

University of Dundee

Association between a common missense variant in LOXL3 gene and the risk of non-syndromic cleft palate

Khan, Mohammad Faisal Jamal; Little, Julian; Mossey, Peter; Steegers-Theunissen, Regine P. M.; Bonsi, Martina; Andreasi, Rita Bassi

Published in:
Congenital Anomalies

DOI:
[10.1111/cga.12288](https://doi.org/10.1111/cga.12288)

Publication date:
2018

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Khan, M. F. J., Little, J., Mossey, P., Steegers-Theunissen, R. P. M., Bonsi, M., Andreasi, R. B., & Rubini, M. (2018). Association between a common missense variant in LOXL3 gene and the risk of non-syndromic cleft palate. *Congenital Anomalies*, 58(4), 136-140. <https://doi.org/10.1111/cga.12288>

General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 **Article type.** Original article

2 **Full Title.** Association between a common missense variant in lysyl oxidase like 3
3 (*LOXL3*) gene and the risk of non-syndromic cleft palate.

4 **First author's surname.** Khan

5 **Short title.** LOXL3 variant in ns-cleft palate

6 **Full name of all authors.** Mohammad Faisal J Khan¹, Julian Little², Peter A Mossey³,
7 Régine PM Steegers-Theunissen⁴, Martina Bonisi⁵, Rita Bassi Andreasi⁶, Michele
8 Rubini⁷.

9 **Address at which the work was carried out.** Department of Biomedical and Specialty
10 Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics,
11 University of Ferrara, Ferrara, Italy.

12 1. Department of Biomedical and Specialty Surgical Sciences, Section of Medical
13 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.
14 Email: khnmmm@unife.it

15 2. School of Epidemiology and Public Health, University of Ottawa, Ottawa, Ontario,
16 Canada. Email: jlittle@uottawa.ca

17 3. Craniofacial Development at the World Health Organization–collaborating Centre for
18 Oral and Craniofacial Research, Dental Hospital and School, University of Dundee,
19 Dundee, Scotland. Email: p.a.mossey@dundee.ac.uk

This is the peer reviewed version of the following article: Khan, M.F.J, et al. (2018) 'Association between a common missense variant in lysyl oxidase like 3 (*LOXL3*) gene and the risk of non-syndromic cleft palate', *Congenital Anomalies*, which has been published in final form at <https://doi.org/10.1111/cga.12288>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

20 4. Department of Obstetrics and Gynaecology, Department of Pediatrics, Division
21 Neonatology Erasmus MC, University Medical Center, Rotterdam, The Netherlands.
22 Email: r.steegers@erasmusmc.nl.

23 5. Department of Biomedical and Specialty Surgical Sciences, Section of Medical
24 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.
25 Email: martina.bonsi@student.unife.it

26 6. Department of Biomedical and Specialty Surgical Sciences, Section of Medical
27 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.
28 Email: bssrti@unife.it

29 7. Department of Biomedical and Specialty Surgical Sciences, Section of Medical
30 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.
31 Email: rub@unife.it

32 **Corresponding author.**

33 **Michele Rubini**, Department of Biomedical and Specialty Surgical Sciences, Section
34 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Via
35 Fossato di Mortara 74 I-44121, Ferrara, Italy. Fax No: [+39.0532.236157](tel:+390532236157) Telephone
36 No:

37 Email: rub@unife.it

38

39 **ABSTRACT**

40 To investigate possible association between functional common variants in the lysyl
41 oxidase like 3 (*LOXL3*) gene and non-syndromic cleft palate (nsCP) we selected a
42 common missense variant p.Ile615Phe (rs17010021), which was predicted to have a
43 probably damaging effect on the *LOXL3* enzyme. We genotyped 258 nsCP case-parent
44 triads of European origin and tested genetic association using the transmission
45 disequilibrium test (TDT) and log-linear regression analyses of genotypic relative risks
46 (RR) and of parent-of-origin effects. The observed genotype frequency in parents was in
47 Hardy-Weinberg equilibrium. Compared with wild-type Ile/Ile homozygotes, the RR for
48 Phe/Phe homozygote infants was 6.87 (p-value 3.0×10^{-3}), while that for Ile/Phe
49 heterozygotes was not significant. Assuming an autosomal recessive model, the RR for
50 Phe/Phe genotype resulted 10.54 (p-value 2.9×10^{-5}), with a 3.6% population attributable
51 risk. No parental-of-origin effect was observed. The identification in *LOXL3* of a
52 missense variant which under a recessive model associates with ten-fold increased risk
53 of nsCP supports the hypothesis that the genetic etiology of this congenital anomaly
54 includes relatively uncommon recessive variants with moderate penetrance and located
55 in genes which are also involved in syndromes that include CP as part of the phenotype.
56 Our findings require functional validation and replication in a larger independent
57 genetic association study.

58 **Key Words:** lysyl oxidase like 3, non-syndromic, cleft palate, missense variant.

59

60

61 **INTRODUCTION**

62 The programming of palatal development starts early in the 4th week, with the
63 formation of facial primordia that involves a complex series of closely coordinated
64 events that includes proliferation, differentiation and morphogenetic movement (Dixon
65 et al. 2011; Mossey et al. 2009). By the end of the 6th week, the primary palate is
66 formed by the fusion of the medial nasal process with the maxillary process. The
67 secondary palate arises as bilateral, medially directed outgrowths of the maxillary
68 processes (palatal shelves) that initially grows vertically on either side of the tongue but
69 later elevate to a horizontal position above the tongue. This horizontal growth of the
70 adjacent palatal shelves leads to their contact with one another and fusion to form the
71 secondary palate (Dixon et al. 2011). Disruption or perturbations at any step during this
72 process that includes elevation, migration or fusion is likely to induce cleft palate.

73 Cleft palate (CP) is a common congenital orofacial malformation. Its prevalence at birth
74 varies with geography and ethnicity between 1 and 25 per 10,000 live births, highest in
75 non-Hispanic Whites and lowest in Africans (Burg et al. 2016). The sex ratio
76 (male:female) of CP is 1:2. This might possibly be explained by differential gene
77 expression as observed between sexes in animal models (Suazo et al. 2011), differential
78 effects of female hormones (Miura et al. 1990), or delayed fusion of palate in females
79 (Burg et al. 2016). Approximately 50% of CP cases are non-syndromic (nsCP), (Mai et
80 al. 2014; Watkins et al. 2014), and are generally considered multifactorial conditions,
81 due to interplay between genetic and environmental factors (Dixon et al. 2011; Mangold
82 et al. 2011).

83 nsCP shows strong familial aggregation, which suggests a genetic component to
84 etiology (Marazita & Leslie 2016). Analyses of nationwide records from Norway and
85 Denmark show an increase in risk of recurrence among first-degree relatives of affected
86 individuals (Sivertsen et al. 2008; Grosen et al. 2010). The environmental factors
87 contributing to CP etiology so far identified are largely the same as those for cleft of the
88 lip and palate (CL/P) including tobacco smoke and alcohol (Little et al. 2004; Sabbagh
89 et al. 2015; Bell et al. 2014) and an inverse association with reported maternal use of
90 vitamin supplements (Butali et al. 2013).

91 A number of genetic factors associated with non-syndromic oral clefts have been
92 identified, mainly for CL/P rather than CP. Based on these genetic findings, non-
93 syndromic CL/P and non-syndromic CP are considered to have only very limited
94 overlap in terms of their genetic etiology (Cura et al. 2016). This is further supported by
95 genome-wide association studies (GWASs) or meta-analyses of GWAS data in different
96 populations that have identified 37 risk loci for non-syndromic CL/P (Birnbaum et al.
97 2009; Mangold et al. 2010; Beaty et al. 2010, 2013; Leslie et al. 2015, 2017; Yu et al.
98 2017; Ludwig et al. 2017; Ludwig et al. 2017; Ishorst et al., 2018), but just one
99 replicated finding for non-syndromic CP (Leslie et al. 2016; Mangold et al. 2016).

100 Although GWAS have helped detect and replicate associations between common gene
101 variants and orofacial clefts, the proportion of heritability accounted for by these
102 variants is relatively low, with inconsistencies across studies (Beaty et al. 2016). GWAS
103 have typically been designed to minimize the risk of false positive genetic associations
104 in common chronic disease, but it is known that there is a substantial risk of false
105 negatives (Ioannidis et al. 2011). Moreover, it has been observed that common variants

106 of genes that are involved in Mendelian disorders have been associated with non-
107 Mendelian forms of the same disorders (Blair et al. 2013). In addition, variants of some
108 genes involved in syndromic clefts have been found to have replicated associations with
109 non-syndromic clefts, reflecting the two forms of clefting as parts of a single spectrum
110 (Stanier & Moore 2004; Dixon et al. 2011). An excellent example is *GRHL3*, the second
111 gene associated with Van der Woude syndrome and its recent identification as
112 associated with nsCP (Leslie et al. 2016; Mangold et al. 2016).

113 The human *LOXL3* gene located on chromosome 2p13.1 has been associated with
114 Stickler syndrome (MIM #108300), which includes CP as a phenotype (Alzahrani et al.
115 2015). Additionally, deletion of this gene impairs collagen assembly and crosslinking
116 during palate development in mouse model (Zhang et al. 2015). However, no evidence
117 of linkage or association with nsCP was reported for this gene in a recent GWAS
118 (Leslie et al. 2016) or an imputation based meta-analysis of GWAS data (Ludwig et al.
119 2017). Of note, the ability to detect common variants with very weak effects, or less
120 common variants with small to modest effects, is strongly dependent on assumptions
121 concerning linkage disequilibrium, allele frequency and genotype certainty (Bomba et
122 al. 2017).

123 We therefore examined the potential association between putative functional variants of
124 *LOXL3* and nsCP, a disorder that is less common than other nsCL/P in humans, and less
125 investigated, in European case-parent triads.

126

127 **MATERIALS AND METHODS**

128 **Participants**

129 The study includes 258 nuclear families of infants with nsCP identified through the
130 EUROCRAN and ITALCLEFT biobanks, which include case-parent trios from 9
131 European countries (Mossey et al. 2017; Ghassibe-Sabbagh et al. 2011), including the
132 United Kingdom, Netherlands, Italy, Spain, Slovenia, Slovakia, Hungary, Estonia and
133 Bulgaria. The case-parent trio design of the present study makes it less vulnerable to
134 population stratification, a particular concern of multi-centre studies [Mossey et al.
135 2017]. Ethical permission was sought and obtained at surgical centres in each
136 participating countries at the time of first surgical intervention on the index infant.
137 Infants with recognized syndromic clefts or Pierre Robin sequence were excluded.
138 Peripheral blood or buccal cell samples were used to obtain genomic DNA from infants
139 and their parents. The use of data and DNA samples from EUROCRAN and
140 ITALCLEFT biobanks was approved by MREC Scotland (Dec 7th 2011, #MREC/1/0/7)
141 and S. Paolo Hosp. E.C. (Mar 2nd, 2012, #3503) respectively.

142 **Exposure information**

143 In both the EUROCRAN and ITALCLEFT studies mothers were asked to respond to a
144 specific questionnaire that was administered by personal interview when the index
145 affected infant was brought in to the surgical centre to undergo the primary surgery.
146 Major areas about which information was sought included use of nutritional
147 supplements and tobacco smoking. Folic acid supplementation was defined as having
148 taken folic acid or folic acid-containing supplements (at least 0.4 mg/day) for at least
149 one month during the periconceptual period (3 months before to 3 months after
150 conception). Maternal smoking during pregnancy was defined as having smoked at least
151 one cigarette per day during the periconceptual period (Mossey et al. 2017).

152 **Selection of putative functional single nucleotide variants in *LOXL3***

153 We screened the exons of the *LOXL3* gene for nucleotide substitutions and insertions
154 and deletions using the UCSC Genome browser GRCh38/hg38 assembly
155 (<https://genome-euro.ucsc.edu/>) and identified 336 missense and 139 synonymous
156 variants. Of these, according to dbSNP build 150
157 (www.ncbi.nlm.nih.gov/projects/SNP), only three are polymorphic, with minor allele
158 frequency (MAF) >1%: rs17010022, rs17010021, and rs77706750. The first SNP,
159 rs17010022, is a synonymous p.Leu371Leu variant located in exon 7 of *LOXL3* gene,
160 with putative no effect on conformation of the encoded peptide, and therefore was
161 discarded. The other two variants, rs77706750 in exon 7, and rs17010021 in exon 11,
162 cause substitutions (p.Arg375His and p.Ile615Phe, respectively) both predicted to be
163 “probably damaging” by PolyPhen-2 (Adzhubei et al. 2013). However, considering the
164 available sample size, the MAF of rs77706750 was too low (1.46%) to provide enough
165 power (0.80) under dominant or recessive genetic models (Quanto 1.2.4,
166 biostats.usc.edu), and hence was not included in the present study. However, the MAF
167 of rs17010021 was much higher (8.23% reported in dbSNP), granting sufficient power
168 for a genetic association study.

169 **Genotyping**

170 For most individuals included in the study, genomic DNA (gDNA) was extracted from
171 peripheral blood specimens using the Nucleon BACC1 kit (Amersham Biosciences, part
172 of GE Healthcare Europe, CH). For around 5% of participants, gDNA was extracted
173 from buccal swab specimens using QIAamp DNA Blood Mini Kit (Qiagen, Hilden DE)
174 according to the manufacturer’s instructions. All gDNA samples were quantified using
175 Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA).

176 Genotypes of p.Ile615Phe variant were obtained by TaqMan allelic discrimination assay
177 using an ABI 7300 real-time thermocycler according to the standard protocol of
178 manufacturer (Applied BioSystems, Foster City, CA). In 15% of samples, genotyping
179 was repeated for quality testing.

180 **Statistical analysis**

181 The χ^2 test for the Hardy-Weinberg equilibrium (HWE) were computed for genotypes
182 of parents and case-infants. The genetic association of the missense variant in nsCP
183 case-parent triads was calculated using the transmission disequilibrium test (TDT),
184 (Spielman et al. 1993). We estimated relative risk (RR) and 95% of confidence interval
185 (CI) for the independent effects of mother and infant genotypes using a log-linear
186 regression model that incorporates an expectation-maximization algorithm to allow
187 inclusion of triads for which both parent genotype were missing (Weinberg et al. 1998;
188 Wilcox et al. 1998). The analyses were implemented using the Stata package
189 (<http://www.biostat-resources.com>, StataCorp LP, College Station, TX). As exploratory
190 analyses, we carried out subgroup analyses stratifying on the sex of the infant and
191 maternal smoking and use of supplements containing folic acid.

192 We further investigated a possible parent-of-origin effect, by assessing the risk
193 increment (I_M) in the offspring associated with receiving the allele transmitted from the
194 mother as compared to the father in log-linear regression analysis (Weinberg et al. 1998;
195 Wilcox et al. 1998).

196

197 **RESULTS**

198 The study included 258 nsCP case-parent trios from 9 European countries. As expected,
199 female cases outnumbered the males, and male:female sex ratio was 0.78 (95% C.I.
200 0.74-0.84).

201 The allele and genotype frequency of the triads included in the study is shown in Table
202 1. Among the 516 parents included in the study the frequency of Phe allele was 4.7%
203 (95%CI 2.9-6.5%), a value lower than the 8.23% reported in dbSNP. Genotype
204 frequency among cases was significantly out of Hardy-Weinberg equilibrium (p-value =
205 2.27×10^{-6}), while both parents resulted not in disequilibrium (p-value = 0.68).
206 Remarkably, the frequency of Phe/Phe homozygotes, predicted to be only 0.22% on the
207 basis of allele frequency in the parents, was 7-fold higher (1.55%) than predicted among
208 nsCP cases.

209 Application of TDT showed no **significant** evidence of asymmetric segregation of Phe
210 allele from parents (Transmitted:Non-transmitted = 21:23, p-value = 0.673).

211 **Considering the observed low frequency of Ile/Phe genotype among parents (Table 1),**
212 **and being the power of the TDT heavily dependent on the number of heterozygous**
213 **parents (Sebro, Rogus, 2010), we performed the calculation of genotype-associated RR**
214 **using a log-linear regression model (Weinberg et al. 1998; Wilcox et al. 1998).**

215 **Calculation** of genotype-associated **RR** showed significant association between Phe/Phe
216 homozygous infant genotype and nsCP risk (RR = 6.9, p-value = 0.003), whereas there
217 was no significant association with the heterozygous genotype. Mother's genotype was
218 not associated with increased risk of nsCP in the offspring (Table 2).

219 **Considering that the Ile/Phe genotype provided no increased risk of nsCP compared to**
220 **wild type Ile/Ile homozygotes, while Phe/Phe genotype associated with increased risk,**

221 we assumed a recessive genetic model. Under this model, log-linear regression analysis
222 showed that infant's Phe/Phe genotype associated with a significant ten-fold increased
223 risk of nsCP (RR = 10.54 (95% C.I. 3.34-33.30, p-value = 2.85×10^{-5}). No parental of
224 origin effect was observed ($I^M = 0.58$, p-value = 0.455).

225 Considering the genotypic frequencies of parents as reference and a birth prevalence of
226 nsCP of 1:2216 among Europeans (Calzolari et al., 2004), the population attributable
227 risk of Phe/Phe genotype was 3.6%, whereas the penetrance was 0.48%.

228 Although we are aware of the limited sample size of our study, we conducted subgroup
229 analyses and report these. Among the four Phe/Phe infants, three females and one male,
230 only one girl was born from a mother exposed to folic acid supplementation during the
231 periconceptional period. As regards periconceptional exposure to tobacco smoking, all
232 four Phe/Phe infants were born from non-smoking mothers. RR did not significantly
233 differed between male and female cases.

234

235 **DISCUSSION**

236 In the present study, we investigated a potential association between functional common
237 variants in lysyl oxidase like 3 (*LOXL3*) gene and the risk of developing nsCP. Rare
238 variants in *LOXL3* have been detected in patients with Stickler syndrome, which may
239 present with CP (Alzahrani et al. 2015), and in mouse model a crucial role of *Loxl3*
240 gene in palate development has been demonstrated (Zhang et al. 2015). Among the
241 hundreds of missense variants annotated in *LOXL3* gene we selected p.Ile615Phe, which
242 is the only one that is predicted to be probably damaging and has relatively high MAF,
243 sufficient to provide enough statistical power considering the sample size of the study.

244 Although Phe/Phe homozygotes are very uncommon, we identified four Phe/Phe
245 homozygotes among the 258 cases included in the study, and detected a significant
246 association between infant's homozygote Phe/Phe genotype and the risk of nsCP,
247 compared to common Ile/Ile homozygotes. Heterozygous Ile/Phe genotype was not
248 significantly associate with nsCP. Therefore, assuming an autosomal recessive model,
249 the Phe/Phe genotype turned out to associate with around ten-fold increased risk of
250 nsCP (p-value = 2.85×10^{-5}). Autosomal recessive genetic model is typical for enzyme-
251 encoding genes, and fits well with the nature of *LOXL3*. As the p.615Phe enzyme is
252 predicted to have lost most or all catalytic activity, we presume that Phe/Phe
253 homozygotes are severely deficient of the amine oxidase activity of *LOXL3* enzyme,
254 and consequently have impaired collagen fiber assembly in palatal mesenchyme. We
255 hypothesize that this impairment could have played a role in determining the failure of
256 fusion of palatal shelves during embryogenesis, and ultimately caused CP. The lack of
257 efficient catalysis of collagen crosslinking associated with p.615Phe enzyme may
258 resemble the effect of LOX's inhibitor β -aminopropionitrile, which determine reduced
259 collagen fibres density and development of CP in animal model (Pratt & King 1972).
260 Functional studies using animal models are awaited to confirm the phenotypic effect of
261 p.615Phe enzyme.

262 The failure of TDT to detect association could rely on the fact that, due to the relative
263 low MAF of the studied *LOXL3* variant, among the cases most of p.615Phe alleles are
264 carried by heterozygotes, which are not at risk of nsCP, and therefore distortion of
265 transmission from parents would not be expected.

266 As might be anticipated for a gene expressed in palate shelves during embryonic
267 development, maternal p.Ile615Phe genotype was not associated with the infant's risk
268 of nsCP. Moreover, no preferential transmission of minor allele from one of two parents
269 was observed. Due to the low frequency of Phe/Phe homozygotes, the statistical power
270 of the study was not sufficient to detect interaction with infant sex, periconceptual
271 folic acid supplementation, or exposure to tobacco smoking.

272 The infant's Phe/Phe genotype seems to strongly increase the risk of nsCP, but its actual
273 weight among the multiple genetic and environmental factors as part of the
274 multifactorial etiology of nsCP is relatively small. Due to the relatively low MAF of
275 p.Ile615Phe, the calculated population attributable risk was only 3.6%, **and the**
276 **penetrance modest (0.48%)**. We hypothesize that other functional variants of the *LOXL3*
277 gene, mainly classified as rare variants and less frequent than the p.Ile615Phe variant,
278 might be associated with nsCP risk.

279 The impact of rare or less common variants associated with increased risk of nsCP has
280 begun to emerge from recent exome-wide and genome-wide sequencing studies
281 (Mangold et al. 2016). In particular, a low frequency missense p.Thr454Met variant in
282 *GRHL3* (rs41268753) was significantly associated with nsCP risk (Mangold et al. 2016;
283 Leslie et al. 2016). From the latest genetic investigations on nsCP, a difference in terms
284 of frequency spectrum of susceptibility variants compared to nsCL/P, is becoming
285 evident. While GWAS of nsCL/P identified a number of common polymorphic variants
286 (Birnbbaum et al. 2009; Beaty et al. 2010; Mangold et al. 2010), GWAS of nsCP have
287 detected only one genome-wide significant variant (Leslie et al. 2016), even though
288 sample size were comparable. This evidence suggests that the genetic aetiology of nsCP

289 may mainly rely on relatively rare variants, or less common variants that act under
290 recessive model, which may present moderate penetrance, tending to escape detection
291 by genome-wide studies and to be located within genes involved in syndromes that
292 include CP as part of the phenotype. *LOXL3* p.Ile615Phe may be one of these variants.

293 In conclusion, using a candidate gene approach, we identified a missense variant in
294 *LOXL3* gene, p.Ile615Phe, which under a recessive model is associated with a
295 significant ten-fold increased risk of nsCP. This finding should be replicated in a larger
296 cohort of case-parent trios, and joint effects with environmental exposure factors
297 investigated. We suggest that *LOXL3* p.Ile615Phe, along with *GRHL3* p.Thr454Met, are
298 part of a constellation of low frequency variants that compose the genetic background of
299 nsCP.

300

301 **ACKNOWLEDGMENTS**

302 This study was partly supported by UNIFE FAR-2016 grant. We acknowledge the
303 support received from the European Science Foundation within the “Network for
304 Orofacial Clefts Research, Prevention and Treatment” (EUROClleftNet, 09-RNP-023)
305 programme (MFJK, ESF exchange visit grants, n. 5023, 5152). Our sincere thanks to
306 Dr. Houda Oudouche, Department of Humanities, University of Ferrara, Italy and Lab.
307 Group members, Valentina Aleotti, Amin Ravaei, Luca Dall’Olio, Vincenzo Aiello,
308 Gianni Astolfi, Ilenia Lombardo and Ilaria Cestonaro for their assistance. JL holds a tier
309 1 Canada Research Chair.

310

311 **DISCLOSURE** None.

312

313 **REFERENCES**

- 314 Adzhubei I, Jordan DM, Sunyaev SR. 2013. Predicting functional effect of human
315 missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* Chapter 7: Unit7
316 20.
- 317 Alzahrani F, Al Hazzaa SA, Tayeb H, Alkuraya FS. 2015. LOXL3, encoding lysyl
318 oxidase-like 3, is mutated in a family with autosomal recessive Stickler syndrome.
319 *Hum Genet* 134: 451-3.
- 320 Beaty TH, Murray JC, Marazita ML et al. 2010. A genome-wide association study of
321 cleft lip with and without cleft palate identifies risk variants near MAFB and
322 ABCA4. *Nat Genet* 42: 525-9.
- 323 Beaty TH, Taub MA, Scott AF et al. 2013. Confirming genes influencing risk to cleft
324 lip with/without cleft palate in a case-parent trio study. *Hum Genet* 132: 771-81.
- 325 Beaty TH, Marazita ML, Leslie EJ. 2016. Genetic factors influencing risk to orofacial
326 clefts: today's challenges and tomorrow's opportunities. *F1000Res* 5: 2800.
- 327 Bell JC, Raynes-Greenow C, Turner RM, Bower C, Nassar N, O'Leary CM. 2014.
328 Maternal alcohol consumption during pregnancy and the risk of orofacial clefts in
329 infants: a systematic review and meta-analysis. *Paediatr Perinat Epidemiol* 28: 322-
330 32.
- 331 Birnbaum S, Ludwig KU, Reutter H et al. 2009. Key susceptibility locus for
332 nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nat Genet*
333 41: 473-7.
- 334 Blair DR, Lyttle CS, Mortensen JM et al. 2013. A nondegenerate code of deleterious
335 variants in Mendelian loci contributes to complex disease risk. *Cell* 155: 70-80.

336 Bomba L, Walter K, Soranzo N. 2017. The impact of rare and low-frequency genetic
337 variants in common disease. *Genome Biol* 18: 77.

338 Burg ML, Chai Y, Yao CA, Magee W, 3rd, Figueiredo JC. 2016. Epidemiology,
339 Etiology, and Treatment of Isolated Cleft Palate. *Front Physiol* 7: 67.

340 Butali A, Little J, Chevrier C et al. 2013. Folic acid supplementation use and the
341 MTHFR C677T polymorphism in orofacial clefts etiology: An individual
342 participant data pooled-analysis. *Birth Defects Res A Clin Mol Teratol* 97: 509-14.

343 Calzolari E, Bianchi F, Rubini M, Ritvanen A, Neville AJ, EUROCAT Working group.
344 2004. Epidemiology of cleft palate in Europe: implications for genetic research.
345 *Cleft Palate Craniofac J* 41: 244-9.

346 Cura F, Bohmer AC, Klamt J et al. 2016. Replication analysis of 15 susceptibility loci
347 for nonsyndromic cleft lip with or without cleft palate in an italian population. *Birth*
348 *Defects Res A Clin Mol Teratol* 106: 81-7.

349 Dixon MJ, Marazita ML, Beaty TH, Murray JC. 2011. Cleft lip and palate:
350 understanding genetic and environmental influences. *Nat Rev Genet* 12: 167-78.

351 Ghassibe-Sabbagh M, Desmyter L, Langenberg T et al. 2011. FAF1, a gene that is
352 disrupted in cleft palate and has conserved function in zebrafish. *Am J Hum Genet*
353 88: 150-61.

354 Grosen D, Chevrier C, Skytthe A et al. 2010. A cohort study of recurrence patterns
355 among more than 54,000 relatives of oral cleft cases in Denmark: support for the
356 multifactorial threshold model of inheritance. *J Med Genet* 47: 162-168.

357 Ioannidis JP, Tarone R, McLaughlin JK. 2011. The false-positive to false-negative ratio
358 in epidemiologic studies. *Epidemiology* 22: 450-6.

359 Ishorst N, Franceschelli P, Böhmer AC et al. 2018. Nonsyndromic cleft palate: An
360 association study at GWAS candidate loci in a multi-ethnic sample. *Birth Def. Res*
361 **Part A.**

362 Leslie EJ, Taub MA, Liu H et al. 2015. Identification of functional variants for cleft lip
363 with or without cleft palate in or near PAX7, FGFR2, and NOG by targeted
364 sequencing of GWAS loci. *Am J Hum Genet* 96: 397-411.

365 Leslie EJ, Liu H, Carlson JC et al. 2016. A Genome-wide Association Study of
366 Nonsyndromic Cleft Palate Identifies an Etiologic Missense Variant in GRHL3.
367 *Am J Hum Genet* 98: 744-754.

368 Leslie EJ, Carlson JC, Shaffer JR et al. 2017. Genome-wide meta-analyses of
369 nonsyndromic orofacial clefts identify novel associations between FOXE1 and all
370 orofacial clefts, and TP63 and cleft lip with or without cleft palate. *Hum Genet* 136:
371 275-286.

372 Little J, Cardy A, Munger RG. 2004. Tobacco smoking and oral clefts: a meta-analysis.
373 *Bull World Health Organ* 82: 213-8.

374 Ludwig KU, Ahmed ST, Böhmer AC, et al. 2017. Meta-analysis reveals genome-wide
375 significance at 15q13 for nonsyndromic clefting of both the lip and the palate, and
376 functional analyses implicate GREM1 as a plausible causative gene. *PLoS Genet*
377 **12: e1005914.**

378 Ludwig KU, Böhmer AC, Bowes J et al. 2017. Imputation of orofacial clefting data
379 identifies novel risk loci and sheds light on the genetic background of cleft lip +/-
380 cleft palate and cleft palate only. *Hum Mol Genet* 26: 829-842.

381 Mai CT, Cassell CH, Meyer RE et al. 2014. Birth defects data from population-based
382 birth defects surveillance programs in the United States, 2007 to 2011: highlighting
383 orofacial clefts. *Birth Defects Res A Clin Mol Teratol* 100: 895-904.

384 Mangold E, Ludwig KU, Birnbaum S et al. 2010. Genome-wide association study
385 identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft
386 palate. *Nat Genet* 42: 24-6.

387 Mangold E, Ludwig KU, Nothen MM. 2011. Breakthroughs in the genetics of orofacial
388 clefting. *Trends Mol Med* 17: 725-33.

389 Mangold E, Bohmer AC, Ishorst N et al. 2016. Sequencing the GRHL3 Coding Region
390 Reveals Rare Truncating Mutations and a Common Susceptibility Variant for
391 Nonsyndromic Cleft Palate. *Am J Hum Genet* 98: 755-62.

392 Marazita ML, Leslie EJ. 2016. Genetics of Nonsyndromic Clefting. In: Losee J,
393 Kirschner R, editors. *Comprehensive Cleft Care*. Boca Roton FL: CRC Press,
394 Taylor & Francis Group. p 207–24.

395 Miura S, Natsume N, Horiuchi R. 1990. Experimental study on cleft lip and palate.
396 Preventive effects of estradiol on cleft lip and/or palate in A/J mice. *J Jpn Cleft*
397 *Palate Assoc* 15:122–131.

398 Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. 2009. Cleft lip and palate.
399 *Lancet* 374: 1773-85.

400 Mossey PA, Little J, Steegers-Theunissen R et al. 2017. Genetic Interactions in
401 Nonsyndromic Orofacial Clefts in Europe-EUROCRAN Study. *Cleft Palate*
402 *Craniofac J* 54: 623-630.

403 Pratt RM, Jr., King CT. 1972. Inhibition of collagen cross-linking associated with beta-
404 aminopropionitrile-induced cleft palate in the rat. *Dev Biol* 27: 322-8.

405 Sabbagh HJ, Hassan MH, Innes NP, Elkodary HM, Little J, Mossey PA. 2015. Passive
406 smoking in the etiology of non-syndromic orofacial clefts: a systematic review and
407 meta-analysis. PLoS One 10: e0116963.

408 **Sebro R, Rogus JJ. 2010. The power of the Transmission Disequilibrium Test in the**
409 **presence of population stratification. Eur J Hum Genet 18:1032-8.**

410 Sivertsen A, Wilcox AJ, Skjaerven R, Vindenes HA, Abyholm F, Harville E, Lie RT.
411 2008. Familial risk of oral clefts by morphological type and severity: population
412 based cohort study of first degree relatives. BMJ 336: 432-4.

413 Spielman RS, McGinnis RE, Ewens WJ. 1993. Transmission test for linkage
414 disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus
415 (IDDM). Am J Hum Genet 52: 506-16.

416 Stanier P, Moore GE. 2004. Genetics of cleft lip and palate: syndromic genes contribute
417 to the incidence of non-syndromic clefts. Hum Mol Genet 13 Spec No 1: R73-81.

418 Suazo J, Tapia JC, Santos JL, Castro VG, Colombo A, Blanco R. 2011. Risk variants in
419 BMP4 promoters for nonsyndromic cleft lip/palate in a Chilean population. BMC
420 Med Genet 12: 163.

421 Watkins SE, Meyer RE, Strauss RP, Aylsworth AS. 2014. Classification, epidemiology,
422 and genetics of orofacial clefts. Clin Plast Surg 41: 149-63.

423 Weinberg CR, Wilcox AJ, Lie RT. 1998. A log-linear approach to case-parent-triad
424 data: assessing effects of disease genes that act either directly or through maternal
425 effects and that may be subject to parental imprinting. Am J Hum Genet 62: 969-
426 78.

427 Wilcox AJ, Weinberg CR, Lie RT. 1998. Distinguishing the effects of maternal and
428 offspring genes through studies of "case-parent triads". *Am J Epidemiol* 148: 893-
429 901.

430 Yu Y, Zuo X, He M et al. 2017. Genome-wide analyses of non-syndromic cleft lip with
431 palate identify 14 novel loci and genetic heterogeneity. *Nat Commun* 8: 14364.

432 Zhang J, Yang R, Liu Z et al. 2015. Loss of lysyl oxidase-like 3 causes cleft palate and
433 spinal deformity in mice. *Hum Mol Genet* 24: 6174-85.

434

435 **Table 1** Allele and genotype frequencies of p.Ile615Phe (rs17010021) in 258
 436 nsCP case-parent triads, and p-value of difference from Hardy-Weinberg (H-W)
 437 equilibrium.

438

Alleles/Genotypes	Cases n (%)	Mothers n (%)	Fathers n (%)
Ile	493 (95.5)	498 (96.5)	486 (94.2)
Phe	23 (4.5)	18 (3.5)	30 (5.8)
Ile/Ile	239 (92.6)	240 (93.0)	230 (89.1)
Ile/Phe	15 (5.8)	18 (7.0)	26(10.1)
Phe/Phe	4 (1.6)	0 (0.0)	2 (0.8)
H-W p-value	2.27 x 10 ⁻⁶	0.85	0.44

439

440 **Table 2** Genotype-associated relative risk of p.Ile615Phe (rs17010021) in 258
441 nsCP case-parent triads assuming the common Ile/Ile homozygous genotype as
442 reference.

443

Mother's genotypes	RR (95% C.I.)	p-value
Ile/Phe	0.54 (0.28-1.05)	0.071
Phe/Phe	n.c.	-
Infant's genotypes	RR (95% C.I.)	p-value
Ile/Phe	0.61 (0.31-1.17)	0.136
Phe/Phe	6.87 (1.97-23.98)	0.003

444