Imputation of orofacial clefting data identifies novel risk loci and sheds light on the genetic background of cleft lip ± cleft palate and cleft palate only

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Abstract

Nonsyndromic cleft lip with or without cleft palate (nsCL/P) is among the most common human birth defects with multifactorial etiology. Here, we present results from a genome-wide imputation study of nsCL/P in which, after adding replication cohort data, four novel risk loci for nsCL/P are identified (at chromosomal regions 2p21, 14q22, 15q24 and 19p13). On a systematic level, we show that the association signals within this high-density dataset are enriched in functionally-relevant genomic regions that are active in both human neural crest cells (hNCC) and mouse embryonic craniofacial tissue.
This enrichment is also detectable in hNCC regions primed for later activity. Using GCTA analyses, we suggest that 30% of the estimated variance in risk for nsCL/P in the European population can be attributed to common variants, with 25.5% contributed to by the 24 risk loci known to date. For each of these, we identify credible SNPs using a Bayesian refinement approach, with two loci harbouring only one probable causal variant. Finally, we demonstrate that there is no polygenic component of nsCL/P detectable that is shared with nonsyndromic cleft palate only (nsCPO). Our data suggest that, while common variants are strongly contributing to risk for nsCL/P, they do not seem to be involved in nsCPO which might be more often caused by rare deleterious variants. Our study generates novel insights into both nsCL/P and nsCPO etiology and provides a systematic framework for research into craniofacial development and malformation.

Introduction

Nonsyndromic cleft lip with or without cleft palate (nsCL/P) is among the most common of all human birth defects (1). The genetic components of the underlying multifactorial etiology have been investigated extensively, with substantial recent progress due mainly to advances in large-scale genotyping technologies. Using genome-wide association studies (GWAS) together with replication assays, candidate gene and linkage studies, 20 genetic risk loci have been identified to date (2–15). A substantial fraction of these loci confer their effects in diverse populations (10–14), although the strength of the associations for single variants in different populations varies as a function of differences in risk allele frequencies and locus heterogeneity; also reflected by different prevalence rates for nsCL/P observed in different populations (1,11,15). Despite these successes it remains unclear how much of the variance in nsCL/P risk can be explained by the risk loci identified to date or common genetic variation in general, and the identification of additional genetic factors contributing to nsCL/P is to be expected.

In addition, one interesting question is the extent to which genetic factors overlap between nsCL/P and isolated (nonsyndromic) cleft palate where the lip is unaffected (nsCPO); the second most common type of orofacial clefting (OFC), after nsCL/P (15). There is ongoing discussion as to whether both disorders represent different OFC subphenotypes with shared genetic factors or whether they are genetically distinct conditions. The latter hypothesis is supported by epidemiological studies showing larger recurrence risks in either of the nsCL/P and nsCPO groups respectively, with cross-over phenotypes showing recurrence risks only slightly above the population background rates (16–18). Moreover, while GWAS have identified numerous associations reaching genome-wide significance for nsCL/P, it was only recently the first common variant reaching genome-wide significance for nsCPO was identified (19,20). Notably, this missense variant in grainyhead-like-3 (GRHL3 [MIM 608317]) did not show any association with nsCL/P (19). Despite these observations, candidate gene approaches have revealed variants nominally associated with both nsCL/P and nsCPO, the most conclusive being markers at the FOXE1 locus (8,21), suggesting that some regions might contribute to both traits with small effect sizes. However, it has remained unclear whether or not shared polygenic components might be involved in risk for both malformations.

Given the recent advances in sequence and haplotype annotation of numerous human populations by projects such as 1000 Genomes (22) or Genomes of the Netherlands (23), improved imputation and statistical methods as well as increasingly large sample sizes, it has now become possible to generate and comprehensively analyze high-density imputed datasets for complex phenotypes including nsCL/P and nsCPO. Such datasets have the power to identify novel associations that had escaped prior detection due to insufficient coverage of that region and/or low linkage disequilibrium (LD) between the genotyped markers and causal variants. In addition, this approach harbours enormous potential for the identification of allelic heterogeneity between populations and (sub-)phenotypes, and overlap with functionally annotated regions. Therefore, in the present study, we used data from previously published GWAS datasets for both nsCL/P (2,7) and nsCPO (24) from European and Asian populations, part of which we obtained from dbGaP with approved data access, to increase our understanding of the genetic architecture. Our results provide systematic novel insights in both genetic etiology and underlying biology of both types of facial malformation.

Results

Genome-wide imputation in nsCL/P and meta-analysis (discovery phase)

We used genotypes from our previously published meta-analysis of nsCL/P (6) which comprised 399 cases, 1318 controls (Bonn GWAS cohort (7)) and 1461 case-parent trios (Baltimore study: 666 European, 795 Asian (2), Supplementary Material, Fig. S1). After imputation and quality control (QC), 8.01 million variants were retained in the analysis. The genomic inflation factor was 1.044 for the analysis of European individuals only (metaEuro), and 1.047 for the overall analysis including Europeans and Asians (metaall). Quantile-quantile (QQ)-plots including the imputed data were similar when compared to QQ-plots including genotyped variants only (Supplementary Material, Fig. S2), indicating the absence of a technical bias due to the imputation performed. A total of 2556 SNPs showed P-values of at most $1 \times 10^{-8}$ in at least one of the analyses, 2002 of which mapped to 17 of the 20 nsCL/P risk loci that were known at the time of analysis (no variants mapped to the FOXE1 region and the chromosomal regions 17q23 and 19q13). Of the remaining 554 variants, two (rs6740960, rs4952552; both at chromosome 2p21) reached $P_{\text{meta}_\text{Euro}} < 5 \times 10^{-8}$ in the European analysis, while three additional variants at two loci met this threshold when Asian samples were included (metaall, Supplementary Material, Figs. S1 and S3, Table S1). These variants included two at chromosome 14q22 (rs4901118, rs60454187) and rs115681412 which maps to the major histocompatibility complex (MHC) region at chromosome 6. To exclude imputation errors at the highly variable MHC region (25), we performed a specific HLA imputation using TIDGC reference data, yielding $P = 4.61 \times 10^{-8}$ for rs115681412 in metaall. Notably, none of these five variants was present on the 550k/610k arrays used in the original studies.

The lead SNP at the newly identified risk region at chromosome 2p21 (abbreviated as 2p21PKDCC), rs6740960, maps 1.36 Mb away from rs7590268, the lead SNP located within the THADA gene at the previously identified locus within
chromosome 2p21 (2p21THADA, (6)). We used conditional analyses in the Bonn case-control GWAS data to show that both effects are independent from one another: the P-value for rs6740960 ($P_{\text{cond}} = 1.38 \times 10^{-04}$, $P_{\text{cond,rs6740960}} = 3.01 \times 10^{-04}$) increased only marginally when conditioned on rs7590268, and also for rs7590268 ($P_{\text{cond}} = 1.68 \times 10^{-05}$) when conditioned on rs6740960 ($P_{\text{cond,rs6740960}} = 3.62 \times 10^{-05}$).

### Replication and combined analysis

To confirm the findings obtained by imputation and in an attempt to identify further risk loci, 44 SNPs were selected for replication in an independent case-control cohort of mixed ethnicities (574 cases, 1635 controls, Supplementary Material, Fig. S1). Of the 42 SNPs passing QC, five showed $P < 0.05$ (Supplementary Material, Table S3), including those at chromosomes 2p21PKDCC and 14q22. No additional variant reached genome-wide significance when replication and imputed data were combined (Table 1, Supplementary Material, Table S1). Accounting for potential ethnic-specific factors, we additionally combined meta_Euro plus the European part of the replication cohort (i.e. the Bonn replication sample ($n = 224$ cases/921 controls); Supplementary Material, Table S1 and Fig. S1). In that analysis a missense variant at chromosome 19p13 (rs3746101; MKNK2, p.Q10K) reached genome-wide significance ($P = 2.44 \times 10^{-08}$, Table 1). This variant was significantly associated with nsCL/P in the Central European (Bonn) component of the replication sample only ($P = 0.03$) while it did not show association in either Mexican or Yemeni samples ($P > 0.3$, Supplementary Material, Table S1).

The high-density dataset also allowed us to combine results directly with summary statistics available from other studies on nsCL/P without the need to use proxy SNPs. We assessed published data on 227 SNPs from a recent multiethnic study on nsCL/P (11), and combined these data with the imputed Bonn GWAS data (meta-analysis of the entire dataset was not possible due to individuals overlapping between studies (2) and (11)). In the combined analysis of European individuals (Supplementary Material, Table S4), no novel associations were detected. In the multiethnic combination (Supplementary Material, Table S5), we observed one locus at chromosome 15q24, previously reported as suggestive (11), which reached genome-wide significance (rs28689146, $P = 6.61 \times 10^{-09}$, Table 1, Fig. 1).

### Associations with other traits

The present dataset on nsCL/P allows further analysis of related and/or non-disease traits in silico. For instance, it has been shown that unaffected family members of patients with nsCL/P show differences in facial morphometrics such as a broader nasal cavity, shorter upper and longer lower faces (27), and seven genetic loci have been found to be associated with various craniofacial morphometric traits (28–30). Analyzing these variants in our dataset did not reveal any significant association ($P > 0.2$, Supplementary Material, Table S6), nor did any variants at plus/minus 500 kb withstand correction for multiple testing. We also searched for yet unknown trait associations with nsCL/P risk regions by assessing the GWAS catalogue (31) using both the list of credible SNPs and all SNPs in strong linkage disequilibrium (i.e. $r^2 > 0.8$) in the European population. Three SNPs at two loci were associated with two non-clefting traits, i.e. the FOXE1 locus with thyroid cancer (32), and the chromosome 3p11 locus with ocular axial length, a quantitative measure for myopia (33) (Supplementary Material, Table S7). At the FOXE1 locus, the risk allele of two variants mapping close to a previously identified craniofacial enhancer hsCNE +22.6 (34) showed decreased risk for thyroid cancer (32). In addition, two common variants located within this particular enhancer (rs12542417, rs10984103) showed association with risk for nsCL/P and are listed among the credible SNPs (Supplementary Material, Table S8, see next paragraph).

### Identification of credible SNPs

We next aimed at identifying SNPs which are highly probable to have a causative contribution to nsCL/P, i.e. the "credible SNP" set, for each of the 24 risk loci reaching genome-wide significance in our present study ($n = 4$) and previous research ($n = 20$, Supplementary Material, Table S9, (2–11)). We herefore used a Bayesian framework analysis in the meta_Euro dataset (Table 2, Supplementary Material, Table S8). In five of the nsCL/P regions, including chromosome 8q24, the set of credible SNPs did not encompass the genotyped lead SNP identified in the respective GWAS where these loci were originally identified. For two regions, at chromosomes 2p21PKDCC and 17q13, only one SNP had a posterior probability of 95%, suggesting these were causative in the European population.

### Table 1. Association results for novel nsCL/P risk loci detected by imputation and replication

| Locus         | Lead SNP position | Chromosomal position | Alleles | GWAS_imputed | P_Replication | Combined analysis |
|---------------|-------------------|----------------------|---------|--------------|---------------|-------------------|
|               |                   |                      |         | $P_{\text{meta}}$ |              |                   |
| (a) Loci identified with genome-wide significance by imputation and replication | | | | | | |
| 2p21          | rs6740960         | 42181679             | T/A     | 3.29E-09     | 1.68E-09      | 3.32E-04         |
| 14q22         | rs4901118         | 51856109             | A/G     | 7.51E-07     | 5.44E-09      | 2.56E-02         |
| 19p13         | rs3746101         | 2050823              | T/G     | 2.30E-07     | 4.52E-04      | 3.17E-02         |
| (b) Loci identified with genome-wide significance by combining imputed Bonn GWAS dataset and (11) summary statistics | | | | | | |
| 15q24         | rs28689146        | 75005575             | A/T     | 6.54E-03 (3.6E-02) | 2.35E-02 | 5.4E-08          |

- Defined as lowest $P$-value in genome-wide imputation.
- Risk allele in bold.
- $P$-value for Bonn replication cohort only.
- Number in brackets indicates $P$-value for rs28689146 in Bonn GWAS case-control cohort. This cohort was used for in silico combination with replication cohort of Leslie et al. (2016) as there were overlapping individuals between Leslie et al. (11) and Beaty et al. (2).
- $P$-value from Leslie et al. 2016 (11).
- No summary statistics for rs28689146 in Leslie et al. (11) for the European dataset.
To gain insights into the biology of nsCL/P, we next performed in-depth analyses of the 24 recognized risk loci. These approaches included DEPICT analyses to identify enriched gene sets and pathways, as well as conditional analyses to investigate potential secondary effects.

To identify tissues in which genes near all 24 nsCL/P associated SNPs are highly expressed, and gene sets showing significant enrichment among these risk loci, we used DEPICT in two settings; (i) including the 24 risk loci (Supplementary Material, Tables S10 and S11), and (ii) all autosomal SNPs with \( P < 10^{-5} \) in metaEuro (Supplementary Material, Tables S10 and S11). In the tissue enrichment analysis, strong results were observed for tissues related to the stomatognathic system. The result for the MEsH term "dentition" met the false discovery rate (FDR) threshold. At the pathway level, our analysis did not identify any significantly enriched gene set that survived correction for multiple testing (at FDR < 0.05).

We also investigated potential secondary effects at each of the 24 risk loci, by performing conditional analyses on the respective lead SNP (i.e. the SNP with the lowest \( P \)-value except for the regions at chromosomes 2p21PKDCC and 17q13 where the...
| Lead SNP   | Closest gene       | Chr | N SNPs | Length of region (bp) | SNP region 95% posterior probability | SNP region 99% posterior probability | References | Genotyped top SNP includeda |
|------------|--------------------|-----|--------|----------------------|--------------------------------------|--------------------------------------|------------|-----------------------------|
| rs742071   | PAX7               | 1   | 317    | 110514               | 21 18939509 18992553 53044            | 33 18925512 18992583 67071           | Ludwig et al. (6) | Yes |
| rs560426   | ABCA4              | 1   | 124    | 41257                | 6 94539386 94551450 12064            | 9 94539386 94570218 30832           | Beaty et al. (2) | No |
| rs642961   | 1RF6_DIEXF         | 1   | 1373   | 584708              | 18 209950760 210022893 72133         | 25 209950760 210022893 72133         | Rahimov et al. (9) | Yes |
| rs4441471  | AC104623.2         | 2   | 536    | 210141              | 68 16706771 16735432 28661           | 77 16706771 16735432 28661           | Leslie et al. (11) | – |
| rs6740960  | 2Cof9r1           | 2   | 75     | 174921              | 1 42181679 42181679 0               | 2 42181679 42198217 16538           | Present study | – |
| rs7590268  | THADA              | 2   | 1036   | 406228              | 9 43501585 43670144 168559           | 31 43501585 43670144 168559          | Ludwig et al. (6) | Yes |
| rs7632427  | EPHA3              | 3   | 1902   | 843457              | 158 89273019 89640350 367313          | 238 89273019 89751353 478334         | Ludwig et al. (6) | Yes |
| rs1384062  | FILIP1L_CMSS1      | 3   | 1222   | 722547              | 75 99436424 100088929 652055         | 175 99428998 100079616 66818           | Ludwig et al. (3) | – |
| rs12543318 | DCAF4L2           | 8   | 1446   | 546749              | 2 8868340 8887382 19042              | 2 8868340 8887382 19042              | Ludwig et al. (6) | – |
| rs987525   | LINCO0976          | 8   | 953    | 318710              | 3 129964873 129990382 25509          | 4 129958384 129990382 31998          | Bimbaum et al. (4) | No |
| rs3758249  | FOXE1              | 9   | 716    | 290739              | 88 100596439 10067871 71432          | 123 100585506 100670272 84766         | Moreno et al. (8) | Yes |
| rs4752028  | KIAA1598           | 10  | 466    | 365794              | 23 118786985 118890006 103021        | 28 118786985 118890006 103021         | Mangold et al. (7) | Yes |
| rs8001641  | LINCO1080_SPRY2    | 13  | 498    | 167219              | 6 80697449 80701485 4036            | 11 80691352 80701485 10133           | Ludwig et al. (6) | No |
| rs4901118  | RP11-25SG12.2     | 14  | 476    | 134026              | 2 51856109 51856566 457             | 2 51856109 51856566 457             | Present study | – |
| rs1258763  | GREM1_FMN1         | 15  | 482    | 99614               | 13 33043284 33054523 11239          | 19 33043284 33054523 11239           | Ludwig et al. (5) | Yes |
| rs1873147  | TPM1               | 15  | 385    | 101368              | 13 6331425 63314519 3094            | 14 6331425 63314519 3094            | Ludwig et al. (6) | Yes |
| rs75490152b| CSK                | 15  | 1721   | 818259              | 287 74668398 75231234 544836         | 748 74622343 75446488 814245         | Present study | – |
| rs8049367  | CREBBP_ADCY9       | 16  | 217    | 43706               | 22 3988115 39832627 14512           | 33 3983187 3997415 34228           | Sun et al. (10) | Yes |
| rs1880646  | NTN1               | 17  | 245    | 77284               | 1 8919415 8919415 0                | 1 8919415 8919415 0                | Beaty et al. (3) | No |
| rs227331   | NOG                | 17  | 215    | 87850               | 4 54773238 54777585 4347            | 5 54770864 54777585 6721           | Mangold et al. (7) | Yes |
| rs8071332a | TANC2              | 17  | 1079   | 618627              | 46 61043405 61388844 346439        | 73 61043405 61405359 361954         | Leslie et al. (11) | – |
| rs3746101  | MKNK2              | 19  | 208    | 74094               | 2048281 2051261 2980              | 10 2038819 2051261 12442           | Present study | – |
| rs8113265  | SLC7A9             | 19  | 1410   | 365251              | 185 33132098 33495231 363133         | 691 33132098 33497141 365043         | Leslie et al. (11) | – |
| rs13041247 | MAFB               | 20  | 390    | 146327              | 41 39238736 39286786 48050        | 62 39238736 39286786 48050          | Beaty et al. (2) | Yes |

N—number of SNPs in the SNP region interval.

aThis column refers to whether the originally presented lead SNP from the respective GWAS (i.e. genotyped SNP), is among the credible SNP set. In studies marked by ‘–’, imputation had already been performed.

bIdentified with lowest $P$-value in metaEuro dataset.
Table 3. Co-localization analyses of nsCL/P imputation analyses results in autosomal regions of functionally annotated datasets

| Study name | Annotation | Number SNPs | Enrichment metaall | References |
|------------|------------|-------------|-------------------|------------|
| Neural crest cell line (NCCL) | Active enhancer | 4293 | <0.0001 | Rada-Iglesias et al. (37) |
| Mouse craniofacial tissue E11.5 (MCT) | Active promoter | 4250 | 0.002 | Attanasio et al. (38) |
| Neural crest cell line (NCCL) | Active and primed CRMs | 22 | <0.0001 | Data from Rada-Iglesias et al. (37) reanalyzed |
| Neural crest cell line (NCCL) | Active enhancers | 16 | <0.0001 | Data from Rada-Iglesias et al. (37) reanalyzed |
| Mouse limb tissue E11.5 (CON1) | Active enhancer | 2030 | 0.10 | Visel et al. (40) |
| IRF6 binding sites in keratinocytes (CON2) | IRF6 binding sites | 3893 | 0.6 | Botti et al. (40) |

*P*-values one-sided; if significant after correction for multiple testing (*P* < 0.00156).

Estimation of variance explained in nsCL/P

We estimated the heritability explained by common variants in the imputed Bonn GWAS dataset using the software GCTA (41). In addition, we estimated the proportion of variance in risk for nsCL/P that can be attributed to the recognized 24 risk loci using logistic regression. We found that 32.1% (8.5% standard error) of variance in the GWAS data can be attributed to common risk factors, with 25.5% contributed by these 24 risk regions. We also determined to what extent a polygenic component contributes to nsCL/P. We generated score alleles in the Bonn case-control GWAS cohort (discovery sample) and tested them for enrichment in the nsCL/P target sample (i.e. Baltimore trio cohort of European descent). We observed a highly significant enrichment of score alleles at a variety of *P*-value thresholds (Supplementary Material, Table S15A), with the lowest *P*-value in the Wald test (*P* < 0.00156); the unpruned matched SNP set at *P* < 0.00156 was obtained for the unpruned dataset (maximum *R*² = 6.8%).

Co-localization of association signals in relevant regulatory regions

In contrast to many common phenotypes for which human material is available and has been used by projects such as Epigenomics Roadmap (36), follow-up analyses of genetic findings for nsCL/P are hampered by the early embryonic time-point of facial development where defects occur, and the lack of appropriate human tissue. We therefore annotated the high-density nsCL/P dataset with ChIP-seq data previously generated to map active enhancers in human neural crest cells (hNCC, (37)) and active promoters and enhancers in mouse craniofacial embryonic tissue (38). Since hNCC are the precursors of the mesenchymal progenitors that will go on to generate most of the facial cartilage and bone during craniofacial development, we hypothesized the previously generated ChIP-seq datasets in hNCC (i.e. p300, H3K4me1, H3K27ac and TFAP2A) could be reanalyzed to define a broader set of cis-regulatory elements, including primed enhancers (see Methods). To identify whether nsCL/P association signals from the genome-wide dataset are significantly overrepresented within regulatory regions considered active during craniofacial development, we performed co-localization analyses (Table 3). Similar cis-regulatory maps generated from mouse embryonic limbs (39) and IRF6 binding sites identified in human adult keratinocytes (40) were used as controls. We identified highly significant enrichments in hNCC when either active enhancers alone or a broader set of active and primed enhancers were considered. Similarly, significant overrepresentations were also seen with mouse craniofacial enhancers (Table 3). Lower levels of significance were observed with mouse craniofacial promoter regions, while no enrichment was found in the control datasets of mouse limb-tissue and human adult keratinocytes.
Insights into the genetic etiology of nsCL/P and nsCPO

To investigate the potential etiological overlap between nsCL/P and nsCPO, we generated and analyzed imputed data from a previous genome-wide nsCPO study that included 550 nsCPO trios ([24], see Methods). No variant reached the threshold of genome-wide significance in either the European or in the analysis of mixed ethnicities (Supplementary Material, Figs. S5 and S6). The only genome-wide significant risk locus for nsCPO identified so far [19, 20] had $P_{\text{nsCPO}} = 1.18 \times 10^{-04}$.

The lead SNPs and associated regions of the 24 recognized nsCL/P risk loci were analyzed in the nsCPO dataset (Supplementary Material, Table S16). At lead SNP level, only rs3758249 at the FOXE1 locus showed significant associations in nsCPO after correction for multiple testing ($P_{\text{nsCPO,all}} = 1.84 \times 10^{-03}$), which confirmed prior findings on associations with both traits [8, 21]. This finding was supported by additional variants being strongly associated at this locus (Supplementary Material, Fig. S7).

Three other nsCL/P lead variants (at chromosomes 15q22, 16p13 and 17q22) were significant at the nominal level (Supplementary Material, Table S16). To investigate allelic or haplotype heterogeneity between both traits, the 24 nsCL/P loci (defined as nsCL/P lead SNP ± 500 kb) were extracted from the nsCPO data, revealing 73 078 SNPs passing QC. Fourteen variants had $P$-values lower than $10^{-04}$ in the overall analysis (Supplementary Material, Table S17), eight of which mapped to the 2p21 (THADA) region previously identified [6]. The lowest $P$-value was observed for rs12476091 ($P_{\text{nsCPO,all}} = 8 \times 10^{-05}$) which approaches the threshold of significance (conservative Bonferroni correction for number of SNPs, i.e. $6.84 \times 10^{-05}$). Comparison of the regional association plots revealed two distinct association clusters for nsCPO and nsCL/P. Interestingly, conditional analyses in nsCL/P revealed suggestive association for variants overlapping the nsCPO-associated region as secondary effect (Supplementary Material, Fig. S8).

To address a polygenic contribution between nsCL/P and nsCPO, we finally sought to systematically analyze (1) the joint contribution of the 24 recognized risk loci, and (2) a general polygenic risk score from nsCL/P applied to nsCPO, using genome-wide data. For the first approach we generated dosage values for transmitted alleles and non-transmitted alleles in each of the nsCPO trios, for each of the 24 nsCL/P lead SNPs (Supplementary Material, Table S8). In each trio, both dosages were weighted with the logarithmic relative risks (RR) of the nsCL/P risk allele and summed over all 24 lead SNPs. This approach resulted in a transmitted and non-transmitted score for each nsCPO trio. Subtracting the non-transmitted score from the transmitted score revealed a mean over families of $-0.1351 \pm 1.51$, indicating that in aggregate, these 24 recognized lead SNPs for nsCL/P do not contribute to nsCPO risk.

The difference from a mean of 0 was not significant ($P = 0.195$).

For the second approach, we used the polygenic score generated in the nsCL/P discovery dataset and evaluated its association in the nsCPO dataset of European ancestry trios (Supplementary Material, Table S15B). In none of the settings (pruned, unpruned, matched and non-matched) was a significant enrichment of nsCL/P risk allele scores in nsCPO detected ($P > 0.1$, Supplementary Material, Table S15, Fig. 2).

Discussion

In the first wave of GWAS, analysis of common variants was limited to a subset of ~500 000 variants present on commercially available microarrays. Although these variants had been selected to tag the majority of genetic variation, it has become evident that true associations can be missed in situations where risk variants had a minor allele frequency lower than 5%; were not adequately tagged by the genotyped variants; or failed genotyping for technical reasons. With advances in the fields of high marker-density population reference sets and bioinformatic techniques, accurate prediction of non-genotyped variants has enabled further discoveries of the genetics underlying complex disorders [42-44].

Here, we performed large-scale imputation analyses of previously published GWAS datasets on nsCL/P and nsCPO, two of the most common types of congenital craniofacial anomalies,
to detect novel risk loci. We identified common variants at four new loci as risk factors for nSL/P, while none was identified for nsCPO. The associations at the chromosomal regions 2p21 (PKDCC), 14q22 and 19p13 were replicated by genotyping in independent samples and are therefore deemed as associated with ‘high confidence’, while chromosome 15q24 revealed genome-wide significance in the nSL/P genome-wide dataset but did not replicate in any of our replication samples, suggesting a false-positive result similar to previous findings (11). As with most of the previous GWAS hits in nSL/P and other complex traits, the top-associated variants at chromosomes 2p21 (PKDCC) and 14q22 map in non-coding regions, and might therefore represent regulatory mutations affecting adjacent genes. Among the candidate genes at these loci are the protein kinase domain containing, cytoplasmic gene, (PKDCC [MIM 614150]), whose orthologues are involved in facial development in both mice (45) and zebrafish (46), FERM-domain containing 6 (FMID6 [MIM 614555]) and thioredoxin-related transmembrane protein 1 (TMX1 [MIM 610527]). In contrast, the top-associated variant at chromosome 19p13 is a missense variant in MAP Kinase Interacting Serine/Threonine Kinase 2 (MKNK2 [MIM 605069]). Although modelling MKNK2 protein structure using Phyre2 (47) revealed considerable changes in protein folding (Supplementary Material, Fig. S9), functional annotation using the Variant Effect Predictor (VEP), as well as a low CADD-Phred score (2.72) suggest rs3746101 may be a benign polymorphism. These observations suggest the biological effect in cleft patterning (49).

Notably, one variant in the highly variable MHC region identified with genome-wide significance in the nSL/P genome-wide dataset did not replicate in any of our replication samples, suggesting false-positive results similar to previous findings (11). One of the variant associations at chromosome 19p13 is a missense variant in MAP Kinase Interacting Serine/Threonine Kinase 2 (MKNK2 [MIM 605069]). Although modelling MKNK2 protein structure using Phyre2 (47) revealed considerable changes in protein folding (49), functional annotation using the Variant Effect Predictor (VEP), as well as a low CADD-Phred score (2.72) suggest rs3746101 may be a benign polymorphism. These observations suggest the biological effect in cleft patterning (49).

Our study increases the total number of conclusive common risk factors for nSL/P to 24, and using credible SNP analyses in the comprehensive European data suggest likely causative SNPs at each of these loci. Notably, our results suggest rs6740960 (2p21 (PKDCC)) and rs1880646 (17q13) as likely causative SNPs in European populations. For other regions, such as those at chromosomes 17q22 (NOG) and 1q32 (IRF6), the credible SNP set contained fewer than 20 variants but included SNPs such as rs227727 or rs42961, which have been previously shown to have some functional effect (9,50). It remains unclear whether the same credible SNP sets were identified in non-European populations; therefore, analyses of other distinct genome-wide datasets (e.g. studies (10) or (11)) would be required. Such an approach would also be helpful to delineate causal variants at other loci such as the EPHA3 locus (chromosome 3p11, > 50 SNPs in credible SNP set) using distinct LD patterns in other populations.

We next sought to analyze whether risk alleles of nSL/P in aggregate contribute to risk for nsCPO. The lack of large etiological overlap between these disorders has been suggested by epidemiological studies, and we now support this hypothesis by a polygenic score approach based on actual GWAS data. Interestingly, during preparation of this manuscript, Wen & Lu developed a novel statistical method to account for phenotypic heterogeneity between disease subtypes and, when applying it to nsOCF datasets, confirmed that nSL/P and nsCPO have different genetic etiologies (51). While there is ample evidence for nSL/P being a multifactorial trait, the situation remains less clear for nsCPO. It is possible that nsCPO results from common risk loci which have not yet been detected (maybe due to low effect sizes and/or inadequate sample numbers). Alternatively, there is also a strong possibility that some patients with nsCPO might, in fact, be syndromic cases lacking the usual accompanying signs/symptoms. In this context, we have recently identified truncating mutations in GRHL3, a gene underlying the autosomal dominant Van der Woude syndrome (VWS [MIM 119300], (52)), in four patients with apparent nsCPO (19).

On aggregate, these observations suggest that nsCPO is likely more often caused by rare or low-frequency variants, while nSL/P seems to follow the pattern of complex traits in which numerous common variants in non-coding regions are associated with moderate effect sizes. We suggest about one-third of the variance in risk for nSL/P can be explained by common variants in general, with one-quarter attributable to the 24 risk loci currently recognized. These findings suggest that further common variants are present in the nSL/P dataset and will have to be identified by increasing sample sizes, clinical details and/or approaches to reduce the burden of multiple testing. Also, contributions of rare variants with modest to strong effect sizes, or gene-environment interactions will probably help to explain the remaining missing heritability. Based on this, subsequent analyses of the biological impact of common variants were performed for nSL/P only.

Notably, using our dataset we were able to identify potential shared etiologies of nSL/P. While association with hypothyroidism has been established at the FOXE1 locus, the association of variants with myopia and clefting is a novel observation suggesting shared etiologies. Co-incidence of clefting defects and eye malformations has been reported previously, for instance in Stickler syndrome (53) and anophthalmia (54). Interestingly, the risk allele for nSL/P decreases axial length, indicating that a delayed cell growth or disturbed cell migration might be an underlying shared biological trait of these malformations. Our attempt to replicate single variants identified in craniofacial morphometric studies did not prove successful, however it remains possible that SNPs associated with craniofacial anomalies might contribute in aggregate to nSL/P risk. Along these lines, recent analysis of a combined score of nSL/P SNPs tested for facial traits revealed association for nose-width-related measurements, although with little variance explained (30). This suggests that polygenic risk scores might be helpful for determining whether these two distinct clinical entities share some genetic background.

Despite the advances in our understanding of nSL/P etiology resulting from published GWAS and the current study, a number of limitations should be considered. First, our study did not have sufficient power to identify very low-frequency or rare variants with confidence; this limits its application with respect to following up causative variants suggested by functional or candidate gene studies which are in these frequency ranges. For example, rs138557689 at the FZD6 gene locus was recently shown to be a functionally-relevant variant in an African-American family (55). In our dataset, this variant was excluded due to its low frequency (<1%). Future imputation studies using combined reference panels such the Haplotype Reference Consortium will increase the accuracy and informativity of low frequency variants and provide the opportunity to identify causal variants in this frequency range for nSL/P similar to recent successes in other traits such as bone mineral density (56).
One important unanswered question is the exact functional mechanisms by which the risk alleles contribute to risk of clefting. The most likely scenario is that associated variants reside in cis-regulatory modules (CRM) that control expression of their target genes in a tissue- and time-specific manner. Therefore, the comprehensive annotation of CRMs in tissues contributing to craniofacial and palate development using, for example, epigenomic approaches, would be highly desirable. Considering the moderate sequence conservation of many regulatory elements, CRM maps should be ideally generated in relevant human tissue, which is a major hurdle given the early developmental time-points at which relevant craniofacial tissue should be collected. Alternatively, CRM maps can be generated in human cell types relevant for craniofacial development (e.g. neural crest), which can be derived in large amounts from human embryonic stem cells (hESC) using CRMs which can be isolated from embryonic animal models. Chiefly, our approach of co-localizing strongly associated variants from the nsCL/P GWAS with available CRM maps from murine embryonic craniofacial tissues and hNCC, shows that associated variants are significantly more often located within regions with regulatory potential (e.g. enhancers) than expected by chance. Notably, this enrichment was not observed when control datasets were considered, illustrating the importance of using CRM maps from relevant tissues and developmental stages. Using previously generated epigenomic datasets in hNCC, we defined a broad set of CRMs which included enhancers predicted to display a primed state that precedes and probably facilitates the future activation of these enhancers and their target genes (57,58). Interestingly, this broad CRM map was highly enriched in nsCL/P association signals, suggesting genetic variation might lead to epigenetic alterations at relevant CRMs very early during human embryogenesis, which could then affect the regulatory networks controlling craniofacial development at later prenatal stages. Elucidating if these early epigenetic alterations actually take place and understanding their mechanistic basis might uncover novel causal relationships which are not yet fully understood. To overcome the limitations associated with the tissues and developmental stage specificity of CRMs, further cell types and tissues should be functionally annotated, which could certainly span the number of causative variants that can be identified and molecularly characterized.

In conclusion, our study generates novel insights into the etiology of nonsyndromic orofacial clefting by revealing novel common variants and biological information for nsCL/P and demonstrating its distinct genetic background from nsCPO. Based on this, further work will now focus on identifying the biological mechanisms by which the associated common variants interfere with normal craniofacial development. Joint consortia efforts such as FaceBase (59) and the development of novel technologies such as massively parallel regulatory assays (60, 61) might provide tremendous resources and opportunities in that direction.

Materials and Methods

Genome-wide imputation (including HLA) plus meta-analysis (discovery phase)

In 2012, our group performed the first meta-analysis on nsCL/P using an in-house case-control dataset (Bonn GWAS dataset (7)) and a trio dataset (Baltimore study (2), Supplementary Material, Fig. S1). Three-hundred-ninety-nine cases, 1318 controls and 1461 individuals had been included, and 497 084 observed SNPs were analyzed (6). In the present study, these genotype data from both case-control and trio cohorts were imputed using IMPUTE2 (62), using 1092 individuals from the 1000 genomes project (22) as reference panel. For statistical analysis in the case-control cohort, logistic regression was performed (with SNPTEST and -method expected (63)), by incorporating the first five dimensions of the multi-dimensional-scaling coordinates. For the trio data retrieved from dbGaP (2), a previously published method was used (FBATdosage (64)). P-values were subsequently combined using Z-scores, in two approaches: European individuals only (metaEuro), and European plus Asian individuals (meta_all). Analysis of Asian data only was not covered by the dbGaP request. To account for the limited power of imputation approaches to correctly predict rare and low-frequency variants, we only retained in the analysis SNPs with a SNPTEST info_score > 0.4 in any of the two datasets (i.e. case-control/trio) and a minor allele frequency (MAF) > 1% in the controls and non-transmitting parental alleles.

For the MHC region, a HLA-specific imputation was performed. SNP2HLA (65) was used to impute SNPs, amino acid residues, indels, and two- and four-digit classical alleles for eight HLA genes in the MHC region from 29 to 33 Mb on chromosome 6p21.3. We used the reference panel provided by T1DGC, which included 5225 European samples with classical typing for eight HLA genes (66).

nsCL/P replication study (replication phase and combined analysis)

The replication sample consisted of 610 nsCL/P cases and 1737 controls from three different cohorts (Bonn, Yemen, Mexico, Supplementary Material, Fig. S1). The same sample was previously used in an independent study, please refer to (5) for sample description. Forty-four SNPs were included in the replication assay. Genotyping was performed using Maldi-ToF on an in-house Agena Bioscience Sequenom platform. Two SNPs failed genotyping (rs6030889, rs3091552) and were excluded, leaving 42 SNPs in the analysis (Supplementary Material, Table S1). After genotyping, 138 individuals (36 cases, 102 controls) had to be excluded due to call rates <95%. Final sample numbers were: 224 cases/921 controls (Bonn), 152 cases/323 controls (Mexico) and 198 cases/391 controls (Yemen).

Association statistics were calculated by applying the Armitage-trend test, for each sample cohort separately. For each SNP, relative risks of the three replication cohorts were combined using fixed-effect meta-analysis. For the combination of these datasets with the imputed data of metaEuro and meta_all, Z-score based analyses were used.

In silico replication: combination with Leslie et al. (2016) (11) using summary statistics

In a recent multiethnic GWAS, three novel risk loci for nsCL/P were identified (11). As there was considerable overlap between individuals (n = 317) used in this study (11) and those used in the Baltimore study (2) (i.e. which were part of the meta-analysis (6)), results of this study could not be added to the imputed meta-analysis. In an attempt to combine independent samples, we therefore combined the Bonn-case-control cohort with (a) data from the European analysis of (11), and (b) data from the multi-ethnic cohort of (11). Summary statistics (P-values, risk allele, relative risks and confidence intervals) were extracted from Table 1, Supplementary Tables S4 and S5 (11) and combined with imputed
Bonn GWAS data generated herein. SNPs comprised either imputed SNPs with $P < 10^{-10}$ or genotyped SNPs that had yielded $P < 10^{-5}$ in (11). In the European dataset (comprising 406 trios, 170 cases and 835 controls), 102 SNPs met these quality criteria, 86 of which were also represented in the imputed dataset of the Bonn GWAS cohort. In the multiethnic cohort (comprising 1319 trios, 823 cases and 1700 controls), 238 variants were identified at these $P$-value thresholds, 227 of which were also present in the Bonn GWAS data. These data were combined using fixed-effect meta-analysis.

**Genome-wide imputation of nsCPO data set**

Genotypes for 550 parent-child trios with nsCPO were retrieved from a previous genome-wide nsCPO study (24). Similar to nsCL/P, variants were imputed using 1000 genomes as reference panel, and statistical analysis was performed using FBAT dosage (64) for about 8.38 million variants (info scores $\geq 0.4$, MAF $\geq 1\%$ in the non-transmitting parental alleles). Again, analysis was split into an European analysis only (CPO Euro), and an analysis of mixed ethnicities (CPO_mixed).

**Polygenic score analysis**

We performed polygenic score analysis (a) to analyze whether a polygenic component contributes to the genetic susceptibility of nsCL/P, and (b) to determine whether this polygenic component of nsCL/P contributes to risk for nsCPO. Based on (67), the polygenic score analysis was performed using the imputed Bonn GWAS data as the discovery cohort and two target cohorts, i.e., European nsCL/P trios from the Baltimore study (2) for analysis (a) as well as European nsCPO trios (24) for (b). We used SNPs with MAF $\geq 1\%$ in the Bonn GWAS data and info-scores $\geq 0.8$ in each of the target cohorts.

We generated 11 SNP sets using different thresholds from the association test $P$-value ($P_T$) in the discovery sample. For each SNP set and each individual, a score in the target sample was calculated as the number of risk alleles which were weighted using the log relative risks obtained from the discovery sample. The polygenic score analysis was performed for all SNPs and also after pruning on linkage disequilibrium. The latter approach enabled analysis of a collective contribution of independent risk alleles ($r^2 < 0.25$). These polygenic scores were tested to determine their association with disease status using logistic regression (unmatched) and conditional logistic regression (matched) which accounts for correlation status between cases and pseudo-controls. Nagelkerke’s pseudo $R^2$ was used to estimate the proportion of the variance of risk to disease in (a) nsCL/P-trios and (b) nsCPO-trios explained by the polygenic risk score. The Wald test was used to test for the effect of the polygenic score on risk for nsCL/P and nsCPO.

**Co-localization analysis**

The tissue-specific mode of action of regulatory elements and the very narrow developmental time range of cleft development (during $6^{th}$ to $8^{th}$ week post-conception) suggest that causative variants in nsCL/P could be identified by overlapping high-density genetic datasets with epigenetic datasets from relevant embryonic tissue or cell types. Given the limitations associated with embryonic tissue datasets from humans, we used publicly available datasets for both human neural crest cells (hNCC, (37)) and mouse facial tissue (38). In these datasets, active enhancer and promoters were identified based on different combinations of epigenetic marks. Moreover, we re-analyzed the ChIP-seq datasets previously generated in hNCC (37) to define broader sets of CRMs in which promoter regions were not explicitly excluded. These additional CRMs were expected to include primed enhancers that are inactive but pre-marked in hNCC and which might become active later in hNCC development, for example when differentiating into craniofacial mesenchyme. To define these larger CRM maps, hNCC ChIP-seq datasets were first re-analyzed with MACS2 (68) using the following criteria:

- $p300$ and TFAP2A peaks were identified using $q \leq 10^{-4}$.
- H3K4me1: peaks were identified using broad settings and $q \leq 10^{-4}$.
- H3K27ac: peaks were identified using broad settings and $q \leq 10^{-6}$.

Next, these peaks were analyzed in a combinatorial manner to define two sets of CRMs:

- Active CRMs (16 177 regions): regions bound by p300 or TFAP2A and enriched in H3K27ac within 1Kb.
- Active and Primed CRMs (22 792 regions): it included the Active CRMs defined above, plus regions enriched in both H3K27ac and H3K4me1.

In addition, two control cohorts containing similar datasets generated from mouse embryonic limbs (39) and IRF6 binding sites identified in human adult keratinocytes (40) were used.

For the co-localization analysis, two different categorization schemes for $P$-values were applied. The first scheme ($P$ groups) consisted of the nine categories $10^{-k+1} > P > 10^{-k}$ for $k = 1, \ldots, 8$ and $10^{-8} \geq P$. The second scheme ($P$ nominal) only distinguished between $P > 0.05$ and $P \leq 0.05$. For both schemes, the frequencies of SNPs being located within and outside annotated regions were counted within each category. The one-sided version of the Cochran-Armitage test for trend was applied to test for an enrichment of smaller $P$-values within annotated regions on the basis of the resulting $9 \times 2$ contingency table.

**Statistical refinement of genetic associations at 24 loci**

A Bayesian refinement method was applied at each of the recognized risk loci to determine the subset of most informative SNPs, referred to as the credible SNP set (69). This approach has previously been demonstrated to be more efficient at selecting putatively functional SNPs compared to linkage disequilibrium based approaches (70,71). A genomic interval around each index SNP was defined by a genetic distance of 0.1 centimorgans (cM) upstream and downstream of each index SNP using HapMap fine-scale recombination rate estimates. An assumption of the Bayesian refinement method is that each locus contains a single association, therefore each interval was tested for multiple independent associations by including the index SNP as a covariate in the logistic regression and repeating the analysis conditional on this SNP. Where multiple associations exist the credible SNP set was calculated for one SNP while fixing the effect of the other by including it as a covariate in the logistic regression. To calculate the credible SNP set for each independent effect the posterior probability that any particular SNP is the causal SNP was calculated based on the Bayes factor for the SNP as a proportion of the mean Bayes factor for all SNPs in the genomic interval. Posterior probabilities were aggregated to define the smallest set of SNPs with a total posterior probability of $\geq 95\%$ and $99\%$ (69).
Estimation of variance explained

The GCTA (41) analysis of the explained variance in nsCL/P risk was performed using the imputed data of the Bonn case-control GWAS dataset. As GCTA requires genotypes as input, individual genotypes were called using a threshold of 90%. The resulting dataset was filtered by keeping SNPs with genotype call rates > 95%, MAF > 1%, HWE P-value > 10^{-6} and non-significant differences in missingness between cases and controls (PLINK (72) P-value > 0.05). In total, 5.61 million SNPs, both genotyped and imputed, respectively, remained after this quality control of the dataset. Using GCTA and a relationship cutoff of 5% estimated from the genome-wide markers, we obtained the Genetic Relationship Matrix (GRM) for 1711 individuals (i.e. 399 cases and 1318 controls). The same first five dimensions of the multi-dimensional-scaling coordinates as for the association analysis were included in the estimation of the explained variance by GCTA. A disease prevalence of 0.1% was assumed.

DEPICT

We applied DEPICT (35) to systematically analyze most likely causal genes at the given associated loci, explore pathways enriched for nsCL/P associations and reveal tissues and cell types where genes from associated loci are highly expressed. The DEPICT framework relies on publicly available datasets and uses large and diverse gene expression data, allowing annotation of potential functional connections between genes at associated loci. Here, we downloaded the beta version of DEPICT_v1 (http://www.broadinstitute.org/mpg/depict/; date last accessed July 15, 2016) and performed the analysis in two settings: (i) all autosomal SNPs with P < 5 x 10^{-5} plus three top SNPs for each of the known 24 loci that did not have genome-wide results in our analyses (n=816 SNPs); and (ii) all autosomal SNPs with P < 5 x 10^{-5} in the metaEuro data (n=1470 SNPs). The latter setting was chosen because limiting the analysis to known risk loci only may result in loss of power because SNPs that have not yet met appropriate thresholds of significance due to limited statistical power. In each setting, gene prioritization, gene set enrichment and tissue/cell type enrichment analyses respectively were performed.

Supplementary Material

Supplementary Material is available at HMG online.

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