periderm differentiation (de la Garza et al., 2013). In a genome-wide association study, Leslie et al. (2016) identified an association between the rs41268753 SNP and NSCPO in two independent populations. The importance of GRHL3 in NSCPO etiology was independently confirmed by Mangold et al. (2016), who collected evidences supporting the non-synonymous polymorphism rs41268753 as a susceptibility factor for NSCPO and described rare mutations of this gene in patients. COL2A1 is a cartilage-specific marker involved in cranial neural crest differentiation by complex mechanisms that involve epithelial–mesenchymal transition, migration, and differentiation (Ghassibe-Sabbagh et al., 2011). Mutations in genes coding for cartilage collagens II cause syndromes that are often associated with cleft palate or micrognathia, such as Pierre-Robin sequence and Stickler syndrome (Kannu et al., 2012). Evidence of an association between COL2A1 alleles and NSCPO risk was found (Nikopensius et al., 2010), though not confirmed by others (Machado et al., 2016).

Cell Migration
The two Rho kinase isoforms (ROCK1 and ROCK2) play essential roles in fundamental cellular processes such as contraction, adhesion, migration, apoptosis, and proliferation. ROCK regulates stress fiber and focal adhesion assembly (Phillips et al., 2012) and modulates cytoskeleton organization by phosphorylating different substrates, mainly myosin light chain and myosin phosphatase, involved in the contractility pathway that leads to normal palate development. A recent family-based association study carried out in two cohorts from Italy and Iran, showed a significant level of association between ROCK1 rs35996865-G and NSCPO (Palmieri et al., 2020). Currently, there are no reports demonstrating the involvement of ROCK2 in the onset of orofacial cleft, however, some studies show that the loss of both chromosome 18q and 2p (where ROCK1 and ROCK2 reside, respectively) determines a series of anomalies including defects of the palate and micrognathia (Suzuki et al., 2016).

FLNB belongs to a family of actin-binding proteins. Filamins are also able to interact with receptors and intracellular proteins involved in cytoskeleton-dependent cell proliferation, differentiation, and migration (Hu et al., 2014). Mutations in this gene cause malformations that include cleft palate as a feature, as reported by Jugessur et al. (2010) who suggest a maternal effect for variants mapping in this gene, in their NSCPO cohort (Jugessur et al., 2010).

Genes Involved in Folate and Homocysteine Metabolism
Vitamin B9 or folate is not synthesized by our organism but is present, for example, in legumes, leafy green vegetables, some fruits, and eggs. Folic acid is the most stable form of folate, synthetically produced and usually used in vitamin supplements and fortified foods (Bailey et al., 2015). Adequate folate intake is essential for cell division and homeostasis. Indeed, the folate pathway plays a crucial role in several strategic biochemical processes such as DNA biosynthesis, methionine regeneration, amino acid metabolism, mitochondrial protein translation, and DNA methylation. Folate deficiency and/or aberrant folate metabolism during embryogenesis were therefore assumed to cause congenital malformations because of the alteration of the precise interplay of cell proliferation and death, migration, and differentiation needed in this delicate moment of development. However, as above documented, there is no strong association between folate supplementation during the periconceptional period and a decreased risk of having an NSCPO baby (Johnson and Little, 2008). Nevertheless, the folate pathway, with its enzymes and substrates, was suspected to have a role in the NSCPO etiology and several studies investigated associations between polymorphisms in genes related to one-carbon metabolism and cleft palate risk. The most investigated gene is MTHFR, with its C677T polymorphism. However, in a meta-analysis based on five studies, with a total of 576 CP cases and 2587 controls, no statistical significances were observed for the risk of NSCPO for heterozygotes, neither for homozygotes (Luo et al., 2012).

Table 2 reports information on folate and homocysteine metabolism gene polymorphisms investigated for their potential association with cleft palate, published until June 2020 in PubMed. Only single studies with data from a cohort or a subset of non-syndromic cleft palate cases have been considered in the table.

Genes Responsible for Syndromic Forms as Candidate for NSCPO
As mentioned above, referring to Burg et al.’s (2016) work, the responsible genes have been identified in a number of syndromes that include cleft palate among their features. Based on the assumption that genes implicated in syndromic forms of cleft could also have a role in non-syndromic phenotypes, the variant association of such genes has been questioned in case control and family based studies on different NSCPO worldwide cohorts.

The attempts of researchers to confirm this hypothesis are summarized in Table 3, where genes already mentioned in a previous section (FLNB) or in Table 1 (TBX22, FOXE1 COL2A1, IRF6, TCOF1) are omitted.

Genome-Wide Analysis
The rapid improvement, in recent years, of technological tools for genotyping and sequencing has allowed researchers
| Gene/  | Locus  | SNP information               | Country                      | Sample size | P-value† | OR (95% CI) | References                        |
|-------|--------|-------------------------------|------------------------------|-------------|----------|-------------|----------------------------------|
| AHCY  | 20q11.22 | rs819142; rs819133             | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| BMT   | 5q14.1 | rs3733890 – G742A – R239Q       | Norway                       | 191 triads  | NS       |             | Boyles et al. (2008)            |
|       |        | rs567754; rs585800             | Norway                       | 93 triads   | 0.022   |             | Boyles et al. (2009)            |
| BMT2  | 5q14.1 | rs626105                       | Norway, Hispanics, others    | 236 cases – 201 controls | 0.9 (0.6–1.3) |             | Zhu et al. (2005)                |
| CBS   | 21q22.3 | 844ins68                      | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs234706 – C699T               | Norway                       | 191 triads  | NS       |             | Boyles et al. (2009)            |
|       |        | rs234705; rs234709; rs4920037  | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs2124459                      | Norway                       | 191 triads  | NS       |             | Boyles et al. (2009)            |
|       |        | rs4920037                      | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs663649                       | Norway                       | 93 triads   | 0.047   |             | Boyles et al. (2009)            |
| CTH   | 1p31.1 | rs681475; rs1145920; rs515064; | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs1021737                      | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs663649                       | Norway                       | 93 triads   | 0.003   |             | Boyles et al. (2009)            |
| DHR   | 5q14.1 | rs2618372; rs380691             | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| DMGDH | 5q14.1 | rs250513; rs479405; rs642013;   | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs2034899; rs1805074; rs248386;| Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
|       |        | rs185077; rs532964             | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| FOLH1 | 11p11.12 | rs6485963; rs11040270; rs7113251; rs202720; rs10839236; rs202676 | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| FOLR1 | 11q13.4 | rs3016432; rs11235468           | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| FOLR2 | 11q13.4 | rs514933                       | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| FOLR3 | 11q13.4 | rs5323207; rs555306; rs575341  | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| FTCD  | 21q22.3 | rs1900983; rs9978174; rs4819208; rs2277820 | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| FTHFD | 3q21.3 | rs1127717; rs3772430; rs2290053; rs2365004; rs2886059; rs1823213 | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| GNMT  | 6q21.1 | rs2296805; rs2274514            | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |
| MATA  | 10q22.3 | rs1556894; rs29935763; rs9285726 | Norway                       | 93 triads   | NS       |             | Boyles et al. (2009)            |

(Continued)
| Gene | Locus | SNP Information | Country                  | Sample size | P-value | OR (95% CI) | References |
|------|-------|----------------|--------------------------|-------------|---------|-------------|------------|
| MAT2A | 2p11.2 | rs6739015; rs2028900; rs2028898; rs7568458 | Norway                  | 93 triads | NS      |             | Boyles et al. (2009) |
| MAT2B | 5q34  | rs10515861; rs4869087; rs4869089; rs725352 | Norway | 93 triads | NS | | Boyles et al. (2009) |
| MTHFD1 | 14q23.3 | rs2236225 – G1968A – R653Q | Norway | 191 triads | NS | | Boyles et al. (2008) |
|        |       | rs3783731; rs8003379; rs1950902; rs2236224; rs1256146 | | | | | |
|         |       | | Norway | 93 triads | NS | | Boyles et al. (2009) |
|         |       | | Ireland | 321 triads | NS | 1.31 (0.94–1.82) | Mills et al. (2008) |
|         |       | | Ireland | 321 mothers – 1599 controls | 0.02 | 1.41 (1.05–1.09) | Mills et al. (2008) |
|         |       | | Norway | 93 triads | NS | | Boyles et al. (2009) |
|         |       | | Europe | 242 triads | NS | 0.93 (0.55–1.57) | Mossey et al. (2017) |
| MTHFD2 | 2p13.1 | rs1667627; rs702462 | Norway | 93 triads | NS | | Boyles et al. (2009) |
| MTHFR  | 1p36.3 | rs1801133 – C677T – A222V | California | 117 cases – 383 control | NS | 0.8 (0.5–1.2) | Shaw et al. (1999) |
|        |       | | Norway | 63 triads | | 2.4 (1.2–4.6) | Jugessur et al. (2003b) |
|        |       | | France | 56 triads | NS | 1.31 (0.6–3.0) | Chevrier et al. (2007) |
|        |       | | Norway | 191 triads | NS | | Boyles et al. (2009) |
|         |       | | UK | 47 triads + 19 dyads | NS | 0.9 (0.44–1.77) | Little et al. (2008) |
|         |       | | Ireland | 321 triads | NS | 0.96 (0.64–1.43) | Mills et al. (2008) |
|         |       | | Ireland | 321 mothers – 1599 controls | 0.03 | 1.5 (1.05–2.16) | Mills et al. (2008) |
|         |       | | Norway | 93 triads | NS | | Boyles et al. (2009) |
|         |       | | Europe | 292 triads | NS | 0.53 (0.31–0.92) | Mossey et al. (2017) |
|         |       | | Italy | 129 triads | NS | 0.98 (0.68–1.42) | Carinci et al. (2019) |
|         |       | | Iran – Tibet – Bangladesh | 65 triads | NS | 1.17 (0.54–2.52) | Carinci et al. (2019) |
| MTHFS  | 15q25.1 | rs885487; rs6495452; rs2562744 | Norway | 93 triads | NS | | Boyles et al. (2009) |
| MTR    | 1q43  | rs1805067 – A2756G | Norway | 191 triads | NS | | Boyles et al. (2009) |
|        |       | | Norway | 93 triads | NS | | Boyles et al. (2009) |
| MTRR   | 5p15.31 | rs1801394 – A66G | Norway | 191 triads | NS | | Boyles et al. (2009) |
|        |       | | Norway | 93 triads | NS | | Boyles et al. (2009) |
| Gene   | Locus | SNP information                                   | Country     | Sample size¹ | P-value‡ | OR (95% CI) | References                     |
|--------|-------|---------------------------------------------------|-------------|--------------|----------|-------------|--------------------------------|
| NOS3   | 7q36.1| rs1800779 – A(922G)                               | California  | 99 cases – 588 controls | 0.9 (0.6–1.4) | 1.1 (0.7–1.7) | Boyles et al. (2005) |
|        |       | rs1799983 – G894T – E296D                         |             |              |          |             |                                |
|        |       | rs1532268                                        | Norway      | 93 triads    | 0.021§   |             | Boyles et al. (2009) |
|        |       | rs3776455                                        | Norway      | 93 triads    | 0.038§   |             | Boyles et al. (2009) |
|        |       | rs162031; rs162036; rs10380                       |             |              |          |             |                                |
| POV1   | 7q21.3| rs662 – A575G                                     | Norway      | 191 triads   | NS       |             | Boyles et al. (2008) |
|        |       | rs854547; rs8491; rs854549; rs2237582; rs3917498; | Norway      | 93 triads    | NS       |             | Boyles et al. (2009) |
|        |       | rs2074351; rs854565; rs2299261; rs705382           |             |              |          |             | Boyles et al. (2009) |
| SHMT1  | 17p11.2| rs2168781                                         | Norway      | 93 triads    | NS       |             | Boyles et al. (2009) |
|        |       | rs7207306                                        | Norway      | 93 triads    | NS       |             | Boyles et al. (2009) |
| SHMT2  | 12q13.3| rs7311958                                         | Norway      | 93 triads    | NS       |             | Boyles et al. (2009) |
| SLC19A1/RFC1 | rs1051266 – A90G            | California  | 123 cases – 364 controls | 0.8 (0.5–1.3) | 1.1 (0.7–1.7) | Boyles et al. (2008) |
|        |       | rs1051266; rs1051268; rs1011361; rs185422          | Norway      | 191 triads   | NS       |             | Boyles et al. (2008) |
|        |       | rs705382                                          | Norway      | 93 triads    | NS       |             | Boyles et al. (2009) |
| SLC46A1| 17q11.2| rs9894260                                         | Utah        | 109 triads   | NS       | 0.75 (0.32–1.78) | VanderMeer et al. (2016) |
|        |       | rs739439                                          |             |              | NS       | 1.40 (0.62–3.15) |                               |
|        |       | rs2239907                                         |             |              | NS       | 0.73 (0.42–1.27) |                               |
| TCN2   | 22q12.2| rs1801198 – C776G – P259R                         | Norway      | 191 triads   | NS       |             | Boyles et al. (2008) |
|        |       | rs61061198; rs1907551; rs1907553; rs1907555; rs1907557 | Ireland     | 321 triads   | NS       | 1.05 (0.73–1.52) | Mills et al. (2008) |
|        |       | rs1801198; rs1801198; rs1907551; rs1907553; rs1907557 | Norway      | 93 triads    | NS       |             | Boyles et al. (2009) |
|        |       | rs1801198; rs1801198; rs1907551; rs1907553; rs1907557 | Italy       | 129 triads   | NS       | 0.98 (0.69–1.40) | Carinci et al. (2019) |
|        |       | rs9606756 – A67G                                  | Iran – Tibet – Bangladesh | 65 triads   | NS       | 1.10 (0.60–2.02) | Carinci et al. (2019) |
| TYMS   | 18p11.32| rs502396; rs2244500; rs1502190; rs1502289          | Norway      | 93 triads    | NS       |             | Boyles et al. (2008) |
|        |       | rs16430 – 1494del6                                |             |              |          |             | Boyles et al. (2009) |
|        |       | rs45445694 – 28-bp VNTR                           | California  | 123 cases – 581 controls | 1.3 (0.8–2.0) | 1.8 (1.1–3.1)* | Boyles et al. (2009) |
|        |       | rs45445694 – 28-bp VNTR                           | California  | 123 cases – 581 controls | 1.3 (0.8–2.0) | 1.8 (1.1–3.1)* | Boyles et al. (2009) |

²Further information about the molecular function, biological process and relative pathway is given in Supplementary Table S2.

¹Studies with a cohort bigger than 50 cases are reported.

²NS, not significant.

§When the maternal association is considered.

*OR for homozygous variant genotype.

¥In the sample study are included 11 cases of Pierre Robin sequence.
TABLE 3 | Genes responsible for syndromic cleft palate, studied in NSCPO cohorts.

| Gene | Locus | Syndrome with cleft palate | SNP information | Country | Sample size | P value † | OR (95% CI) | References |
|------|-------|----------------------------|-----------------|---------|-------------|-----------|-------------|------------|
| BCOR | Xp11.4| Oculofaciocardiodental     | Haplotype rs4076107-rs6520620-rs5963158-rs4308866; rs6609051; rs12887359 42 genotyped SNPs | Asia    | 253 triads  | <10^{-41} |            |            |
| COL11A1 | 1p21.1 | Pierre Robin Sequence and Stickler | rs213209 | Norway–Denmark | 114 + 69 triads | NS |            |            |
| COL11A2 | 6p21.32 | Pierre Robin Sequence and Stickler | rs9277928 | Baltic regions | 104 cases – 182 controls | NS |            |            |
| EFN1 | Xq13.1 | Craniofrontonasal | rs877818 | Norway–Denmark | 114 + 69 triads | NS |            |            |
| FGFR1 | 8p11.23 | Kallman type 2 | rs7829058 | Baltic regions | 104 cases – 606 controls | NS | 0.0451 | 0.637 (0.408–0.993) |
| FGFR2 | 10q26.13 | Apert and Crouzon | rs1047100 | Brazil | 41 triads | NS |            | Machado et al. (2016) |
| FLNA | Xq28 | Otopalatodigital spectrum disorders | rs766419; rs2070822; rs7829058 | Ireland | 293 cases – 902 controls | NS | 0.0049 | 1.789 (1.189–2.720) |
| OFD1/CXORF5 | Xp22.2 | Oral-facial-digital syndrome 1 | rs2285635; rs2283707 | Norway–Denmark | 114 + 69 triads | NS |            |            |
| PHF8 | Xp11.22 | Siderius type | rs6521788; rs12115965; rs7876951; rs5960612 | Norway–Denmark | 114 + 69 triads | NS |            | Jugessur et al. (2012) |
| PQBP1 | Xp11.23 | Renpenning | rs4824733; rs2016813; rs741932 | Norway–Denmark | 114 + 69 triads | NS |            | Jugessur et al. (2012) |
| SMS | Xp22.11 | Snyder-Robinson | rs2040357; rs5951678 | Norway–Denmark | 114 + 69 triads | NS |            | Jugessur et al. (2012) |
| SOX9 | 17q24.3 | Pierre Robin sequence and Campomelic dysplasia | rs12941170 | China | 46 triads | 0.03 | 0.56 (0.3–0.96) | Jia et al. (2017) |
| TGFB2 | 1q41 | Loey-Dietz | pPC-27 probe | Philippines | 48 cases – 214 controls | NS | 0.06 | 0.57 (0.3–1.03) | Lidal et al. (1997) |

*Further information about the molecular function, biological process and relative pathway is given in Supplementary Table S3.*

† NS, not significant.

‡ Association among 100 males.
to approach whole genome studies. The aim is to identify as many susceptibility genes as possible, while the challenge is to collect and analyze dataset large enough to deliver the required statistical power. The genome-wide association studies (GWAS) approach is an indirect mapping technique, able to identify associated polymorphisms, while exome/genome sequencing (WES/WGS) studies are more focused on rare genetic mutations. Table 4 reports genes and polymorphisms associated with NSCPO detected by GWAS. Besides IRF6, an already known genetic risk factor for orofacial clefts, the list includes several new candidate genes. Among these GRHL3, whose involvement in NSCPO etiology was confirmed in an independent European cohort (Leslie et al., 2016) and by Mangold et al. (2016) in a subsequent investigation in which they evidenced common and rare causative mutations.

Whole exome sequencing (WES) searching for NSCPO mutations was performed in affected individuals from multiplex families with NSCPO (Hoebel et al., 2017). A probable deleterious mutation in ARHGAP29, a gene expressed in developing palate, was found in four individuals of a single family affected by cleft of the soft palate (Liu et al., 2017). The WES of 16 individuals from 8 multiplex families allowed Hoebel et al. (2017) to select 26 candidate genes that were sequenced in additional 132 NSCPO cases. The investigation failed to identify genes with recurrent deleterious mutations but produced data that may be useful for subsequent investigations.

The potential role of epigenetic regulation in NSCPO has been recently explored by two groups (Sharp et al., 2017; Xu et al., 2019). In these investigations, epigenome-wide association studies (EWAS) were conducted to test whether DNA methylation of blood, or other relevant tissues, was associated with orofacial clefting. These preliminary investigations suggest differences in methylation profiles of patients and unaffected controls, as well as between different types of clefts. However, future result replications may be difficult because methylation status may consistently vary as a function of kind of investigated tissue, and of patient age at the moment of sampling.

**Gene x Gene Interaction**

Gene–gene epistatic interaction (GxG) analysis has been successfully adopted for other diseases in order to narrow a critical region (Cox et al., 1999) or to identify additional loci (Pankratz et al., 2003). A small number of researchers tested for GxG in cohorts that do not include syndromic forms of CPO nor patients with CL. In detail, Jugessur et al. (2003a) found a significant 6.3-fold increased risk of NSCPO in patients carrying the TGFA Taq I A2A2 genotype in combination with one or two copies of the T-allele at MTHFR C677T polymorphism. This same gene–gene interaction was investigated by Mossey et al. (2017), evidencing an opposite effect when considering the combination Taq I A2 and one or two copies of MTHFR variant for the child genotype, with a 6 and 12.5-fold reduced risk, respectively. Lastly, Duan et al. (2017) evidenced an epistatic interaction between rs4844913
(43 kb 3’ of DIXEF) and rs11119388 (SYT14) and between rs6072081 (53 kb 3’ of MAFB) and rs6102085 (33 kb 3’ of MAFB).

**Gene x Environment Interactions**

Orofacial clefts, including NSCPO, are considered as typical complex diseases caused by several genetic and environmental factors. Although many candidate gene studies and GWAS have been performed to reveal genetic factors of NSCPO, the genetic variants identified so far can explain only a small fraction of heritability of this common malformation. This has opened the hypothesis that the effect of genetic factors on a disorder can be modulated by environmental factors and *vice versa.* Several different models of gene–environment interactions (GxE) were postulated; in some cases it could be easier to detect the effect of genetic factors if the effect of environmental factors is also considered (Ottman, 1996).

The first attempts to investigate GxE interaction in OFC were reviewed by Murray (2002). Most of these studies included NSCL/P cases only, or NSCL/P cases together with a small fraction of NSCPO cases. Some possible GxE interactions were suggested but not confirmed (Murray, 2002).

An interaction between the rs7205289 polymorphism and passive smoking in NSCPO was observed in the Chinese population (Li et al., 2011). The rs7205289 lies in the microRNA-140 gene and can reduce its expression (Li et al., 2011). Interestingly, tobacco smoke had the same effect in cultured mouse palatal mesenchymal cells (Li et al., 2011). This data is particularly relevant because dysregulation of miRNA-140 was suggested but not confirmed (Murray, 2002).

In a GxE study on OFC cases, Shi et al. (2007) investigated the interaction between maternal smoking and 16 candidate genes involved in detoxification. An interaction was found with GSTT1 among OFC, with a higher effect in the NSCPO subgroup. In particular, the combination of fetal GSTT1-null genotype and maternal smoking provided the highest risk of cleft. This finding was important, because it confirmed a previous study involving an OFC sample from The Netherlands (van Rooij et al., 2001).

A GWAS conducted with 550 patient-parent triads supported the importance of a GxE interaction investigation. Indeed, while no individual gene reached the statistical threshold of significance in the test of association with NSCPO, several genes demonstrated an association when their interaction was considered. In particular, polymorphisms of MLLT3 and SMC2 interacted with maternal alcohol consumption; TBK1 and ZNF236 with maternal smoking; and BAALC with multivitamin supplementation (Beaty et al., 2011). Data were further examined by stratifying the sample by European and Asian ancestry (Wu et al., 2014). Several polymorphisms of a chromosome 4 region that includes SLC2A9 and WDR1 genes were associated with NSCPO in Asians when the interaction with environmental tobacco smoke exposure was tested.

The same dataset was investigated with an alternative model that includes the parent of origin (PoO) effect, i.e., the risk in the child varies on whether the allele is inherited from the mother or the father. The hypothesis of interaction between PoO and maternal exposure (PoOxE) provided the evidence for new genes involved in NSCPO etiology. Indeed, several polymorphisms in ICE2 and NAALDL2 showed PoOxSmoke interaction in European families (Haaland et al., 2017).

**DISCUSSION**

Defects of the closure of the secondary palate only are, among orofacial clefts, the rarest, the least studied, and those with the least obvious nature. CPO can occur as a feature of Mendelian single gene disorders or of several chromosomal syndromes. On the other hand, the isolated form of CPO is considered to have a multifactorial etiology, and in the last decades researchers have attempted to elucidate all the teratogenic and genetic factors causing this common malformation. The identification of causal factors and understanding of the molecular mechanisms of cleft formation would be an invaluable aid for clinicians in optimizing prevention approaches, providing genetic counseling, and planning personalized surgical and medical treatments.

Several evidences have highlighted the fact that NSCPO and NSCL/P are different malformations with different, or slightly overlapping, causes. Scientific research has been mainly focused on NSCL/P, while NSCPO study has been marginal, maybe owing to the lower incidence that makes harder to collect cohorts of comparable size. In recent years, this gap has been reducing, but new insights on CPO are accumulating more slowly than expected, especially with regards GWAS. A possible explanation is a limited contribution of common genetic variants in NSCPO etiology, which lower the success rate of association analyses. Genetic investigations have provided convincing evidences for a limited number of heritable factors that do not account for the high level of hereditability of NSCPO. These include FOXE1, GRHL3, and PAX7, while a number of investigations provided support for transcription and regulatory factors necessary for the normal development of neural crests. If rare variants play a major role in CPO etiology, future large-scale sequencing efforts may represent a promising approach to detect genetic factors. Emerging trends of research regard more complex models, including gene–gene interaction, gene–environment interaction, and epigenetic analyses, which could be more powerful to explain missing heritability, but all of these require larger sample sizes. However, such approaches need a deeper investigation, in order to earlier predict NSCPO risk and to plan healthcare strategies aimed at improving environmental conditions and reducing exposure to potential epigenetic factors.

Considering the long, but not exhaustive, list of potential susceptibility factors for NSCPO, a wide range prevention could appear unrealistic, also because palate closure occurs during a gestation period in which the mother is often unaware she is pregnant. It is in any case crucial to identify exposure risks acting around the conception time (1 month before, 3 months
after) that may impact embryonic development. A consistent bulk of data supports an epidemiological role for both active and passive tobacco smoking, while contrasting evidences have been collected for moderate alcohol consumption. On the contrary, folate fortification and multivitamin supplementation seem to decrease the risk of NSCPO.

**AUTHOR CONTRIBUTIONS**

MM conceived the idea and design of the manuscript, acquired most of the references and data, and contributed to the drafting, revision, and final approval of the manuscript. AP and FC contributed to the manuscript design and critical revisions and final approval of the manuscript. LS contributed to the design, drafting, acquisition of statistical data, revision, and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.592271/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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